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Gut-tropic T Cells that Express Integrin α4β7 and CCR9 are Required for Induction of Oral Immune Tolerance in Mice

Barbara Cassani[1,*], **Eduardo J. Villablanca**[1,*], **Francisco J. Quintana**[2], **Paul E. Love**[3], **Adam Lacy-Hulbert**[4], **William S. Blaner**[5], **Tim Sparwasser**[6], **Scott B. Snapper**[7], **Howard L. Weiner**[2], and **J. Rodrigo Mora**[1,**]

[1]Gastrointestinal Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

[2]Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

[3]Eunice Kennedy Schriver, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, 20892

[4]Department of Pediatrics, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

[5]Department of Medicine, Columbia University, New York, NY 10032

[6]Institute of Infection Immunology, Centre for Experimental and Clinical Infection Research, Twincore, 30625 Hannover, Germany

[7]Gastrointestinal Unit, Children's Hospital & Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

Abstract

BACKGROUND & AIMS—Induction of oral immunological tolerance (OT) blocks proinflammatory responses to orally administered antigens; this approach might be used to treat autoimmune conditions. We investigated whether gut-tropic T cells that express the integrin α4β7 and the chemokine receptor CCR9 are required for OT.

METHODS—Skin delayed-type hypersensitivity and experimental autoimmune encephalomyelitis were used to monitor OT in mice. To assess the role of receptors that mediate localization of lymphocytes to the gut (gut-homing receptors) in induction of OT, we studied *CCR9*−/− and β*7*−/− mice, and also blocked the α4β7 ligand MAdCAM-1 in wild-type mice. We

**Correspondence and requests for materials should be addressed to J. Rodrigo Mora (j_rodrigo_mora@hms.harvard.edu)..
*BC and EJV contributed equally to this work

Author's Contributions: BC and EJV designed and performed most of the experiments, analyzed the data and wrote the paper. PEL provided CCR9$^{-/-}$ mice. FJQ and HLW performed the experiments and analyzed the data in the EAE model. ALH helped designing some experiments. WSB provided LRAT$^{-/-}$ mice. TS provided DEREG mice. SBS provided IL-10Rβ$^{-/-}$ mice and helped designing some experiments. JRM conceived the study, designed experiments, analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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Online Supplemental Material: Supplementary Figures S1–S10 plus supplementary methods.

used DEREG and Scurfy mice to assess the role of Foxp3$^+$ regulatory T (T_{REG}) cells, and *IL-10*−/− and *IL-10R*β−/− mice to examine the role of IL-10, in induction of OT.

RESULTS—OT could not be induced in *CCR9*−/− or β*7*−/− mice, or when MAdCAM-1 was blocked in wild-type mice, indicating that gut-homing receptors are required for oral tolerization. Consistent with the role of all-trans retinoic acid in inducing gut homing T cells, OT could not be induced in mice depleted of vitamin A. OT was rescued in *CCR9*−/− mice following adoptive transfer of wild-type T cells, but not *CCR9*−/− or β*7*−/− T cells. Gut-homing T cells are therefore necessary and sufficient to induce OT. Wild-type T_{REG} cells and IL-10 were required to restore OT to *CCR9*−/− mice, indicating that homing and functional differentiation of IL-10-producing T_{REG} cells in the gut is required for OT. Conversely, transfer of *CCR9*−/− or β*7*−/− T cells to wild-type mice partially inhibited OT.

CONCLUSIONS—Expression of CCR9 and α4β7 on T cells and their subsequent localization to the gut is required for induction of oral immunological tolerance in mice. Therapies designed to block gut-homing receptors might, under some conditions, interfere with normal tolerogenic mechanisms in the intestinal mucosa.

Keywords

immune regulation; autoimmunity; allergy; intestine; Peyer's patch

Two general mechanisms have been proposed to induce OT. Feeding high doses of antigen typically results in T cell clonal deletion and/or anergy[1], while low doses/repeating regimes of oral antigen administration lead to active immunosuppression by inducing regulatory T cells[2, 3]. Although orally administered antigens can be presented to T cells by dendritic cells (DC) in Peyer's patches (PP) and mesenteric lymph nodes (MLN), it has been shown that OT requires MLN[4, 5] but not PP[4]. In this setting, orally administered antigens are captured by DC in the intestinal lamina propria (LP) and transported to MLN via the lymph. DC from MLN, PP and small intestine lamina propria (gut-associated DC) induce high expression of gut-homing receptors integrin α4β7 and chemokine receptor CCR9 on lymphocytes upon activation[6]. The capacity of gut-associated DC to imprint gut-tropism on lymphocytes is explained by their selective capacity to metabolize vitamin A into RA[7], and RA is necessary and sufficient to induce gut-tropic α4β7$^+$CCR9$^+$ T and B cells in mice and humans[6, 7]. Moreover, RA, in the presence of TGF-β, potentiates the induction of Foxp3$^+$ regulatory T cells (T_{REG})[8, 9], which are required for the establishment of OT[2]. We hypothesized that RA-dependent induction of gut-homing T cells is critically required for OT by promoting the differentiation of tolerogenic T_{REG} in the gut lamina propria and by re-directing potentially pro-inflammatory T cells to the gut mucosa.

Methods

Mice

Wt, β7$^{-/-}$, *Scurfy*, and IL-10$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). CCR9$^{-/-}$ and LRAT$^{-/-}$ mice have been described[10, 11]. DEREG mice[12] were provided by Dr. Thorsten Mempel (MGH, Boston). Experiments using IL-10Rβ$^{-/-}$ mice were done in collaboration with Dr. Scott Snapper (Children's Hospital, Boston). All mouse strains were used between 8–13 weeks of age. Mice were maintained in SPF/VAF animal facilities at MGH and used in accordance with the guidelines from the Subcommittee on Research Animal Care at MGH and HMS.

OT induction

Mice were fed daily either 2.5 mg OVA or 0.25 mg MOG peptide ($MOG_{35–55}$) via oral gavage for 5 days, as described[3, 13].

DTH

Mice were injected s.c. with 200 μl OVA/CFA emulsion (250 μg OVA plus 100 μl CFA) in the tail base. Seven days later the mice were challenged with 50 μl of aggregated OVA (10 μg/ml) injected into the left footpad. The right footpad (control) received 50 μl of PBS. The thickness of both hind footpads was measured 24 hours later with a caliper. Specific DTH was calculated as the difference between the left and the right footpad.

Statistical analysis

Data are presented as mean ± SEM and were analyzed using GraphPad Prism Software 5.0c (La Jolla, CA). Significance was determined using unpaired Student's t test or one-way ANOVA with Bonferroni's post-hoc tests, as appropriate.

Results

CCR9 is required for OT induction

To test the role of gut-homing lymphocytes in OT we used $CCR9^{-/-}$ mice, whose T cells are significantly impaired in their capacity to migrate to the small bowel[6], we used the classical ovalbumin (OVA) skin DTH model as OT readout[3, 13]. If gut-homing is required for the establishment of OT we predicted that mice lacking CCR9 would develop DTH despite of being pre-treated orally with OVA. DTH was induced equally efficiently in *wt* and $CCR9^{-/-}$ mice that received oral PBS, indicating that CCR9 is not required for T cell recall responses or DTH induction. However, oral OVA administration prevented DTH responses only in *wt* mice, whereas $CCR9^{-/-}$ mice developed DTH regardless of whether they were pre-treated orally with OVA (Fig. 1A). Moreover, oral OVA did not decrease *ex vivo* OVA-specific cell proliferation in splenocytes from $CCR9^{-/-}$ mice, while it efficiently suppressed systemic recall responses in *wt* mice (Fig. 1B). As expected, polyclonal T cell activation induced similar levels of cell proliferation and IFN-γ production in orally tolerized *wt* and $CCR9^{-/-}$ mice (Fig. S1). IL-10 is critical for OT[14, 15] and IL-4 seems to play a role in OT under some conditions[15]. Accordingly, systemic IL-10- and IL-4-producing cells were readily induced in *wt* mice, but not in $CCR9^{-/-}$ mice supplemented with oral OVA (Fig. 1C and Table S1). Reciprocally, the production of IL-2 and IFN-γ was reduced in splenocytes from *wt* mice upon oral OVA administration, while it was not affected in $CCR9^{-/-}$ mice.

Similar to spleen, $CD4^+$ T cells isolated from draining popliteal PLN of orally tolerized *wt* but not $CCR9^{-/-}$ mice displayed lower OVA-specific proliferative responses and IFN-γ production than $CD4^+$ T cells from mice given oral PBS (Fig. S2A, B), an effect that was mirrored by reduced cellularity in PLN of *wt* as compared to $CCR9^{-/-}$ mice (Fig. S2C). Moreover, increased IL-10 production was consistently observed at the protein and mRNA levels in OVA-stimulated $CD4^+$ T cells from PLN of orally tolerized *wt* but not $CCR9^{-/-}$ mice (Fig. S2D, E). Immunomodulation in OT also involves TGF-β-producing cells[16]. Correspondingly, enhanced levels of *Tgfb1* mRNA were detected in $CD4^+$ T cells from PLN of orally tolerized *wt* but not $CCR9^{-/-}$ mice (Fig. S2F).

To extend our results to another OT experimental system we used the EAE model, in which OT can be generated by orally supplementing mice with a peptide derived from myelin oligodendrocyte glycoprotein ($MOG_{35–55}$)[1, 17]. EAE developed similarly in *wt* and $CCR9^{-/-}$ mice supplemented orally with PBS (Fig. 1D), indicating that CCR9 is not required for EAE pathogenesis. However, orally administered $MOG_{35–55}$ prevented EAE only in *wt* but not in

$CCR9^{-/-}$ mice. Accordingly, cell proliferation upon *ex vivo* re-stimulation with $MOG_{35–55}$ was significantly reduced in splenocytes from MOG-tolerized *wt* mice, but not in cells from $CCR9^{-/-}$ mice (Fig. 1E). Th17 and Th1 cells are involved in EAE and DTH pathogenesis and OT induction abrogates the generation of these pro-inflammatory T cells[18, 19]. Consistently, MOG-tolerized *wt* mice showed lower frequencies of IL-17- and IFN-γ-producing cells as compared to non-tolerized controls. However, $CCR9^{-/-}$ mice did not show a decrease in the proportions of Th17 or Th1 cells upon oral $MOG_{35–55}$ administration (Fig. S3). In summary, our results using two independent experimental systems indicate that CCR9 is critical for OT generation.

Blocking MAdCAM-1 blocks migration of antigen-specific T cells to the gut and abrogates OT in wt mice

As a complementary approach we assessed the role of α4β7-MAdCAM-1, the other gut-homing receptor pair[6], in OT. Treatment of *wt* mice with a blocking mAb against MAdCAM-1 (clone MECA-367) increased the frequencies of systemic $\alpha4\beta7^{+}$ effector/memory CD4 T cells, an effect that was significantly more pronounced in mice that were orally supplemented with OVA (Fig. 2A, B), suggesting that oral OVA administration generated OVA-specific gut-homing CD4 T cells and that blocking α4β7-MAdCAM-1 interaction prevented the migration of these cells to the gut, hence increasing their peripheral frequency. Given the low frequencies of endogenously generated OVA-specific CD4 T cells in OVA-tolerized mice, we could not reliably detect these cells in the small intestine lamina propia (LP). Nevertheless, adoptively transferred OT-II T cells ($V\beta5.1^{+}$) were readily detected in the LP upon oral OVA supplementation (days 3 and 6) and they were markedly decreased by anti-MAdCAM-1 mAb (Fig. S4), indicating that OVA-specific T cells traffic to the small bowel LP upon activation in the MLN and that their homing to the LP is efficiently blocked by anti-MAdCAM-1 treatment.

Importantly, similar to $CCR9^{-/-}$ mice, anti-MAdCAM-1 mAb prevented OT in *wt* mice, as evidenced by increased skin DTH, higher *in vitro* OVA-specific proliferation, enhanced *ex vivo* IL-2 and IFN-γ production, and lack of induction of systemic IL-10-producing cells as compared with mice treated with control rIgG (Fig. 2C–E). Thus, our data indicate that both gut-homing receptor pairs, CCR9-CCL25 and α4β7-MAdCAM-1, are required for OT.

OT is abolished in vitamin A depleted (VAD) mice

Since RA is required for imprinting gut-tropic $\alpha4\beta7^{+}$ $CCR9^{+}$ lymphocytes, we predicted that vitamin A-depleted (VAD) mice should be impaired in OT induction. For vitamin A depletion we used mice deficient in lecithin:retinol acyltransferase ($LRAT^{-/-}$), which cannot store vitamin A in the liver and therefore can readily become VAD after only 2–3 weeks on a VAD diet, hence avoiding potential side effects of long-term vitamin A depletion[10]. DTH was similarly induced in mice on a control or VAD diet, indicating that RA is not required for recall T cell responses during DTH induction. However, OT was abrogated in VAD mice, as shown by increased skin DTH, higher *in vitro* OVA-specific proliferation and IL-2 production, and lack of systemic IL-10-producing cells when compared to mice on a control vitamin A-sufficient diet (Fig. 3A–C and Table S2). As expected, *ex vivo* polyclonal T cell activation induced similar levels of cell proliferation and IFN-γ production in control and VAD mice (Fig. S5A). Of note, although RA has been shown to promote T_{REG} differentiation[8, 9], VAD mice exhibited only a mild decrease in T_{REG} frequencies in MLN (Fig. S5B), which is in line with recent data indicating that VAD mice do not show a decrease in total T_{REG}[20].

Adoptive transfer of wt naïve T cells or gut-tropic T cells rescues OT in CCR9$^{-/-}$ and VAD mice, respectively

Next, we assessed which cell types need to express gut-homing receptors during OT induction. In addition to T cells, plasmacytoid DC (pDC) also play an important role in OT[2, 13]. Therefore, we explored whether *wt* T cells or pDC were sufficient to rescue OT in CCR9$^{-/-}$ mice. For this purpose, we adoptively transferred T cells or pDCs from non-tolerized *wt* or CCR9$^{-/-}$ donors into CCR9$^{-/-}$ recipient mice prior OT induction. Remarkably, adoptive transfer of *wt* T cells (CD45.1^{+}) (Fig. S6A) was sufficient to fully restore OT in CCR9$^{-/-}$ mice, as indicated by reduced DTH responses, decreased *ex vivo* OVA-specific T cell proliferation and increased numbers of IL-10 producing cells upon oral OVA administration as compared to CCR9$^{-/-}$ mice adoptively transferred with a similar number of CCR9$^{-/-}$ T cells (Fig. 4A–C and Fig. S6B). Of note, transfer of β7$^{-/-}$ CCR9$^{+/+}$ T cells did not rescue OT in CCR9$^{-/-}$ mice (Fig. 4D and Fig. S6C), indicating that T cells need both CCR9 and α4β7 in order to rescue OT in CCR9$^{-/-}$ mice. On the other hand, adoptive transfer of *wt* pDC from BM was not sufficient to rescue OT in CCR9$^{-/-}$ mice (Fig. S6D, E).

We then asked whether adoptive transfer of T cells could also rescue OT in VAD mice. However, gut-homing induction is abrogated in mice lacking RA[7, 21]. Therefore, to bypass the lack of gut-homing imprinting in VAD mice, we generated gut-tropic T cells *ex vivo* by activating naïve CD4 T cells from Thy1.1^{+} OT-II mice in the presence of RA[7]. Notably, *ex vivo*-generated gut-tropic OT-II CD4 T cells were sufficient to rescue OT induction in VAD mice (Fig. S7), suggesting that the most critical role of RA in OT is the induction of gut-tropic T cells, rather than other potential immune effects of RA, such as promoting T_{REG} and IgA-ASC differentiation[8, 9].

Gut-homing receptors on T_{REG} are required to rescue OT in CCR9$^{-/-}$ mice

We assessed the role of T_{REG} in our experimental system. For depleting T_{REG} we used DEREG mice, which express diphtheria toxin (DT) receptor (DTR)-GFP under the control of the Foxp3 promoter. In line with previous work[2, 3], T_{REG} depletion in DT-treated DEREG mice abrogated OT (Fig. S8A–C). As expected, DT treatment did not interfere with DTH or OT generation in *wt* (non-DEREG) mice (Fig. S8B–C). Interestingly, despite that endogenous CCR9$^{-/-}$ T_{REG} are found in CCR9$^{-/-}$ recipient mice (Fig. S8D), they were not sufficient for allowing OT induction in the absence of adoptively transferred *wt* T cells (Fig. 1), suggesting that T_{REG} need to express CCR9 in order to allow for OT induction. Consistent with this possibility, specific depletion of CCR9$^{+/+}$ T_{REG} derived from adoptively transferred DEREG CD4 T cells abolished OT rescue in CCR9$^{-/-}$ mice (Fig. 4E, F and Fig. S8E). Analogous to CCR9$^{-/-}$ mice, OT was also abolished in β7$^{-/-}$ mice. Whereas *wt* CD4 T cells partially recued OT in β7$^{-/-}$ mice, CD4 T cells from *Scurfy* mice, which lack functional Foxp3[22], did not rescue OT in these mice (Fig. S9), confirming the key role of gut-tropic T_{REG} in OT.

Previous reports suggested that naturally occurring thymus-derived T_{REG} (nT_{REG}) are not required for OT[16], whereas adaptive/induced T_{REG} (iT_{REG}) play a critical role in OT generation[23, 24]. In line with these data, CD4 T cells from DEREG mice depleted of nT_{REG} rescued OT in CCR9$^{-/-}$ mice, whereas additional T_{REG} depletion during oral OVA supplementation prevented OT rescue in CCR9$^{-/-}$ mice (Fig. S10). Thus, our data suggest that newly generated OVA-specific iT_{REG} need to express CCR9 in order to generate OT.

Gut-homing and IL-10 signaling are required for the generation of tolerogenic IL-10^{+} T_{REG}

T_{REG}[2, 23, 25] and IL-10 are critical for OT[14, 15]. Our results indicate that upon OT induction the production of IL-10 and TGF-β1 is mostly confined to Foxp3^{+}CD4^{+} T_{REG} (Fig. S11A).

Moreover, in agreement with a critical role of gut-homing during OT, anti-MAdCAM-1 treatment abrogated IL-10 production by T_{REG} (Fig. S11B). In addition, adoptive transfer of naïve CD4 T cells from IL-$10^{-/-}$ mice failed to rescue OT and did not induce OVA-specific IL-10-producing cells in CCR$9^{-/-}$ mice upon oral OVA administration (Fig. 5A). Therefore, our data suggest that gut-tropic *wt* (IL-$10^{+/+}$) CD4 T cells are required for the generation of IL-10^{+} T_{REG} in OT.

It has been proposed that IL-10 signaling is required on T_{REG} to maintain their Foxp3 expression and suppressive function[26, 27]. Interestingly, similar to IL-$10^{-/-}$ T cells, adoptive transfer of CD4 T cells from IL-10Rβ$^{-/-}$ mice did not induce OVA-specific IL-10-producing cells in CCR$9^{-/-}$ mice upon oral OVA administration (Fig. 5B), suggesting that gut-homing CD4 T cells need to be exposed to IL-10 signals in the gut to become fully tolerogenic IL-10-producing cells in OT.

Transfer of CCR$9^{-/-}$ or β$7^{-/-}$ T cells impairs OT in wt mice

Since oral antigen directs antigen-specific effector T cells to the gut mucosa by inducing α4β7 and CCR9[6], we reasoned that, analogous to clonal deletion/anergy[17], targeting T cells to the gut could be an alternative mechanism for excluding effector T cells from peripheral immune responses. Thus, we predicted that T cells deficient in gut-homing capacity might interfere with OT generation in *wt* mice. Consistent with this possibility, adoptive transfer of CCR$9^{-/-}$ or β$7^{-/-}$ T cells partially abrogated OT induction in *wt* mice, as determined by enhanced DTH responses and an increased OVA-specific T cell proliferation as compared with control *wt* mice (Fig. 6).

Discussion

The prevailing view indicates that OT relies on the induction of antigen-specific T_{REG} in the MLN, which then reach the blood and suppress pro-inflammatory responses in different tissues[2, 3, 5, 23, 25, 28]. However, although priming of naïve T cell in MLN is required for OT[4, 5], our data indicate that T cell activation in MLN is not sufficient for OT induction. This is despite the fact that T_{REG} are readily generated in MLN upon oral immunization and that MLN-DC are sufficient to induce T_{REG} *ex vivo*[9, 29, 30]. We propose that T_{REG} require a second activation step in the small intestine LP to complete their differentiation into fully tolerogenic IL-10-producing T_{REG}, which can then suppress pro-inflammatory responses (Fig. 7). While the first step in MLN involves naïve T cells and therefore does not require gut-homing receptors, we propose that the second step in the small intestine LP depends on the induction of retinoic acid (RA)-dependent[6] gut-homing receptors α4β7 and CCR9 on differentiating T_{REG}. Of note, homing to the gut is also required for further T_{REG} expansion in the lamina propria[24, 31] and our results indicate that gut-homing induction might also target effector T cells to the intestinal mucosa, making them unavailable to participate in pro-inflammatory responses at peripheral sites.

IL-10[14, 15, 31] and TGF-β[16] are required for OT induction in different models. Given that the induction of systemic antigen-specific IL-10- and TGF-β-producing cells was markedly decreased in mice with impaired gut-homing, our data suggest that T cells need to migrate to the gut to become IL-10- and/or TGF-βproducing T_{REG}. In fact, while intestinal T_{REG} secrete IL-10, spleen T_{REG} do not produce this critical tolerogenic cytokine[32], which is consistent with our hypothesis of functional T_{REG} specialization in the gut lamina propria in the setting of OT. Moreover, our results suggest that IL-10 signals on gut-tropic T_{REG} are critical for promoting the differentiation of fully tolerogenic IL-10^{+} T_{REG} during OT. Among the possible sources for IL-10 in the LP are CX3CR1^{+} macrophages[24, 26] and CD11b^{+} DC[33].

Of note, OT induction in an asthma model was recently correlated with the presence of a subset of $CCR9^+$ IL-10-producing T_{REG} in the airways, leading to the speculation that CCR9 might be required for T_{REG} homing to the airway mucosa upon OT induction[31]. However, since CCR9 has not been shown to play a role in lung homing, we propose that CCR9 expression on T_{REG} in the asthma OT setting might reflect T_{REG} priming in MLN by orally administered antigens and their subsequent differentiation in the small bowel LP into IL-10^+ T_{REG}, which then migrate to the lung mucosa to suppress airway inflammation.

Interestingly, a subset of IL-10-producing cells has been specifically found among circulating memory $CCR9^+$ CD4 T cells in healthy human volunteers, whereas memory $CCR9^{Neg}$ CD4 T cells did not produce IL-10[34], suggesting that human T cells might also need to express CCR9 in order to acquire tolerogenic IL-10-producing potential.

Other functions have been proposed for CCR9 in addition to gut-homing, such as effects on T cell apoptosis[35] and on T cell-epithelial cells interaction[36]. Nevertheless, since our data showed that the other gut-homing receptor pair α4β7-MAdCAM-1 is also critical for OT induction, we favour the hypothesis that bona fide α4β7- and CCR9-expressing gut-tropic T cells are required for OT. Moreover, given that the CCR9-CCL25 receptor pair plays a role only in small bowel homing[6], especially in the upper segments[37], we propose that T cell homing to the upper portions of the small bowel might be especially important for generating IL-10^+ T_{REG} and OT. In agreement with this possibility, previous studies showed that antigen administration in the upper intestinal segments was more effective than inoculation in the ileum or colon for inducing OT[38].

Even though RA promotes T_{REG} induction *ex vivo*[9, 29, 30], its *in vivo* role in T_{REG} differentiation remains unclear. In fact, VAD mice did not show a decrease in T_{REG} in MLN or the lamina propria[20]. Moreover, OT could be rescued in VAD mice by adoptively transferring exogenously generated gut-tropic antigen-specific CD4 T cells, suggesting that additional non gut-homing related roles for RA might not be essential during OT. Therefore, our data showing impaired OT in VAD are more likely explained by decreased gut-homing imprinting rather than by a direct effect on T_{REG} generation, which is in line with our data in $CCR9^{-/-}$ mice or in *wt* mice after blocking MAdCAM-1.

Although adoptive transfer of spleen T_{REG} from orally tolerized mice is sufficient to confer tolerance to naïve mice[39], plasmacytoid DC (pDC) have also been proposed to play a key role in OT[2, 13] and adoptive transfer of pDC from orally tolerized mice was also sufficient to tolerize naïve mice in a DTH model[13]. In fact, a subset of immature pDC also expresses CCR9[40] and CCR9 plays a role in pDC homing to the gut[41]. However, in contrast to *wt* T cells, adoptive transfer of *wt* bone marrow $CCR9^+$ pDC was not sufficient to rescue OT in $CCR9^{-/-}$ mice. Nonetheless, it is formally possible that intravenously administered pDC did not home in sufficient numbers and/or to the proper tissue compartments.

In addition to active immunosuppression by T_{REG} and clonal deletion/anergy[17], our data suggest that induction of α4β7$CCR9^+$ gut-tropic T cells is an alternative mechanism for OT by excluding effector, pro-inflammatory T cells from inducing potentially pathogenic immune responses at the peripheral sites. In fact, intestinal effector/memory T cells exhibit a very slow turnover and have a long dwell-time in the gut[42], which might act like sequestering T cells. Conversely, interfering with gut-homing imprinting would allow effector T cells to remain in the periphery, potentially interfering with OT induction. Our experiments transferring T cells genetically deficient in gut-homing receptors emulate this scenario, as those cells are not targeted to the gut mucosa even when they are primed in gut-associated lymphoid compartments. Our findings might offer a partial explanation for the difficulty to mount tolerogenic responses during ongoing inflammatory responses as

compared to prophylactic tolerogenic treatments[43]. In this setting, peripherally primed T cells, induced in the context of extra-intestinal inflammation, would not acquire gut-homing receptors and therefore would not be efficiently targeted to and eventually retained in the gut mucosa.

In summary, our findings highlight a novel and unexpected link between gut-homing imprinting and mucosal immunological tolerance. In addition to establishing a novel role for gut-homing imprinting, our data suggest a need for caution when using immunotherapies aimed at blocking gut-homing receptors during chronic intestinal inflammation[44], because of their potential to interfere with normal homeostatic regulatory mechanisms in the intestinal mucosa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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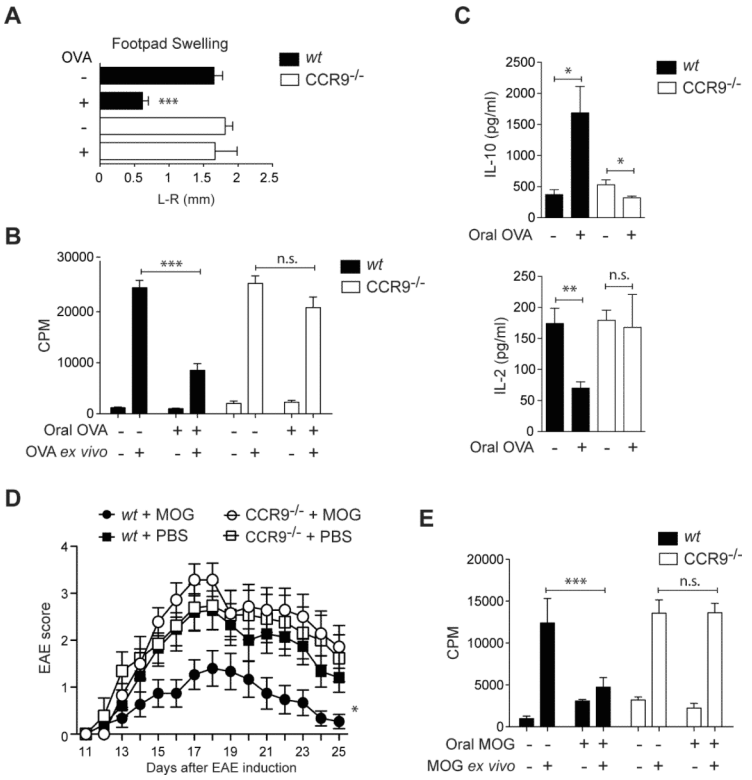

Figure 1. CCR9 is required for OT induction

(**A–C**) Wild type (*wt*) and CCR9$^{-/-}$ mice were supplemented daily with oral PBS or 2.5 mg OVA for 5 days, and then they were immunized s.c. with OVA plus CFA. After 7 days the mice were challenged with OVA in the left footpad and PBS in the right footpad. (**A**) DTH responses were measured after 24 hours (n=7–9 mice/group). (**B, C**) Total splenocytes were stimulated with 1 mg/ml OVA. (**B**) Cell proliferation was assessed after 72 hours. (**C**) Cytokines were measured in the culture supernatant after 24–48 hours. (**D, E**) *Wt* and CCR9$^{-/-}$ mice were supplemented daily with oral PBS or 0.25 mg $MOG_{35–55}$ for 5 days, then immunized s.c. with $MOG_{35–55}$ plus CFA. (**D**) EAE induction and progression was scored from days 11 to 25 (n=8–10 mice/group). (**E**) At day 16, the mice were sacrificed and total splenocytes were stimulated *ex vivo* with 20 μg/ml $MOG_{35–55}$. T cell proliferation was measured after 72 hours (n=4 mice/group). Mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, n.s.: not significant.

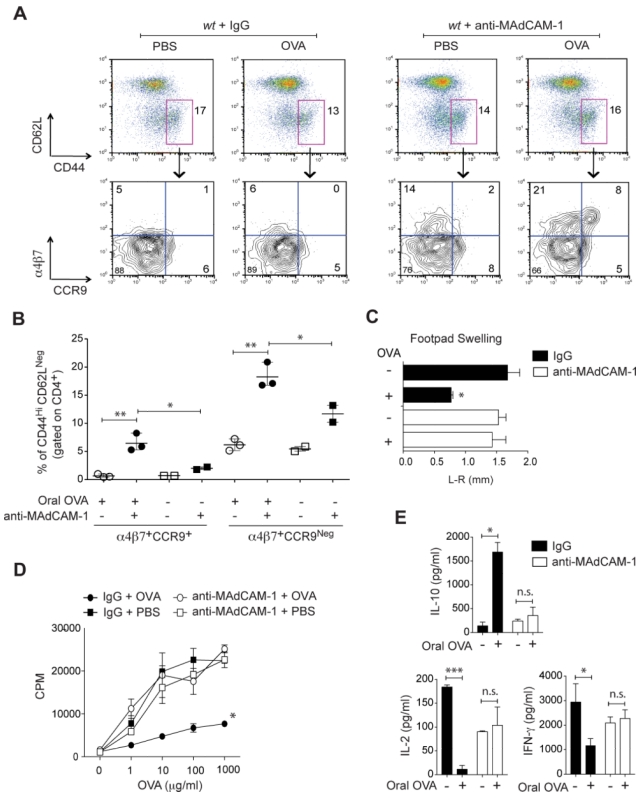

Figure 2. Blocking MAdCAM-1 abrogates OT in *wt* mice

Wt mice were treated i.p. with anti-MAdCAM-1 or control rIgG. OT and DTH were induced as described in Fig. 1. (**A**) Flow cytometry analysis of one representative mouse/group. (**B**) Frequencies of α4β7- and CCR9-expressing cells in the spleen. Cells were gated on $CD44^{Hi}CD62L^{Low}$ effector/memory $CD4^{+}$ T cells (**C**) DTH responses were measured after 24 hours (n=2–3 mice/group). (**D, E**) Total splenocytes were stimulated with the indicated OVA concentrations. (**D**) Proliferation was measured after 72 hours (n=2–3 mice/group). (**E**) Cytokines were measured in the culture supernatant after 24–48 hours. Mean ± SEM, *P<0.05, *P<0.01,***P<0.001, n.s.: not significant.

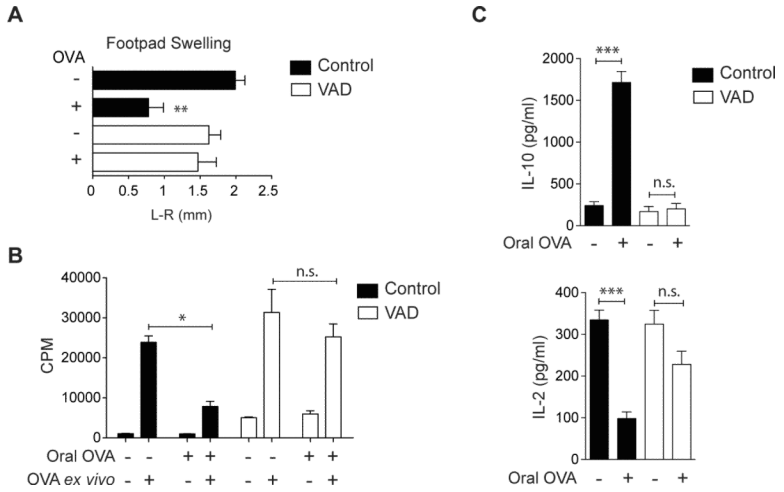

Figure 3. OT is abolished in vitamin A depleted (VAD) mice

Mice were maintained on a control or VAD diet. OT and DTH were induced as described in Fig. 1. (**A**) DTH responses (footpad swelling) were measured after 24 hours, and were expressed as the difference between the left and right footpad (L-R) (n=4 mice/group). (**B, C**) Total splenocytes were stimulated with 1 mg/ml OVA. (**B**) T cell proliferation was measured after 72 hours. (**C**) Cytokines were measured in the culture supernatant after 24–48 hours. Mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, n.s.: not significant.

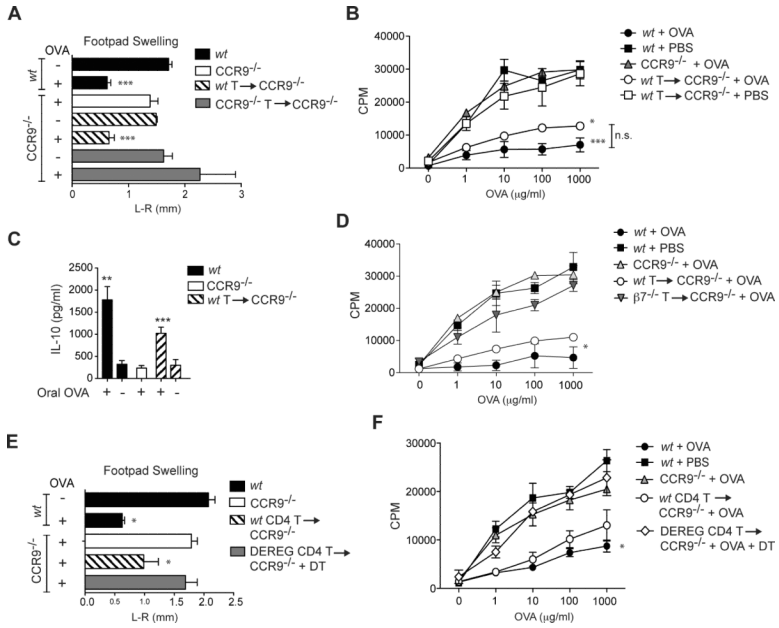

Figure 4. Adoptive transfer of *wt* T cells rescues OT in CCR9$^{-/-}$ mice

T cells from nontolerized *wt* or CCR9$^{-/-}$ mice were adoptively transferred into CCR9$^{-/-}$ recipients. OT and DTH were induced as described in Fig. 1. CCR9$^{-/-}$ mice receiving CD4^{+} T cells from DEREG mice were treated with DT every day. (**A, E**) DTH responses were measured after 24 hours (n=2–5 mice/group). (**B–D, F**) Total splenocytes were stimulated with the indicated OVA concentrations. (**B, D, F**) Proliferation was assessed after 72 hours (n=4–5 mice/group). (**C**) IL-10 was measured in the culture supernatant after 48–60 hours. Mean ± SEM, *p<0.05, ***p<0.001

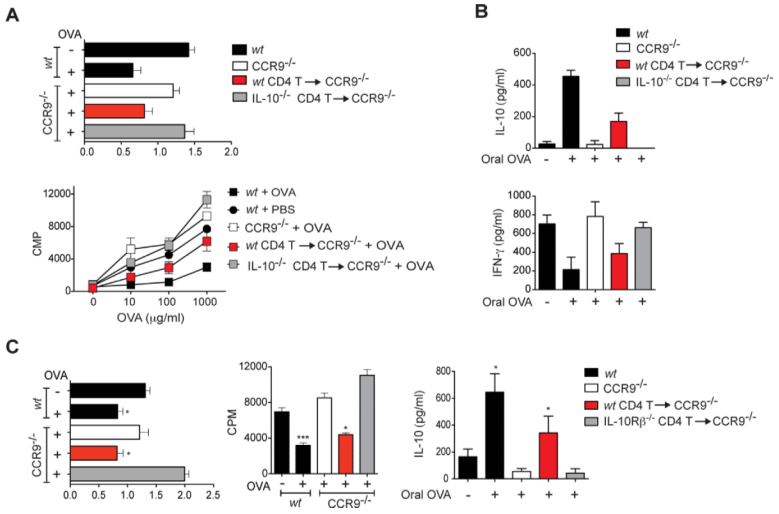

Figure 5. IL-10 signals in gut-tropic T_{REG} are critical for OT

T cells from IL-$10^{-/-}$ or IL-10Rβ$^{-/-}$ mice were adoptively transferred into CCR9$^{-/-}$ mice. OT and DTH were induced as described in Fig. 1. (**A**) Total splenocytes were stimulated with the indicated OVA concentrations and cell proliferation was measured after 72 h. (**B, C**) Cytokines were measured in the culture supernatant after 48–60 hours. n=2 mice/group, Mean ± range

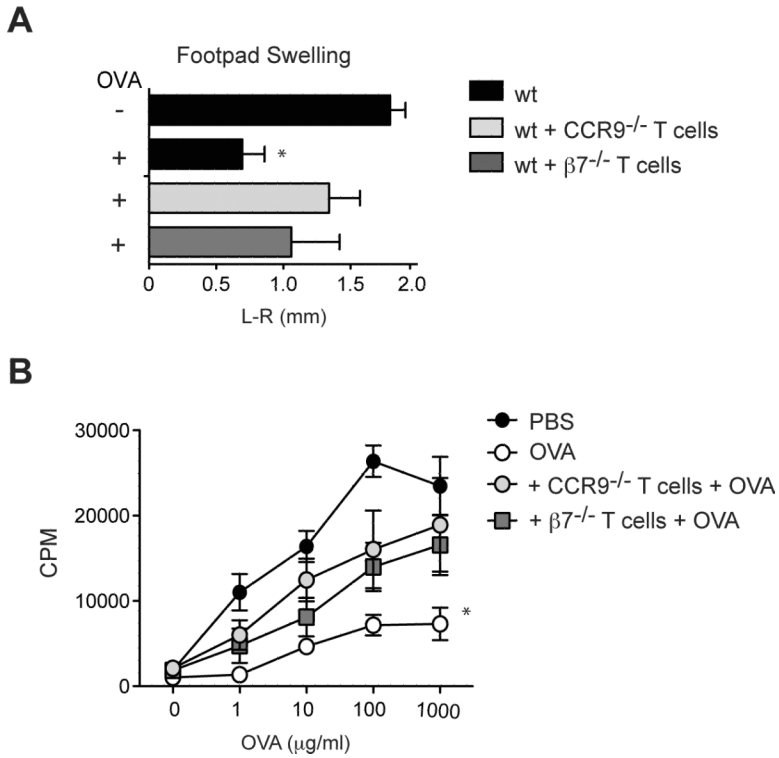

Figure 6. Transfer of CCR9$^{-/-}$ or β7$^{-/-}$ T cells impairs OT in *wt* mice

T cells from CCR9$^{-/-}$ or β7$^{-/-}$ mice were adoptively transferred into *wt* mice. OT and DTH were induced as described in Fig. 1. (**A**) DTH responses were measured after 24h (n= 2 mice/group). Groups were compared versus wild type mice receiving oral PBS. (**B**) Total splenocytes were stimulated with the indicated OVA concentrations and cell proliferation was measured after 72h (n= 2 mice/group). Mean ± SEM, *p<0.05 (ANOVA)

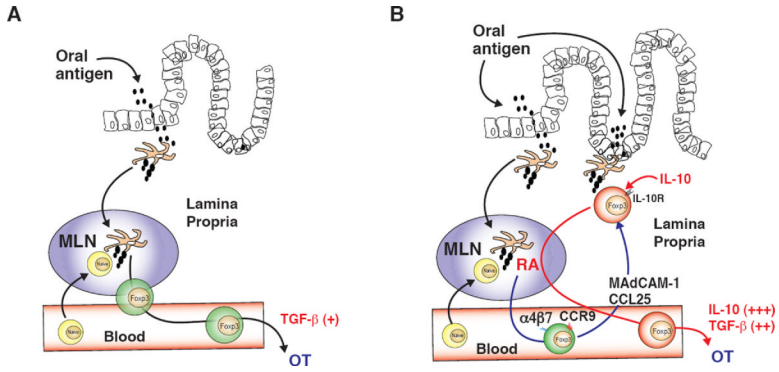

Figure 7. Gut-homing-dependent model for OT

(**A**) The prevailing view indicates that OT relies on the induction of antigen-specific Foxp3$^+$ regulatory T cells (T_{REG}) in the mesenteric lymph nodes (MLN), which then reach the blood and suppress pro-inflammatory responses in different tissues. (**B**) In our new model we propose that T_{REG} require a second activation step in the intestinal lamina propria (LP) to complete their differentiation into fully tolerogenic IL-10-producing T_{REG}, which can then suppress pro-inflammatory responses. While the first step in MLN involves naïve T cells and therefore does not require gut-homing receptors, the second step in the gut LP depends on the induction of retinoic acid (RA)-dependent gut-homing receptors α4β7 and CCR9 on activated T_{REG} and their respective ligands MAdCAM-1 and CCL25 on LP endothelial cells. In the LP T_{REG} are re-stimulated and proliferate in response to oral antigen and they are also exposed to IL-10, which is required to confer T_{REG} with IL-10-producing capacity, a critical property for OT generation. After that, IL-10$^+$ T_{REG} return to the blood and exert their tolerogenic activity in different organs.