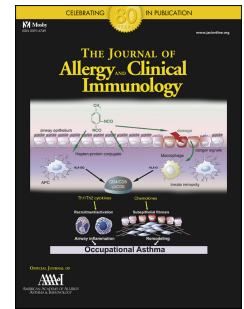


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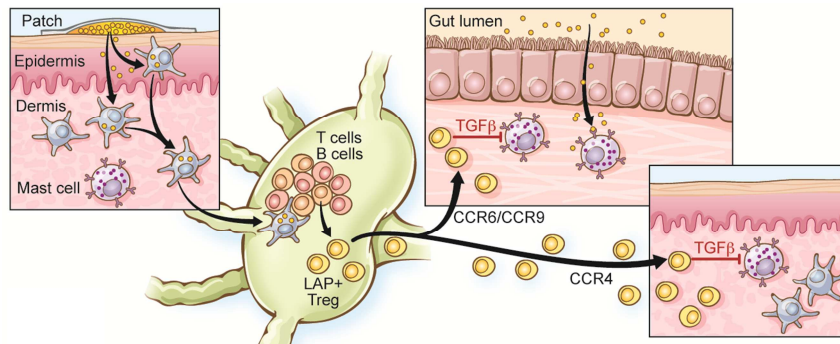
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Epicutaneous immunotherapy induces gastrointestinal LAP+ Tregs and prevents food-induced anaphylaxis

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Abstract

Background: The attempt to induce oral tolerance as a treatment for food allergy has been hampered by a lack of sustained clinical protection. Immunotherapy by non-oral routes, such as the skin, may be more effective for the development of maintained tolerance to food allergens.

Objective: To determine the efficacy and mechanism of tolerance induced by epicutaneous immunotherapy in a model of food-induced anaphylaxis.

Methods: C3H/HeJ mice were sensitized to ovalbumin (OVA) orally or through the skin and treated with EPIT using OVA-Viaskin® patches or OIT using OVA. Mice were orally challenged with OVA to induce anaphylaxis. Antigen-specific Treg induction was assessed by flow cytometry using a transgenic T cell transfer model.

Results: Using an adjuvant-free model of food allergy generated by epicutaneous sensitization and reactions triggered by oral allergen challenge, we found that epicutaneous immunotherapy induced sustained protection against anaphylaxis. We show that the gastrointestinal tract is deficient in *de novo* generation of Tregs in allergic mice. This defect was tissue-specific, and epicutaneous application of antigen generated a population of gastrointestinal-homing LAP⁺Foxp3⁺ Tregs. The mechanism of protection was found to be a novel pathway of direct TGF- β -dependent Treg suppression of mast cell activation, in the absence of modulation of T or B cell responses.

Conclusions: Our data highlights the immune communication between skin and gastrointestinal tract, and identifies novel mechanisms by which epicutaneous tolerance can suppress food-induced anaphylaxis.

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Key Messages:

- Epicutaneous immunotherapy protects against food-induced anaphylaxis independent of the initial route of sensitization
- Treg generation by the oral route is impaired in allergic mice, while epicutaneous antigen delivery induces gut-homing LAP⁺ Foxp3⁺ Tregs
- EPIT prevents food-induced anaphylaxis by a TGF β -dependent mechanism
- Tregs directly suppress mast cell activation in response to a food challenge

Capsule summary: Application of food allergen to intact skin, but not the gastrointestinal tract, of allergic mice generates gastrointestinal-homing LAP⁺ Tregs that directly suppress mast cell activation and protect from food-induced anaphylaxis by a TGF β -dependent mechanism.

Key words: Epicutaneous immunotherapy, oral immunotherapy, food allergy, regulatory T cells, mast cells.

Abbreviations: Epicutaneous immunotherapy (EPIT); Latency-associated peptide (LAP); Mesenteric lymph node (MLN); Oral immunotherapy (OIT).

Introduction

Food allergies are increasing in Western countries and are currently estimated to affect 3-6% of the population¹. There is no cure, and the standard of care is allergen avoidance, which is difficult to maintain and accidental exposures leading to reactions are a common experience for those with food allergies². Subcutaneous allergen immunotherapy has been extensively used for venom allergy and some respiratory allergies. However, its use in food allergy has not been pursued due to the high risk of anaphylactic shock³. Allergen immunotherapy delivered by the oral route (oral immunotherapy, OIT) has been the focus of several recent trials^{4,5}. It was thought that the oral route, in addition to being safer than subcutaneous immunotherapy, would facilitate sustained immune tolerance due to the regulatory tone of the mucosal immune system. While OIT successfully induces a desensitized state, defined as protection while on therapy, permanent tolerance induction after OIT has been more elusive^{4,6-8}.

Oral tolerance is a state of systemic unresponsiveness induced by antigens delivered by oral route. It is an active process mediated by antigen-specific regulatory T cells^{9,10}. The therapeutic potential of oral tolerance has been studied for multiple sclerosis¹¹, arthritis¹², diabetes¹³ and colitis¹⁴. The translation of oral tolerance from prevention to treatment strategy has been limited, suggesting that prior immunity may impair the development of regulatory responses. This is consistent with the lack of sustained tolerance in response to OIT in human trials^{4,6,8} and in mouse models of OIT^{15,16}.

We hypothesized that an altered gastrointestinal immune milieu in food allergy was preventing the generation of Tregs, and that provision of antigen by an alternative route could bypass this defective regulatory response. The skin is a highly active immune site

capable of generating tolerance or immunity. We investigated the epicutaneous route through the use of Viaskin[®] patches that have been shown to suppress inflammation in experimental models of asthma and eosinophilic esophagitis through the generation of regulatory T cells ¹⁷⁻²¹. We show for the first time that the epicutaneous route of antigen delivery protected mice from food-induced anaphylaxis and supported the selective expansion of a population of unique gut-homing LAP⁺ Tregs. These Tregs did not function by suppressing IgE antibodies, but instead directly suppressed mast cell activation, leading to sustained clinical protection against food-induced anaphylaxis. These data show that the unique immune communication between skin and gastrointestinal tract can be used to generate long term tolerance in food allergy.

Methods***Mice***

C3H/HeJ and Balb/c mice were obtained from Jackson Laboratories (Bar Harbor, ME) and NCI (Frederick, MD), respectively. DO11.10 mice (Jackson Laboratories) were maintained as breeding colonies at Mount Sinai. All procedures were approved by the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee.

Sensitization of mice

Mice were sensitized once a week for 6 weeks with ovalbumin (OVA, grade V; Sigma, St Louis, MO) or peanut by epicutaneous or oral exposure. For skin sensitization, abdominal fur was removed with depilatory cream (Veet; Reckitt Benckiser, Parsippany, NJ), immediately followed by application of 100ug OVA or 1mg of peanut extract in 50ul of PBS spread on the skin to dry. Epicutaneous sensitization was performed in the absence of adjuvant or tape stripping. Mice were orally sensitized with 1mg of OVA or 10mg of ground peanut + 10ug of cholera toxin (CT) (List Biologicals, Campbell, CA) by gavage. Mice were passively sensitized by injection with 100ul of pooled serum from actively sensitized mice. Oral challenge was performed as previously described¹⁵. See Supplemental Materials for additional information.

Immunotherapy treatment

After sensitization, mice received epicutaneous or oral immunotherapy. Epicutaneous immunotherapy was performed using EDS Viaskin[®] patches (DBV Technologies, Paris, France) loaded with 100ug OVA, as previously described¹⁸. Briefly, mice were anesthetized, the back shaved with an electric clipper, depilatory cream applied,

and 24h later the patch was placed on the back for 48h. This was repeated once per week for 8 weeks. Oral immunotherapy was performed by the administration of 1mg of OVA per day, in drinking water, daily, for 8 weeks.

Adoptive cell transfer experiments

CD4⁺ T cells from DO11.10 mice were purified by negative selection (StemCell Technologies, Vancouver, British Columbia, Canada), labeled with CFSE (Invitrogen, Life Technologies, Grand Island, NY, USA) and 3-5 x 10⁶ CD4⁺ T cells transferred i.v. into naïve, OVA-sensitized, or peanut-sensitized Balb/c recipients. Twenty-four hours later, mice were exposed to OVA by OVA-Viaskin[®] for 48h, or by daily gavage for 5 days. One week after first exposure, tissues were harvested for assessment by flow cytometry.

TGF β blockade

EPIT-treated mice were injected intraperitoneally with neutralizing anti-TGF β (1D11) (1mg) or isotype control (both from BioXCell, West Lebanon, NH) 24h before challenge.

In vivo Treg transfer

LAP⁺CD25⁻ or LAP⁻CD25⁻ T cells were sorted from DO11.10 mice and transferred (10⁵ cells/mouse) into passively sensitized Balb/c mice. After 24h, recipient mice were orally challenged with OVA. Symptoms were monitored and blood samples were obtained 30 min after challenge. In some experiments, 1mg of anti-TGF β antibody was injected at the same time as the cells.

161

162 ***Serum MCPT-7 ELISA***

163 For detection of MCPT-7, serum was incubated on plates covered with anti-
164 mTryptase β -1/MCPT7 (R&D Systems, Minneapolis, MN), followed by detection with
165 biotinylated anti-mTryptase β -1 (R&D systems), HRP-labeled avidin (eBioscience, San
166 Diego, CA) and TMB substrate (eBioscience).

167

168 ***Statistics***

169 Differences between groups were analyzed by Mann-Whitney U test, ANOVA or
170 Kruskal-Wallis test followed by post-hoc analysis with the Dunn's, Sidak's or Tukey's
171 multiple comparisons test or by multiple t test with Sidak-Bonferroni method for
172 correction of multiple comparisons when appropriate. Data analysis was done by using
173 Prism software (GraphPad, San Diego, CA). Results are expressed as mean $\pm$ SEM. A value
174 of $p < 0.05$ was considered significant.

175

Results*Epicutaneous immunotherapy induces sustained clinical protection in a model of food-induced anaphylaxis*

A lack of permanent tolerance to foods after OIT has been described in both mouse and human studies. We studied the outcome of EPIT and OIT in a model of oral OVA-induced anaphylaxis. To determine if the route of primary sensitization was a factor in the outcome of immunotherapy, we used mice that had been sensitized to OVA through either epicutaneous or oral routes, followed by 8 weeks of EPIT or OIT (Fig 1, A). At the end of the treatment, mice were orally challenged with OVA and anaphylaxis measured by drop in body temperature. Mice sensitized by the epicutaneous route developed anaphylaxis upon oral challenge with OVA, while mice subjected to EPIT or OIT were completely protected (Fig 1, B). When mice were challenged again to test sustained protection after 4 weeks without treatment, mice treated with EPIT were still significantly protected against anaphylaxis while mice treated with OIT regained clinical reactivity. However, there was no statistical difference between OIT and EPIT treated groups at the 12 week time-point. In mice orally sensitized to OVA using cholera toxin adjuvant, mice treated with EPIT were completely protected from anaphylaxis (Fig 1, C) but mice treated with OIT were not protected. However, 4 weeks later, even EPIT-treated mice lost protection against anaphylaxis (Fig 1, C). We hypothesized that this difference in outcome between mice sensitized by the oral or epicutaneous routes could be due to exogenous adjuvant (cholera toxin) needed during oral sensitization. To test that, mice were sensitized by the epicutaneous route using cholera toxin adjuvant and then subjected to EPIT. Similar to the response to EPIT in orally sensitized mice, mice sensitized by the epicutaneous route with exogenous adjuvant were protected from anaphylaxis while on EPIT therapy but not after

200 termination of EPIT (Fig E1). Our results show that independent of the primary route of
201 sensitization, EPIT led to a robust clinical protection against food-induced anaphylaxis.

202 Blood samples were taken to quantify specific immunoglobulins and basophil
203 activation. There was a significant increase in OVA-specific IgG1 and IgG2a after 8 weeks of
204 EPIT (Fig 2, A), that persisted in skin-sensitized mice at week 12. IgE levels also increased
205 during the treatment but remained stable after 12 weeks. Despite these changes in
206 immunoglobulin levels, functional assays did not support a role for antibodies in clinical
207 protection after EPIT. Basophil activation tests showed no significant difference between
208 untreated and EPIT-treated mice (Fig 2, B). Secondly, passive sensitization of naïve mice
209 with sera from untreated or EPIT-treated mice generated similar degrees of anaphylaxis
210 after recipient mice were orally challenged (Fig 2, C). These results suggest that although
211 EPIT modified the humoral response to OVA, the protection against anaphylaxis was likely
212 mediated by a mechanism other than blocking antibodies.

213 214 *Expansion of Tregs by oral antigen is impaired in allergic mice*

215 We hypothesized that the altered gut immune environment in food allergy is poorly
216 tolerogenic and the lack of maintained clinical tolerance after oral immunotherapy is
217 related with defect in Treg generation in the gut when antigen is applied orally. To study
218 the response to immunotherapy of antigen-specific Tregs in the gastrointestinal tract of
219 allergic mice, we used an adoptive transfer model. CFSE-labeled CD4⁺ T cells from DO11.10
220 transgenic mice (recognizing the 323-339 epitope of the egg allergen ovalbumin (OVA))
221 were transferred to naïve or OVA-sensitized recipient mice prior to repeated low-dose
222 feeding with OVA (Fig 3, A). We quantified the expansion of Foxp3⁺ cells and LAP⁺ cells

within proliferating cells (Fig 3, B). After 5 days of oral antigen, there was an expansion of both Foxp3⁺ and LAP⁺ antigen-specific Treg populations in MLN of naïve mice (Fig 3, C), consistent with induction of a tolerance response. LAP⁺ T cells in the proliferating compartment did not express CD25, while non-transgenic LAP⁺ T cells found within the MLN were found to be either negative or positive for CD25. We confirmed in suppression assays that LAP⁺ T cells had regulatory activity similar to LAP-CD25⁺ Tregs independent of the expression of CD25 (Fig E2). The expansion of Tregs after oral antigen was observed in MLN but not in distal sites such as spleen (Fig 3, D). By contrast, in mice that were OVA-sensitized prior to T cell transfer and OVA feeding, the expansion of Foxp3⁺ and LAP⁺ Tregs was completely abrogated (Fig 3, C). The defect in Treg generation after oral antigen feeding was observed in mice sensitized by either the oral or the epicutaneous routes and therefore is a feature of the allergic state. When mice were sensitized to a bystander antigen (peanut), the generation of OVA-specific Tregs was not impaired, demonstrating the antigen specificity of this Treg defect (Fig 3, E).

Epicutaneous immunotherapy expands LAP⁺ Tregs in the gastrointestinal tract of allergic mice

Based on the results of the anaphylaxis model, we hypothesized that antigen delivered via the skin could induce Tregs in sensitized mice. We first examined the induction of antigen-specific Tregs in the skin-draining lymph nodes. There was an increase in proliferating LAP⁺ but not Foxp3⁺ Tregs in comparison with untreated mice in brachial lymph nodes 1 week after applying antigen (Fig 4, B). As with oral exposure, there was no selective expansion of Tregs in the spleen after epicutaneous OVA. Unexpectedly,

there was a significant expansion of LAP⁺ Tregs in the MLN of mice after epicutaneous OVA (Fig 4, B). Furthermore, this expansion of LAP⁺ Tregs in the MLN was not impaired in mice sensitized by either the oral or skin routes (Fig 4, C). There was also an increased frequency of DO11.10 cells in the small intestinal lamina propria after epicutaneous OVA, which were highly enriched in LAP⁺ Tregs (Fig 4, D). This was selective to the gastrointestinal tissues, as other non-draining lymph nodes (inguinal lymph nodes) demonstrated no expansion of LAP⁺ Tregs (data not shown). These data demonstrate that epicutaneous antigen exposure can result in the appearance of Tregs in the gastrointestinal tract, and this is observed in naïve mice and also allergic mice that have impaired Treg generation in response to oral antigen exposure.

LAP⁺ T cells are primed for gut homing in skin-draining lymph nodes

Tissue homing normally reflects the site of priming, such that T cells primed in skin-draining lymph nodes home back to the skin, and T cells primed in the mesenteric lymph nodes home back to the lamina propria. We therefore asked how epicutaneous antigen could induce LAP⁺ T cells to appear selectively in the MLN. We first examined kinetics of cell proliferation at different sites. Two days after patch placement only brachial lymph nodes showed evidence of T cell proliferation (Fig 5, A). After 3 days, CFSE-low cells began to appear in inguinal and mesenteric lymph nodes and in the small intestinal lamina propria. Mice that had received DO11.10 T cells but did not received antigen stimulation had a low frequency of CFSE-high cells in the absence of CFSE-low cells at each of the sites examined. To determine if the appearance of CFSE-low T cells at distal sites was due to migration of cells primed in brachial lymph nodes or due to transport of antigen to distal

sites, we treated mice with FTY720, a sphingosine-1-phosphate receptor agonist that prevents exit of T cells from lymph nodes. Cells were recovered 3 days after patch application. The percentage of CFSE-low cells was decreased by FTY720 treatment in inguinal and mesenteric lymph nodes and the small intestinal lamina propria (Fig 5, B and 5, C), while proliferation of cells in the brachial lymph node was unaffected. These data indicate that T cells are primed with antigen within the brachial lymph node and subsequently migrate to gastrointestinal tissues.

We next studied the expression of tissue-homing markers by T cells primed in skin-draining lymph nodes. After epicutaneous OVA exposure, there was a high expression of the skin-homing marker CCR4²² in all CFSE-low cells and also some induction of the pan-mucosal homing marker CCR6²³, while the gut-homing marker CCR9²⁴ was only slightly increased and $\alpha 4\beta 7$ ²⁵ was not expressed (Fig 6, A). By contrast, during oral OVA exposure, proliferating cells in MLN showed high levels of CCR9 and $\alpha 4\beta 7$. Focusing on the expression of tissue-homing markers in different subsets of T cells, we observed that more than 80% of LAP⁺Foxp3⁻ T cells expressed CCR9 and more than 50% expressed CCR6 in skin-draining lymph nodes after epicutaneous OVA (Figure 6, B and 6, C). This was cell-type specific, as neither Foxp3⁺ nor non-Tregs expressed CCR6 or CCR9 after epicutaneous antigen exposure. LAP⁺ cells did not express the gut-homing marker $\alpha 4\beta 7$. All of the different subsets of T cells expressed high levels of CCR4, which is in agreement with priming through the skin. This data shows that there was a unique imprinting of gut homing capacity upon the LAP⁺ Treg subset.

The surface phenotype of LAP⁺ cells was more similar to non-Tregs than to the Foxp3⁺ Treg population. There was no expression of conventional Treg markers such as

GITR, CD25 and CD103, and lower levels of ICOS than seen in the Foxp3⁺ population (Figure 6, D). The LAP⁺ cells expressed intermediate levels of CTLA-4. The different populations showed similar levels of CD69, CD44 and PD1, and did not express granzyme B.

Clinical protection induced by EPIT occurs through a TGFβ-dependent mechanism

We hypothesized that Tregs induced during EPIT could be acting directly on allergic effector cells to suppress hypersensitivity reactions during anaphylaxis without affecting T and B cell responses. Because TGFβ has been related to the suppression capacity of LAP⁺ T cells, mice treated with EPIT were injected with neutralizing anti-TGFβ antibody or isotype control 24h before challenge (Fig 7, A). Acute suppression of TGFβ completely abrogated the protection induced by EPIT (Fig 7, B). The acute time frame of this neutralization would be too short for modification of B cell responses, and supports the hypothesis that Tregs may directly suppress mast cells. To test this hypothesis, OVA-specific DO11.10 LAP⁺ Tregs or non-Tregs as control were transferred into Balb/c mice that had been passively sensitized with serum containing high levels of OVA-specific IgE. The next day, mice were orally challenged with OVA and the mast cell protease MCPT-7 was measured in sera obtained 30min after challenge (Fig 7, C). Passively sensitized mice responded to oral OVA challenge with an increase in circulating MCPT-7 (Fig 7, D). Mice receiving OVA-specific LAP⁺ cells, but not non-Tregs (LAP⁻,CD25⁻), had decreased MCPT-7 in the serum after oral challenge as well as a trend toward reduced onset of allergic symptoms (7/10 untreated mice versus 2 of 6 mice receiving LAP⁺ Tregs). Transfer of LAP⁺ Tregs without antigen specificity (purified from naive Balb/c mice) did not suppress mast cell activation *in vivo* (data not shown). When mice were injected with anti-TGFβ antibody at the moment of the

transfer, the levels of MCPT-7 were partially restored (Fig 7, E), indicating that the suppression of mast cell activation by Tregs is dependent, at least in part, on TGF β . Although LAP⁺ Tregs showed the potential to release IL-10 (Fig E3, A), degranulation of bone-marrow derived mast cells stimulated with IL-10 for 24h was in fact enhanced, while TGF β suppressed degranulation (Fig E3, B). In summary, we show that antigen-specific LAP⁺ Tregs are induced by epicutaneous immunotherapy, and can directly suppress mast cell activation and downstream type-I hypersensitivity reactions.

Discussion

Oral tolerance is a state of antigen-specific systemic unresponsiveness that is mediated by Tregs educated in the mesenteric lymph nodes by CD103⁺ DCs ²⁶. To restore immune tolerance in food-allergic patients, immunotherapy given through the oral route has emerged as a promising treatment ^{4,5}. Although desensitization, defined as protection from reactions while on therapy, has been achieved in the majority of subjects treated with OIT, a lack of permanent tolerance and recurrence of reactions to foods has been found after OIT is discontinued ^{4,6,8}. Our data suggest that an impaired generation of Tregs in the food allergic gastrointestinal tract underlies this resistance to oral tolerance induction, and we identify skin-gut immune communication as a novel means to induce tolerance. Previous studies have documented the efficacy of this approach in suppression of allergic inflammation¹⁷⁻²¹, and for the first time we demonstrate efficacy in food-induced systemic anaphylaxis.

Allergen-specific immunotherapy has been described as a sequential response, with an early decrease in mast cell and basophil activity associated with a rise in allergen-specific IgG4 antibodies, and a subsequent generation of allergen-specific Tregs that is essential for the development of sustained tolerance. Treg development is believed to be necessary to suppress Th2 responses, and reduce allergen specific IgE and effector cell activation ²⁷. Our data show that the intestine of food allergic mice is not capable of supporting Treg generation in response to fed antigens. This is in agreement with recently reported results ¹⁶, and provides an explanation for the lack of sustained efficacy of OIT in the treatment of food allergy in humans ²⁸ and mice ¹⁵. Our data show that this Treg defect is limited to the sensitizing allergen, which may be due to the suppressive effect of mast cell activation on Treg generation ¹⁶. Mast cell degranulation can induce maturation and

migration of DCs to lymph nodes ²⁹ and mast cell activators have been shown to function as adjuvants ³⁰. Our data show that this defect in Treg generation was tissue-specific, and the skin could support Treg generation in sensitized mice. This Treg generation in skin was paralleled by a trend of increased efficacy of epicutaneous immunotherapy compared to oral immunotherapy in preventing anaphylaxis. Therefore site of immunotherapy is a critical factor in clinical efficacy.

Oral tolerance is mediated by antigen-specific Foxp3⁺ Tregs induced in the periphery ⁹. Th3 regulatory cells, characterized by the expression of LAP on their surface, were also originally described as key players in the development of oral tolerance. Feeding of self-antigens including myelin basic protein induces an expansion of Th3 cells in mice and humans ^{11,31}. These previously described Th3 cells are consistent with the phenotype of gastrointestinal T cells induced by epicutaneous antigen exposure in our study. Th3 cells induced by feeding can suppress experimental models of colitis, systemic lupus erythematosus, multiple sclerosis and diabetes ³²⁻³⁶. Although not specifically termed Th3 cells, LAP⁺Foxp3⁻ cells induced by inhaled antigen can suppress allergic eosinophilia in the lung ³⁷. In humans, Th3 cells have been found in blood ³⁸ and in tumors, with higher immunosuppressive activity than Foxp3⁺ Tregs ^{39,40}. In addition, children with food allergy have reduced numbers of Th3 cells in the duodenal mucosa ^{41,42}. The suppressive effects of Th3 cells are mediated by secreted TGFβ and IL10 ^{34,38,40} and also by contact through membrane-bound TGFβ1 ⁴³. The LAP⁺Foxp3⁻ cells have been described as CD69⁺CTLA-4⁺, with variable reported CD25 expression between studies, possibly indicating differences in the level of activation ^{34,37,38,40,43-45}. In our study, we observed that LAP⁺Foxp3⁻ cells induced by epicutaneous antigen were CD69⁺CD25⁻CTLA-4^{low}. However, they did not

express markers associated with Foxp3⁺ Tregs, such as GITR or CD103. We confirmed the regulatory role of these LAP⁺ Tregs in standard suppression assays, and more relevant to food allergy, showed that they could directly suppress mast cell activation triggered by oral food challenge. Our results emphasize a key role of this overlooked population of Tregs in the protection against food allergy and anaphylaxis.

The selective induction of gastrointestinal homing of LAP⁺ Tregs by epicutaneous antigen was a novel and unexpected finding. The gastrointestinal tract and skin provide tissue-specific T cell imprinting, such that T cells home back to the initial site of priming^{46, 47, 48, 49}. When we examined antigen-specific proliferating T cells as a whole, these paradigms were true. However, when LAP⁺ T cells were examined in skin-draining lymph nodes, CCR9 and CCR6 were highly expressed on LAP⁺ T cells together with CCR4, conferring a phenotype capable of homing to multiple tissues including the gastrointestinal tract. This was unique to this population and not observed on Foxp3⁺ Tregs or non-Tregs. Intranasal vaccination has also been described to elicit migration of antigen-specific T cells to the MLN that can protect against intestinal pathogens^{50, 51}, indicating that tissue-specific imprinting is a flexible and cell-type specific process. Understanding how to manipulate this homing potential has major implications for vaccine and immunotherapy design.

The mechanism by which immunotherapy leads to clinical protection has primarily been thought to be due to changes in the antibody repertoire, with sustained protection requiring a loss of allergen-specific IgE. However, in human immunotherapy trials allergen-specific IgE and basophil activation have not been able to discriminate between those with transient desensitization versus sustained tolerance^{4, 8}. Regulatory T cells are thought to participate in this process by suppressing Th2 responses necessary for the maintenance of

IgE. The suppression of Th2 responses would also be expected to reduce local tissue inflammation. EPIT has previously been described to suppress airway hyperreactivity and allergic esophago-gastro-enteropathy¹⁷⁻¹⁹ through the generation of CD25⁺ Tregs²¹. We show that TGF β is critical for protection against anaphylaxis, but through a novel mechanism involving a direct suppression of mast cell activation by Tregs rather than through modulation of antibody levels. It has been reported that CD25⁺ Tregs can inhibit mast cell degranulation directly through a contact-dependent manner⁵² and that transfer of Tregs can suppress anaphylaxis⁵³. *In vitro* studies identify the suppressive mechanisms as OX40-OX40L interactions⁵⁴ and soluble and surface-bound TGF β ^{53,55,56}. In addition, CD25⁺ Tregs reduce Fc ϵ RI surface expression on mast cells, reducing antigen sensitivity⁵². Our *in vitro* studies support a role for TGF β , but not IL-10, in suppression of mast cells. However, neutralization experiments *in vivo* suggest additional mechanisms beyond TGF β contribute to mast cell suppression. Although our data focus on LAP⁺ Tregs due to our finding that they are the main Treg cells induced early in EPIT, our data do not rule out a role for Foxp3⁺CD25⁺ Tregs in clinical protection. Neutralization of TGF β would also be expected to suppress the activity of Foxp3⁺ induced Tregs. Th3 cells promote the development of Foxp3⁺ Tregs⁵⁷ and therefore there may be a contribution by both Treg subsets after prolonged immunotherapy.

Our data show for the first time that epicutaneous immunotherapy generates gut-homing antigen-specific LAP⁺ Tregs that can directly suppress systemic anaphylaxis without upstream modification of humoral or cellular immunity. This changes the paradigm by which Tregs are thought to contribute to tolerance to allergens. Optimization of antigen delivery and Treg generation by the epicutaneous route may be the most

416 effective means for the generation a safe and effective treatment for food allergy leading to
417 sustained immune tolerance.

418

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Figure Legends

Figure 1. EPIT induces sustained protection against food-induced anaphylaxis. (A) Experimental schematic. (B) Oral challenge with OVA in skin-sensitized mice at weeks 8 and 12. Body temperature 30 min after challenge is shown. (C) Oral challenge with OVA in orally-sensitized mice at weeks 8 and 12. Data are individual mice from 2 independent experiments. x = death. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2. OVA-specific IgG1 and IgG2a generated during EPIT do not modify basophil reactivity or protect against anaphylaxis. (A) IgE, IgG1 and IgG2a levels in serum prior to oral challenge from skin- or orally-sensitized mice at week 8 and 12 after EPIT or OIT. Data are mean $\pm$ SEM. (B) OVA-induced basophil activation measured by upregulation of CD200R. (C) Oral challenge of mice were passively sensitized with sera from naïve, untreated or EPIT-treated mice. Data show individual mice from 2 independent experiments. * $p < 0.05$ vs untreated mice.

Figure 3. Impact of sensitization on Treg generation by oral antigen. (A) Experimental schematic. (B) Gating strategy of OVA-specific Tregs. (C,D) Foxp3⁺ and LAP⁺ T cells generated in MLN (C) and spleen (D) of mice sensitized to OVA by the oral or skin route versus naïve. (E) Foxp3⁺ and LAP⁺ T cells in MLN from naïve, OVA-sensitized mice or peanut-sensitized mice. Data are mean $\pm$ SEM of at least 5 mice/group in 2 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4. Epicutaneous antigen expands LAP⁺ T cells in skin and gastrointestinal lymph nodes. (A) Experimental schematic. (B) Foxp3⁺ and LAP⁺ T cells in skin-draining lymph nodes, MLN and spleen of naïve mice. (C) Foxp3⁺ and LAP⁺ T cells in MLN of mice sensitized by the oral or skin route versus naive. (D) OVA-specific cells in lamina propria after EPIT. Data are mean + SEM of at least 6 mice/group (3 mice/group for lamina propria) in two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 5. T cells are primed by epicutaneous antigen in brachial lymph nodes prior to migration to other tissues. (A) Proliferation of DO11.10 T cells recovered from brachial, inguinal and mesenteric lymph nodes and lamina propria 48h and 72h after application of antigen. (B) Proliferation of DO11.10 T cells in mice treated with FTY720. (C) Summary data showing the impact of FTY720 on proliferation at different sites. Data are mean + SEM of 3 mice/group.

Figure 6. LAP⁺ T cells express CCR9 and CCR6 after activation in skin-draining lymph nodes. (A) Expression of tissue-homing markers in DO11.10 T cells from brachial or mesenteric lymph nodes after epicutaneous or oral antigen, respectively. (B) Representative plots of the expression of tissue-homing markers by T cell subsets activated by epicutaneous antigen. (C) Summary data of chemokine receptor expression in sub-populations. Data are mean + SEM of 5 mice/group in 2 independent experiments. (D) Representative plots showing Treg surface markers. Minus control is staining control.

Figure 7. Tregs can directly suppress mast cell activation. (A) Experimental schematic. (B) Temperature measured 30 min after oral OVA challenge. (C) Experimental schematic. (D) MCPT-7 levels in serum obtained 30 min after challenge. (E) Levels of MCPT-7 in serum from mice injected with anti-TGF β or isotype control calculated as % with respect to untreated mice.. Data are mean $\pm$ SEM of at least 6 mice/group in 3 independent experiments. * $p < 0.05$, ** $p < 0.01$.

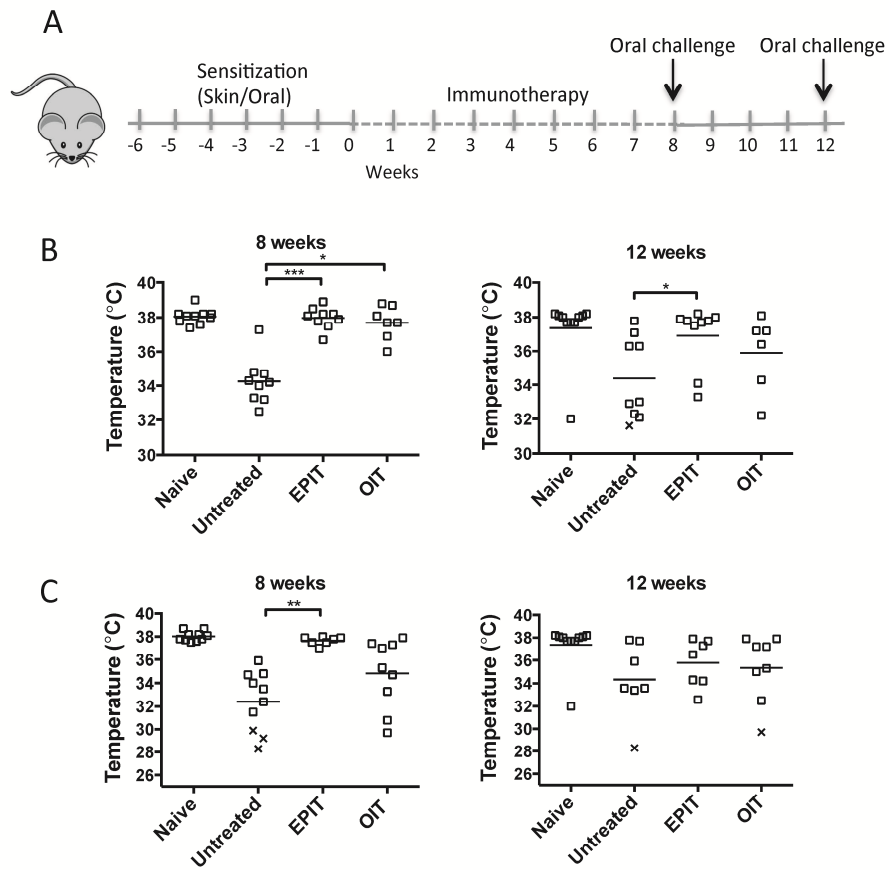


Figure 1

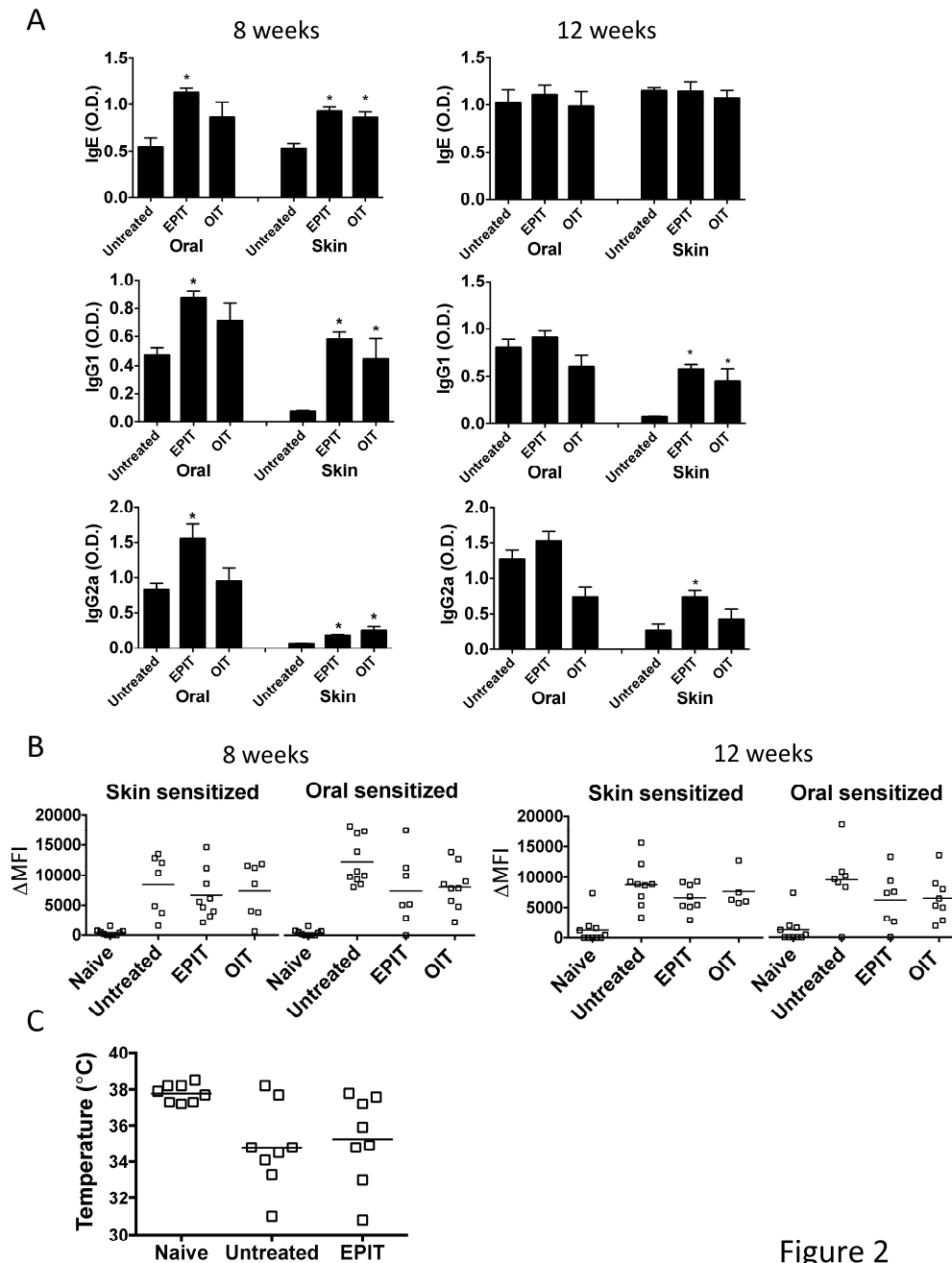


Figure 2

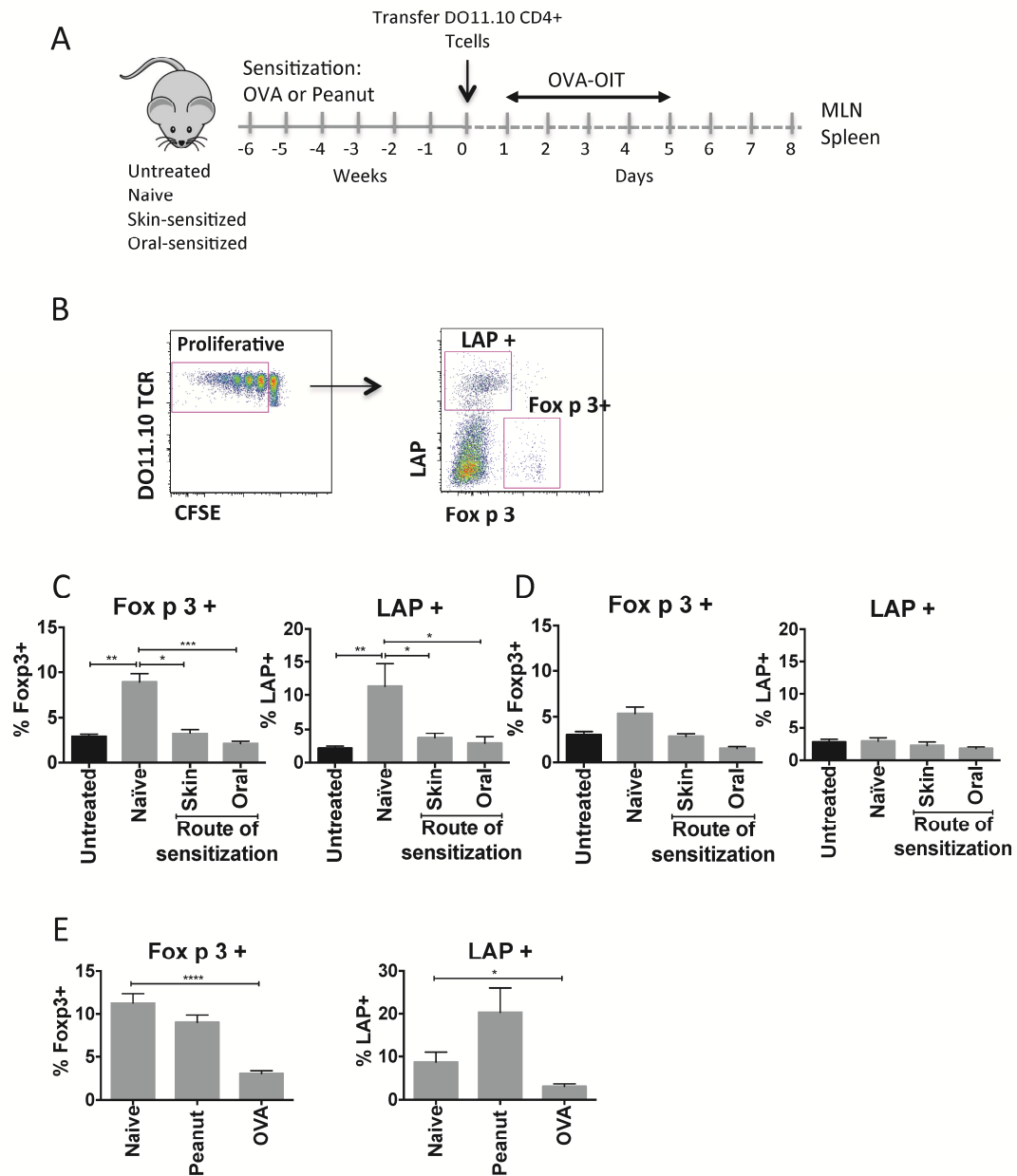


Figure 3

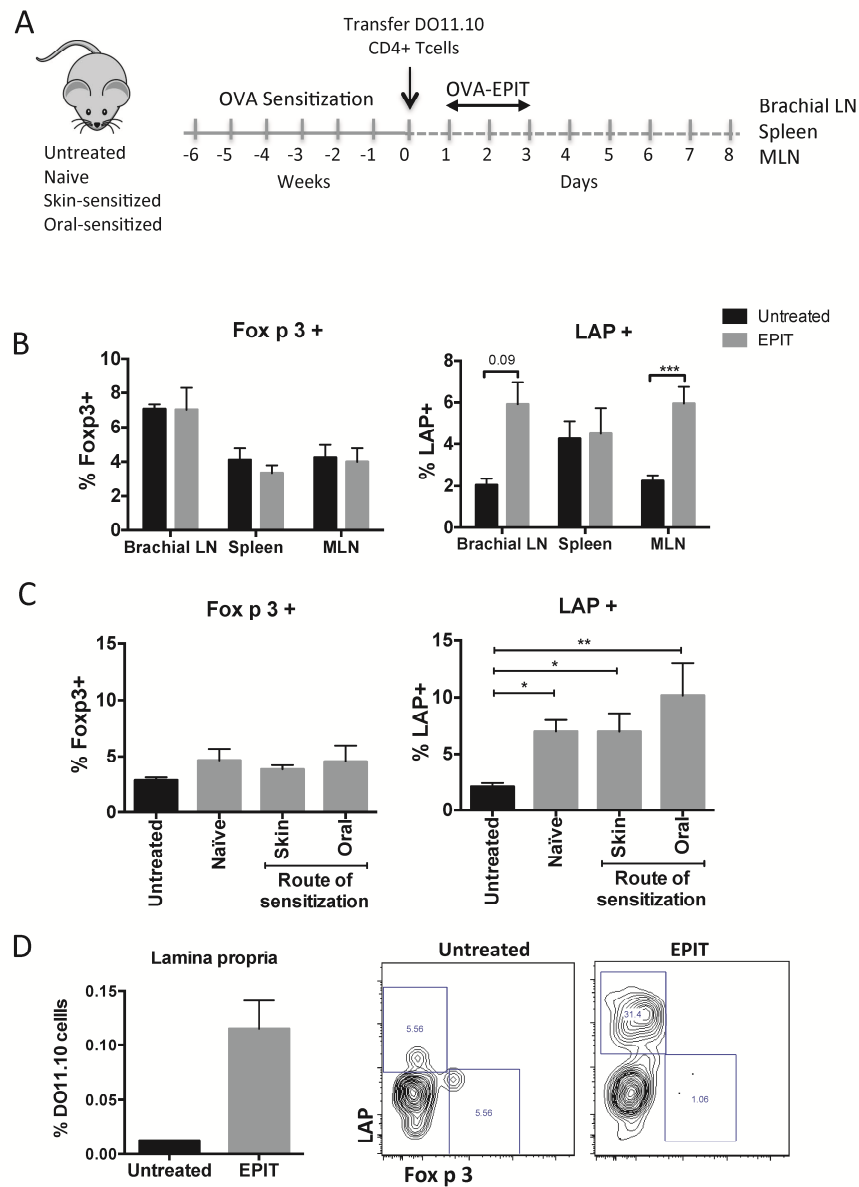


Figure 4

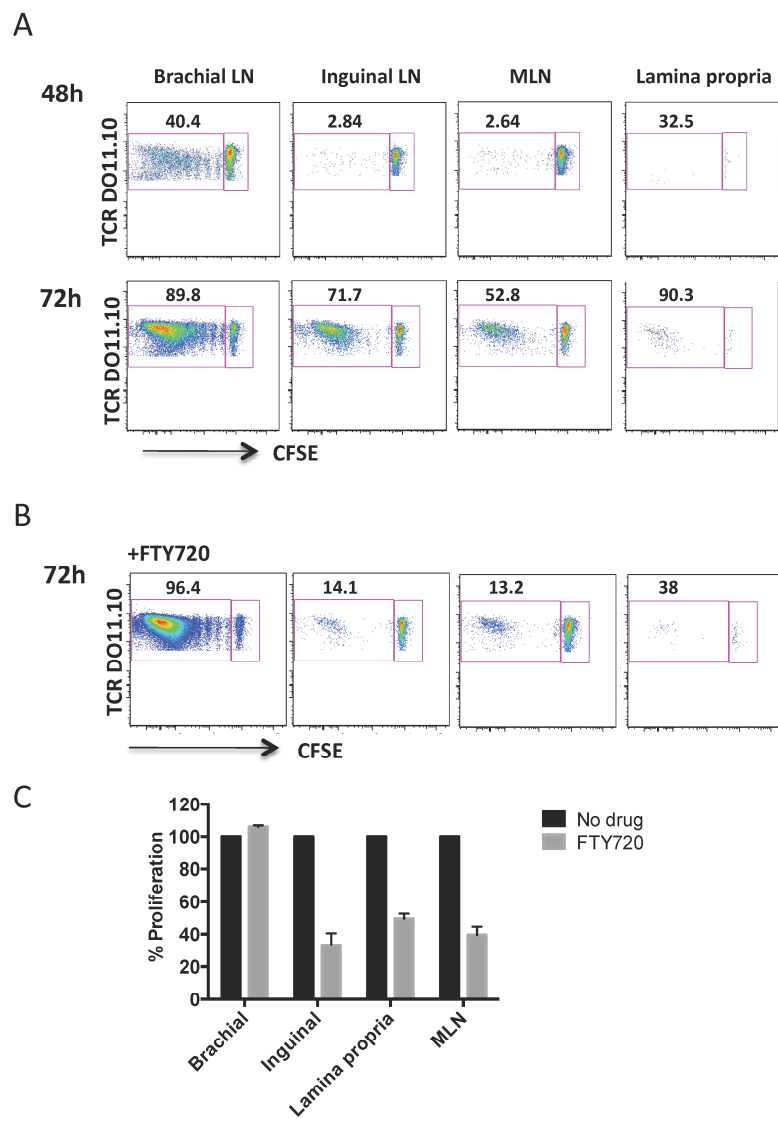


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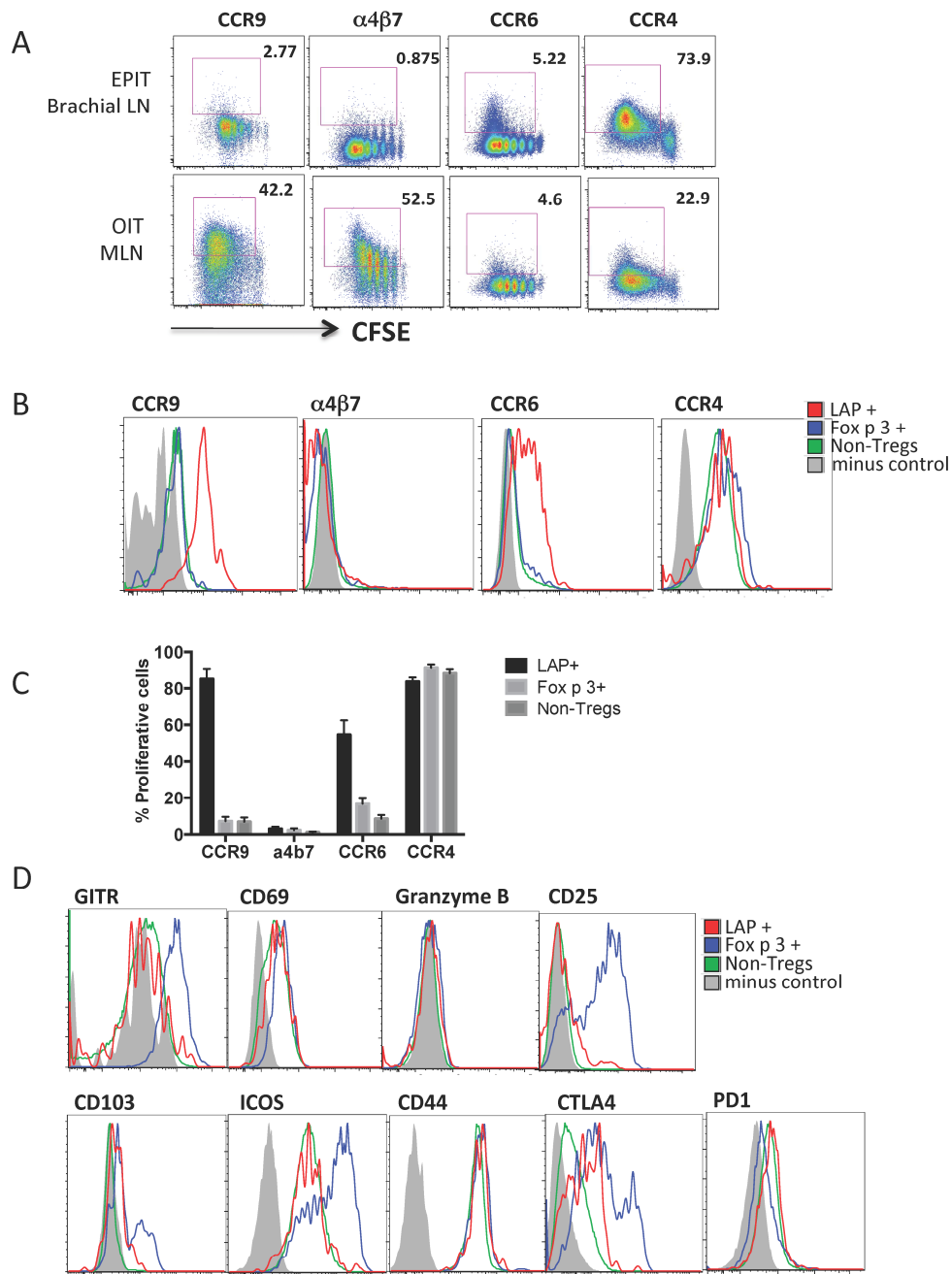


Figure 6

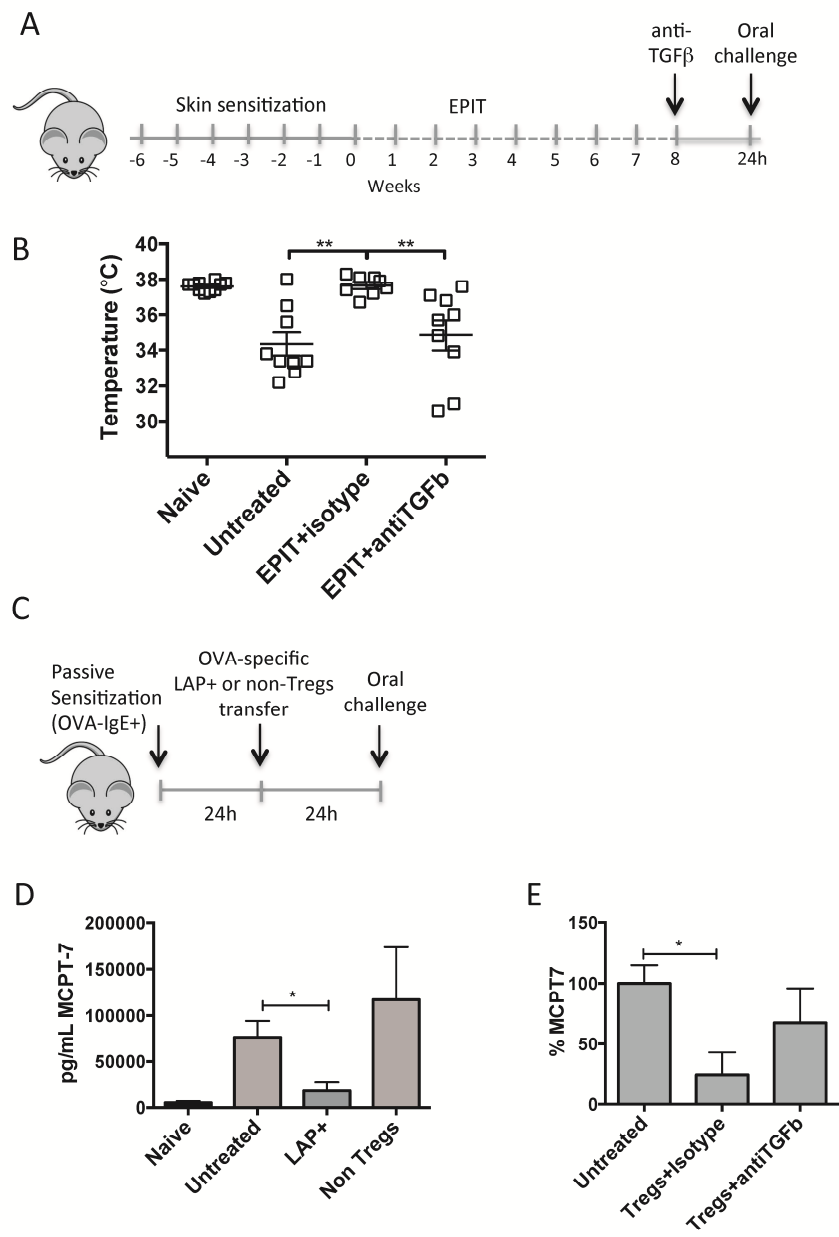


Figure 7

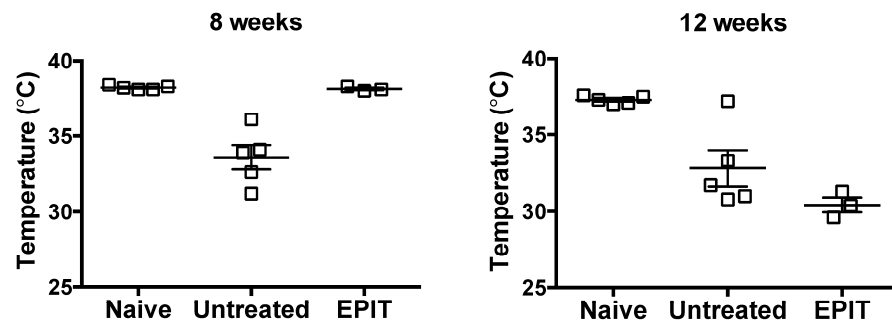


Figure E1

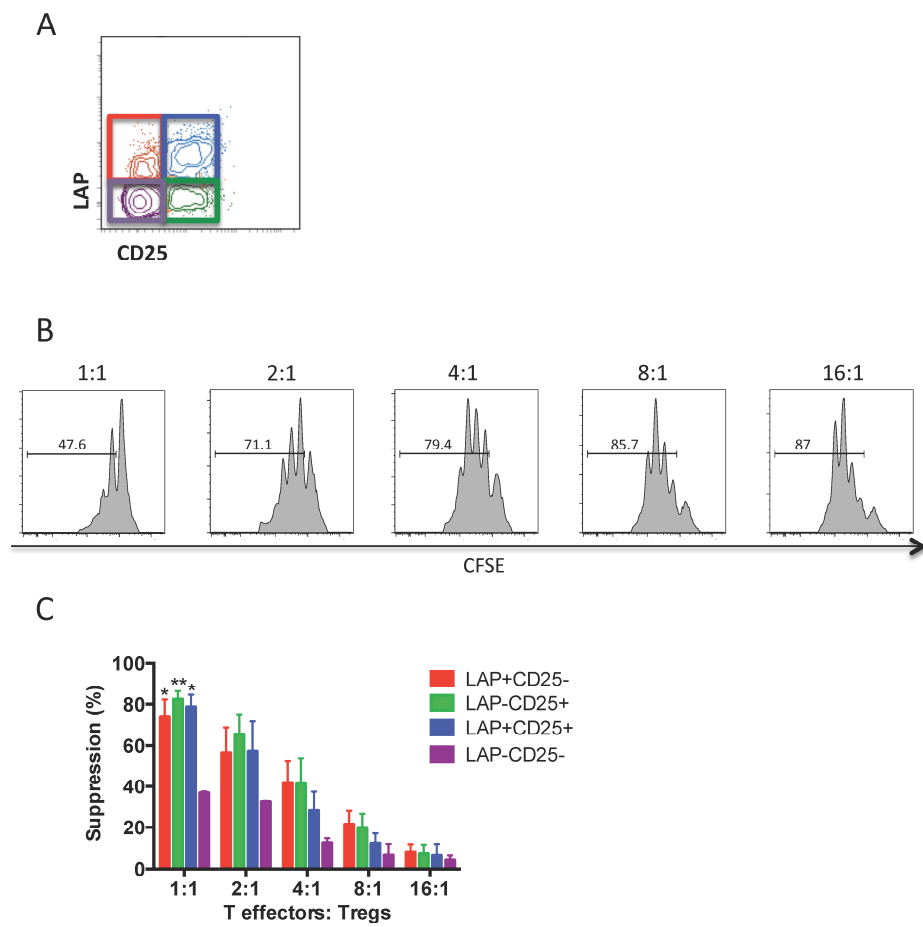
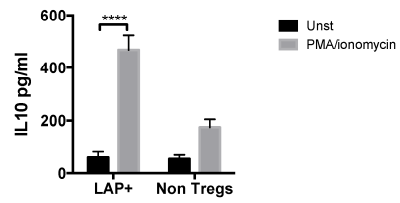


Figure E2

A



B

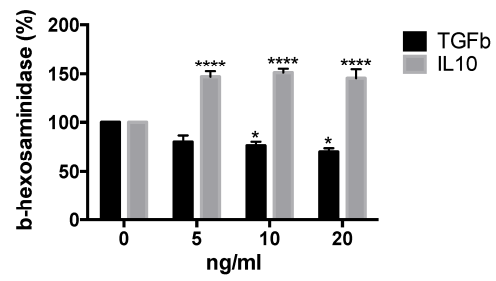


Figure E3

SUPPLEMENTARY MATERIALS

Supplementary Methods

Oral challenge and assessment of anaphylaxis

Mice were orally challenged with increasing doses of OVA (10mg, 20mg and 50mg) by gavage, every 30 min. Mice experiencing anaphylaxis at the lower dose (drop in body temperature below 36°C) were not challenged with the higher dose. Body temperature was monitored by rectal thermometer (WPI Instruments, Sarasota, FL).

Suppression assays

CFSE-CD4⁺ T cells (5×10^4 cells/well) were obtained from naïve Balb/c mice as described in Methods and cultured in the presence of irradiated T-cell depleted splenocytes (5×10^4 cells/well). LAP+CD25⁻, LAP-CD25⁺, LAP+CD25⁺ and LAP-CD25⁻ T cells were FACS sorted from naïve Balb/c mice and added to the cultures in varying proportions. Cells were stimulated with anti-CD3 (0.5ug/mL) (eBioscience) for 3 days and cell proliferation was evaluated by CFSE dilution.

Measurement of antigen-specific immunoglobulins

OVA-specific IgE was measured by capture ELISA by using DIG-labeled OVA for detection, as previously described¹. OVA-specific IgG1 and IgG2a levels were measured by ELISA using biotinylated monoclonal antibodies (all from BD Biosciences, San Diego, CA).

Basophil activation test

Basophil activation tests were performed as previously described ². Blood was diluted in RPMI and incubated at 37°C for 90 minutes with 20ug/mL of OVA. Red blood cells were lysed, and cells were stained with CD49b and IgE to detect the population of basophils, after gating out T and B cells with CD3/CD19 staining. CD200R was used as basophil activation marker (all antibodies were from eBioscience).

Flow cytometry

Lymph nodes and spleen cells were isolated and stained with specific antibodies according to standard techniques. Lamina propria was isolated as previously described ¹. All antibodies were from eBioscience unless otherwise stated. Cells were blocked with FcBlock before staining with surface antibodies and live/dead aqua viability reagent (Invitrogen). Cells were fixed and permeabilized (using Foxp3-optimized buffers from eBioscience), for intracellular staining. For Treg staining, the antibodies used were anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-LAP (TW7-16B4), anti-CD25 (PC61.5), anti-Foxp3 (intracellular) (FJK-16s) and anti-DO11.10 TCR (KJ1-26). Phenotyping of Tregs was performed using anti-GITR (DTA-1), anti-CD103 (2E7), anti-Granzyme B (intracellular)(GB11; BioLegend), anti-CTLA-4 (intracellular) (UC10-4B9; BioLegend), anti-CD44 (IM7) (BioLegend), anti-CD69 (H1.2F3) (BioLegend), anti-ICOS (7E.17G9), anti-PD-1 (RMP1-30) and anti-GATA3 (intracellular)(TWAJ). For migration marker staining, the antibodies used were anti-CCR9 (CW-1.2) (BioLegend), anti-CCR6 (29-2L17)(BioLegend), anti-CCR4 (2G12)

and anti- $\alpha 4\beta 7$ (DATK32). Cells were acquired on a LSR Fortessa cytometer (BD Bioscience) and analysis performed using FlowJo software (Treestar, Ashland, OR).

Measurement of cytokines after PMA/ionomycin stimulation

LAP+CD25⁻ and LAP-CD25⁻ were FACS sorted and stimulated with PMA/ionomycin for 24h. Supernatants were recovered and IL-10 was quantified by ELISA following manufacturer's instructions (eBioscience).

Mast cell activation assays

Mast cells were derived from Balb/c mice as described elsewhere³. To assess β -hexosaminidase release, mast cells were culture in RPMI supplemented with 5ng/mL IL-3 with or without IL-10 or TGF β (Peprotech, Rocky Hill, NJ) for 24h. Then, they were sensitized with 1ug/mL anti-DNP IgE for 3h and activated with 100ng/mL DNP-BSA (both from Sigma) for 30min, at 37°C. After activation, the release of β -hexosaminidase was measured as previously described⁴ and the percent of β -hexosaminidase was determined by dividing the amount of β -hexosaminidase in the supernatant by the total amount present in the supernatant and cell pellet.

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Supplementary Figure Legends**Figure E1. Mice epicutaneously sensitized with OVA plus cholera toxin**

adjuvant do not have sustained protection against anaphylaxis after EPIT. Mice

were sensitized through the skin with OVA in the presence of cholera toxin and

challenged at week 8 and week 12. Temperature was measured 30 min after

challenge. Data are individual mice from 2 independent experiments.

Figure E2. LAP⁺CD25⁻ T cells are able to suppress proliferation of CD4⁺ T cells

in vitro. (A) Graphic showing the four different populations found in naïve mice

attending to the expression of LAP and CD25. (B) CFSE-labeled CD4⁺ T cells from

Balb/c mice were cultured in the presence of irradiated T-cell depleted splenocytes.

LAP⁺CD25⁻, LAP⁻CD25⁺ and LAP⁺CD25⁺ T cells were FACS sorted from naïve mice

and added to the cultures in varying proportions. Cells were stimulated with anti-

CD3 for 3 days and cell proliferation was evaluated by CFSE dilution. Representative

plots of proliferation in cultures with different proportions of CFSE-CD4⁺T

cells:LAP⁺CD25⁻ T cells are shown. (C) Suppression (%) of the different Tregs

subsets is represented. Data are mean + SEM of 3 independent experiments.

Figure E3. IL-10 released by LAP⁺CD25⁻ T cells does not inhibit mast cell

activation. (A) LAP⁺CD25⁻ and LAP⁻CD25⁻ (non-Tregs) T cells were stimulated

with PMA/ionomycin and IL-10 was quantified in the supernatants. (B) β -

hexosaminidase release was calculated for bone-marrow derived mast cells

incubated with IL-10 or TGF β and activated with DNP-BSA. Data are mean + SEM of

109 5 replicates in 2 independent experiments . *p < 0.05, **p < 0.01, ****p < 0.0001 vs

110 untreated mast cells.

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