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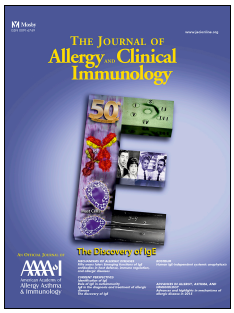

Peanut-specific Tr1 cells induced *in vitro* from allergic individuals are functionally impaired

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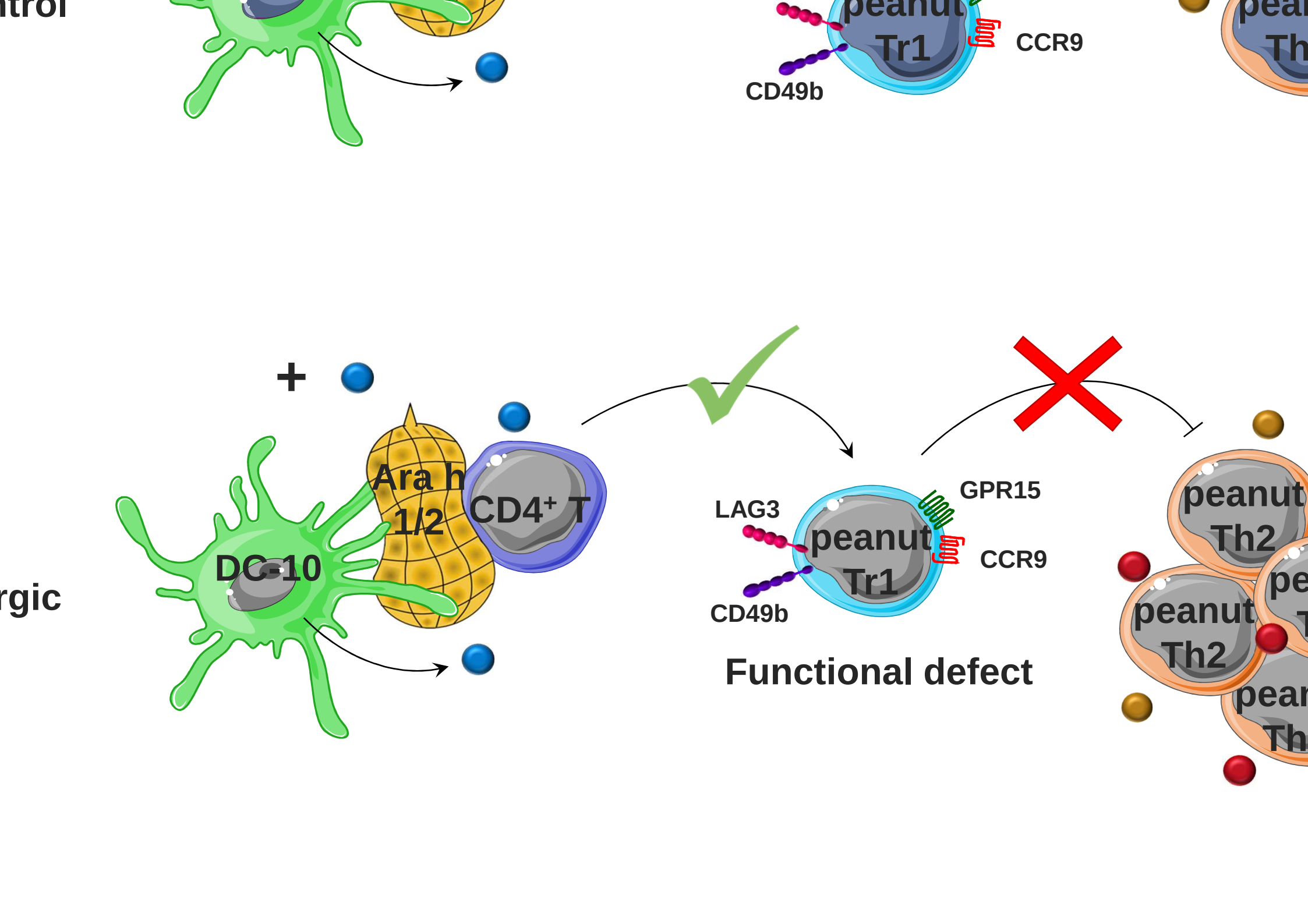
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KEY WORDS

Peanut allergy; Oral immunotherapy; Tr1 cells; LAG3; CD49b; Th2 cells; Ara h 1/2

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ABBREVIATIONS USED

Ara h: Arachis hypogaea
Bas: baseline
DC-10: tolerogenic dendritic cells
HC: healthy control
LT-OIT: long-term oral immunotherapy
mDC: mature dendritic cells
OIT: oral immunotherapy
PA: peanut allergic
pea-: peanut
ST-OIT: short-term oral immunotherapy
pea-T10: $CD4^+$ T cells co-incubated with tolerogenic dendritic cells pulsed with the main peanut allergens
pea-Tm: $CD4^+$ T cells co-incubated with mature dendritic cells pulsed with the main peanut allergens
Tr1: T regulatory type 1 cells
TT: tetanus toxoid

ABSTRACT (250 words or less)

Background: Peanut allergy is a life threatening condition which lacks regulatory-approved treatment. T regulatory type 1 (Tr1) cells are potent suppressors of immune responses and can be induced *in vivo* upon repeated antigen exposure or *in vitro* using tolerogenic dendritic cells (DC-10). Whether or not oral immunotherapy (OIT) leads to antigen-specific Tr1 cell induction has not been established.
Objectives: To determine whether peanut-specific Tr1 cells can be generated *in vitro* from peripheral blood of peanut allergic (PA) individuals at baseline or during OIT, and whether they are functional as compared to peanut-specific Tr1 cells induced from healthy controls (HC).
Methods: DC-10 were differentiated in the presence of IL-10 from peripheral blood mononuclear cells of PA individuals and HC pulsed with the main peanut allergens Arachis hypogaea (Ara h)

1 and 2, and used as antigen presenting cells for autologous $CD4^+$T cells (pea-T10). Pea-T10 cells were characterized by the presence of $CD49b^+LAG3^+$ Tr1 cells, antigen-specific proliferative responses, and cytokine production.

Results: $CD49b^+LAG3^+$ Tr1 cells were induced in pea-T10 cells at comparable percentages from HC and PA individuals. Despite their antigen specificity, pea-T10 cells of PA individuals with or without OIT, as compared to those of HC, were not anergic and had high Th2 cytokine production upon peanut-specific restimulation.

Conclusions: Peanut-specific Tr1 cells can be induced from HC and PA individuals, but those from PA individuals are functionally defective independently of the OIT. The unfavorable Tr1/Th2 ratio is discussed as possible cause of PA-Tr1 cell impairment.

KEY MESSAGES

- Peanut-specific Tr1 cells can be induced *in vitro* from healthy controls and peanut allergic individuals.
- Tr1 cell cultures induced *in vitro* from peanut allergic individuals, unlike those from healthy controls, are not anergic and have high Th2 responses.

INTRODUCTION

Food allergies are an important health issue in developed countries, with a prevalence of 8% amongst children (1). Peanut allergy is one of the most common food allergies, affecting 1 to 2% of children and 0.6% of adults in developed countries (2). As of today, there is no regulatory-approved treatment for peanut allergy, and exposure to trace amounts of the allergen can lead to a potentially fatal anaphylactic reaction (3). Management of the disease consists of avoidance of the allergen that is difficult to achieve, and patients are at risk of accidental exposure to the allergen.

Oral immunotherapy (OIT) is a promising experimental therapy for food allergies. It consists of the ingestion of small and increasing doses of the allergen during a buildup phase that is immediately followed by the daily ingestion of a target amount of the allergen during a maintenance phase that can last for several years (4-6). Even though OIT shows encouraging results and may improve patient quality of life, it carries important risks of adverse reactions. Taken together, these results illustrate a need to improve the safety and efficacy of OIT (7, 8).

'Immunological tolerance', defined as a state of antigen-specific unresponsiveness, is established through different mechanisms that include anergy, exhaustion and active suppression prompted by T regulatory cells (9, 10). In particular, T cell anergy corresponds to the lack of a functional response following exposure to the cell's cognate antigen (11-13). Antigen specific T cell anergy can be induced in the presence of IL-10 during primary antigen stimulation *in vitro* (14). IL-10 anergized T cells have impaired secondary responses to the same antigen and T-cell cloning of these anergic cells allows the isolation of IL-10 producing T regulatory type 1 (Tr1) cells (15). T regulatory cells are essential contributors to the maintenance of peripheral immunological tolerance (16-19). $FOXP3^+$ thymic derived T regulatory cells are well studied $CD4^+$ T regulatory cells involved in regulation of immune responses towards self-antigens, although the demonstration of their antigen specificity *in vivo* and their generation *in vitro* towards specific antigens have been challenging (20). Tr1 cells are a subset of $CD4^+$ T regulatory cells distinct from $FOXP3^+$ T regulatory cells. They are generated towards different antigens encountered in the periphery, including allo-antigens in tolerant transplanted patients (21), gliadin in celiac disease patients (22), and bee venom in beekeepers (23). Tr1 cells can be generated *in vitro* towards different antigens, in the presence of IL-10 independently of the expression of FOXP3 (24). Tr1 cells are identified by the co-expression of the surface markers LAG3 and CD49b (25). IL-10 anergized or polarized T cell cultures, and peripheral blood of tolerant transplanted patients are enriched in $CD49b^+LAG3^+$ Tr1 cells. Tr1 cells produce high levels of IL-10, low IL-2, no IL-4, no IL-17, and variable amounts of IFN-γ (15, 25-27). The suppressive properties of Tr1 cells are primarily mediated by IL-10, and prior to the

identification of the specific Tr1 surface markers LAG3 and CD49b in 2013, Tr1 cells were often referred to as hyporesponsive (i.e. anergic) cells or IL-10 producing $CD4^+$ T cells.

Tr1 cells regulate both innate and adaptive responses underlying allergic reactions by acting on eosinophils, basophils, mast cells and Th2 cells (28, 29). Allergic subjects present disproportionate Th2 responses to innocuous antigens (30, 31) and defects in IL-10 producing T regulatory cells that result in an *in vivo* imbalance between T regulatory cells and Th2 populations (23, 29, 32, 33). The intestinal mucosa is the initial site of interaction between immune cells and dietary antigens, and therefore plays a crucial role in the maintenance of immunological tolerance to foods (34). Gliadin-specific Tr1 cells that possess high suppressive properties have been isolated from the intestinal mucosa of celiac disease patients in remission (22). In addition, repeated ingestion of a specific antigen is responsible for the expansion of IL-10 secreting cells in the gut in mice (35), but it remains to be determined whether similar mechanisms occur in humans. Reinforcing this concept, our group published preliminary findings in a limited patient population which indicate that peripheral $CD49b^+LAG3^+$ Tr1 cells are present at higher frequencies in peripheral blood of patients under OIT compared to untreated patients, but no functional data were obtained at that time (33).

Based on the established role of Tr1 cells in immunomodulation and regulation of allergic responses *in vivo*, we hypothesize that peanut specific Tr1 cells could be generated from healthy controls (HC) but not from untreated peanut-allergic (PA) individuals. As a corollary, we further hypothesized that OIT could favor Tr1 cell differentiation via chronic exposure to low doses of the allergen in the gut, and that peanut-specific Tr1 cells could be generated at higher frequencies from PA individuals under OIT compared to untreated individuals.

Our group has previously published that antigen-specific Tr1 cells can be induced *in vitro* using tolerogenic dendritic cells (DC-10) (36). In particular, functional Der p 2-specific Tr1 cells that present antigen-specific anergy and suppressive activities *in vitro* can be induced from individuals allergic to house dust mite (37).

In this study, we tested whether Tr1 cells could be induced *in vitro* using DC-10 pulsed with the main peanut allergens Arachis hypogaea (Ara h) 1 and 2 (pea-T10 cells) in (1) HC, (2) PA individuals untreated (baseline), (3) PA individuals undergoing OIT for a short period of time (short term/ST-OIT), and (4) PA individuals undergoing OIT for several years (long term/LT-OIT). Our data demonstrate that $CD49b^+LAG3^+$ Tr1 cells can be induced from all 4 groups. Despite their peanut-antigen specificity, pea-T10 cells from PA individuals of all 3 groups were not anergic upon secondary peanut stimulation and produced high levels of Th2 cytokines whereas all pea-T10 cells from HC showed anergy and significantly reduced Th2 cytokine production.

168 METHODS
169
170 **Study subjects**
171 This study included 21 PA individuals (median age= 15.1±9.8years) confirmed by double-blind
172 placebo-controlled oral food challenge, and 7 HC (median age= 16±3.3years) from the IRB-
173 approved protocol 5136 at Stanford University School of Medicine. Seven PA individuals had
174 not undergone OIT (baseline), 7 had been undergoing OIT for more than 3 months and less than
175 1 year (short-term OIT/ST-OIT), and 7 had been under OIT for more than 3 years (long-term
176 OIT/LT-OIT). No withdrawal of maintenance daily peanut therapy had occurred in any of the
177 OIT groups. Clinical reactivity was defined as any sign of allergic reaction (*i.e.*, score >1 on the
178 Book criteria (38)) upon ingestion of the allergen. The clinical symptoms as well as the
179 demographics of the PA individuals are presented in Table 1. Written informed consent was
180 obtained for all participants before entering the study.
181
182 For more information on dendritic cell differentiation, T cell differentiation, Ara h 1/2 antigen
183 specificity assay and cytokine detection, flow cytometry and statistical analyses, see the Online
184 Repository Supplementary Materials & Methods.
185

RESULTS

Peanut-specific Tr1 cells can be induced from HC and PA individuals using pea-DC-10

Tolerogenic DC-10 and mature DC (mDC) were differentiated from peripheral blood of HC and PA individuals using previously published methods and pulsed with Ara h 1 and 2 (36, 37). Experimental scheme is shown in Fig. E1A in the Online Repository. Both pea-DC-10 and pea-mDC from HC and PA individuals expressed high levels of CD11c and CD86 (Fig. 1), showing that the cells displayed a mature myeloid phenotype. DC-10 remained $CD14^+$ whereas mDC were $CD14^-$ (Fig. 1). CD11c, CD86 and CD14 expression in pea-DC-10 or pea-mDC were comparable between HC and all 3 cohorts of PA individuals (Fig E2A-C).

Pea-T10 cells were generated using pea-DC-10 in the presence of IL-10 to induce anergy and Tr1 cells from autologous $CD4^+$ T cells obtained from HC and PA individuals as previously described (37) (Fig. E1A in the Online Repository). Control pea-Tm cells were generated with pea-mDC in the absence of IL-10. At the end of both cultures, we checked the expression of CD25 within the $CD4^+CD45RA^-$ memory population as an indicator of the activation state of the cells. Overall, $CD4^+CD45RA^-$ T cells from HC showed lower percentages of $CD25^+$ cells as compared to $CD4^+CD45RA^-$ T cells from PA individuals (31.2% vs. 38.1%, respectively; p=0.0454; Table E1); pea-Tm from PA individuals showed higher percentages of $CD25^+$ cells as compared to pea-Tm from HC (28.2±6.7% vs. 38.1±12.7%, respectively; p>0.05; Fig. E3A and B in the Online Repository) indicating that peanut-specific activation was overall higher in PA individuals than in HC. The percentage of $CD25^+$ cells was comparable between all 3 cohorts of PA individuals (Fig. E3C).

Pea-T10 cells of both HC and PA individuals contained a higher frequency of $CD49b^+LAG3^+$ Tr1 cells as compared to pea-Tm cells ($CD49b^+LAG3^+$ for pea-T10 cells of HC= 7.4±3.7% vs. 2.6±3% for pea-Tm (Fig. 2A and B; Table E1); p=0.0156; pea-T10 cells of PA individuals= 9.0±4.3%, vs. 2.9±1.0% for pea-Tm; p<0.0001; Fig. 2A-C; Table E1). There was no statistical difference in the percentage of $CD49b^+LAG3^+$ Tr1 cells between pea-T10 cells of HC and of PA individuals (p>0.05; Fig. 2D; Table E1). In addition, there was no statistical difference in the percentage of $CD49b^+LAG3^+$ Tr1 cells present in the pea-T10 cells of the 3 cohorts of PA individuals ($CD49b^+LAG3^+$ Tr1 cells for pea-T10 cells of baseline PA individuals= 8.6±2.7%; for ST-OIT PA individuals= 9.4±6.2%; for LT-OIT PA individuals= 8.8±3.9%, p>0.05; Fig. 2E). Altogether, the frequency of $CD49b^+LAG3^+$ Tr1 cells induced by pea-DC-10 was similar in HC and in PA individuals irrespective of the presence and duration of OIT.

Peanut-specific hyporesponsiveness can be achieved in pea-T10 cells of HC but not of PA individuals

To test whether pea-T10 cells were hyporesponsive to a secondary stimulation with the same antigen (i.e. anergic) as compared to pea-Tm cells, we measured proliferative responses by means of CFSE dilution after 4 or 5 days of secondary stimulation with pea-mDC (Fig. 3A-B, Fig. E1B and E4A in the Online Repository). We considered pea-T10 cells to be anergic when their proliferative response to the antigen was lower than the proliferative response of pea-Tm. Pea-T10 cells from all HC were anergic. Pea-T10 cells of 6 out of 7 HC presented anergy above 67%, and one HC presented anergy of 32% (mean anergy HC= 69%, range 32-87%; Fig. 3A-B and E4A), with an overall proliferation to peanut antigens significantly higher in pea-Tm than in pea-T10 cells ($p=0.0156$). In contrast, pea-T10 cells of only 9 out of 21 PA individuals were anergic (mean anergy PA individuals= 34.3%, range 5-80%; Fig. 3A-B and E4A), and only 2 out of 21 PA individuals presented anergy above 67%, a level of anergy that we previously established acceptable for clinical purposes (39, 40). The ratio of proliferation of pea-Tm/pea-T10 cells was higher in HC vs. PA individuals ($p=0.0007$; Fig. 3C and E4B).

Among the baseline PA individuals, pea-T10 cells of 4 out of 7 tested presented with low anergy (mean anergy baseline PA individuals= 20.5%, range 5-32%; Fig. 3A-B and E4A) and peanut-specific anergy was not detected in pea-T10 of 3 out of 7 baseline PA individuals. We next asked if OIT would modify the level of anergy in pea-T10 cells. Anergy was observed at a level of 80% in pea-T10 cells in 1 out of 7 ST-OIT PA individuals, and low anergy was observed in 2 out of 7 ST-OIT PA individuals (mean anergy ST-OIT PA individuals= 43%, range 15-80%; Fig. 3A-B and E4C), but was absent for the other 4 individuals. Similarly, anergy was observed at a level of 70% for pea-T10 cells in 1 out of 7 LT-OIT PA individuals, and was observed but below 67% for 5 out of 7 LT-OIT PA individuals (mean anergy LT-OIT PA individuals= 39.1%, range 9-70%; Fig. 3A-B and E4A), but was absent for 1 out of 7 individuals. There was no significant difference in proliferation of pea-Tm and pea-T10 cells of baseline PA individuals ($p>0.05$), of ST-OIT PA individuals ($p>0.05$) or of LT-OIT PA individuals re-stimulated by pea-mDC. Overall, there was no statistically significant difference in the pea-Tm/pea-T10 cell proliferation ratio between any of the 3 PA cohorts, although we observed a trend towards higher pea-Tm/pea-T10 cell proliferation ratio in LT-OIT PA individuals ($p>0.05$; Fig. 3D and Fig. E4C). Pea-Tm/pea-T10 cell proliferation ratio was significantly lower in the LT-OIT cohort compared to HC ($p=0.0262$). Of note, there was no correlation between the age of the subjects in the study, the severity of the peanut allergy, the duration of OIT and the induction of anergy.

We next tested whether anergy of pea-T10 was peanut-specific by measuring proliferation elicited by a different antigen, not present during the primary stimulation such as tetanus toxoid (TT). We found that proliferation upon re-stimulation with TT-mDC was comparable in pea-Tm and pea-T10 cells of both HC and PA individuals ($p>0.05$; Fig. E4D-E), confirming that when present, hyporesponsiveness was only towards the peanut antigens used in

the primary stimulation. Altogether, these results indicate that anergy towards the peanut antigens could be induced by pea-DC-10 from HC but not from PA cohorts irrespective of OIT.

Since Tr1 cells from PA individuals were not able to anergize the pea-T10 cells, we analyzed their cytokine secretion profile upon peanut-specific re-stimulation (Fig. E1B in the Online Repository). Pea-T10 cells of HC re-stimulated with pea-mDC secreted significantly less IL-4 (23.4±19.3 pg/mL, p=0.03; Fig. 4A, left panel; Table E1) and less IL-5 (123.1±67.6 pg/mL, p=0.034; Fig. 4B, left panel; Table E1) than control pea-Tm cells (49.9±32.7 pg/mL and 408.5±118.2 pg/mL, respectively). Conversely, pea-T10 and pea-Tm cells of PA individuals secreted comparable amounts of IL-4 (343.5±358.7 pg/mL and 447±449.6 pg/mL, respectively, p>0.05; Fig. 4A, middle panel; Table E1) and IL-5 (784.2±534.8 pg/mL and 1024±556.4 pg/mL, respectively, p>0.05; Fig. 4B, middle panel; Table E1), and both cytokines were higher than those produced by HC cells (Fig. 4A and B, right panels). Pea-Tm and pea-T10 of HC secreted comparable amounts of IL-10 (p>0.05; Fig. E5A in the Online Repository; Table E1) whereas pea-Tm of PA individuals secreted more IL-10 compared to pea-T10 (p=0.06; Fig. E5A in the Online Repository; Table E1). Pea-Tm and pea-T10 of both HC and PA individuals secreted comparable amounts of IFN-γ (p>0.05; Fig. E5B in the Online Repository; Table E1), whereas pea-T10 of PA individuals secreted more IL-10 compared to pea-T10 of HC (p=0.0365; Fig. E5A in the Online Repository). Interestingly, we observed a negative correlation between the levels of IL-4 and IL-5 produced by the pea-T10 cells and the percentage of anergy (p=0.0145, r=-0.5509, and p=0.0181, r=-0.5498, respectively; Fig. E5C-D), but no correlation between the levels of IL-10 produced by the pea-T10 cells and the percentage of anergy (r=-0.2917, p=0.212; Fig. E5E). Severity of peanut allergy and duration of OIT did not influence cytokine production by pea-Tm and pea-T10. Taken together, these results demonstrate that activation of $CD4^+$ T cells with autologous pea-DC-10 promotes the induction of anergic, peanut-specific T cells that secrete low amounts of Th2 cytokines in HC but not in PA individuals. In pea-T10 of PA individuals, responsiveness to the peanut-antigens remains higher than in HC and it could not be impacted by the addition of IL-10. These data suggest that pea-Tr1 from PA individuals either lack antigen-specificity or are functionally defective.

Peanut-specific cells are enriched in $CD49b^+LAG3^+$ Tr1 cells

We next tested whether the general lack of anergy in pea-T10 cells from PA individuals was due to lack of antigen specificity in the Tr1 population. Pea-T10 cells were re-stimulated with pea-mDC, and the expression of the activation markers CD69 and CD137 (41) was quantified in the $CD45RA^-$ memory and $CD49b^+LAG3^+$ Tr1 populations (Fig. 5A-B and Fig. E1B in the Online Repository). Background expression of CD69 and CD137 from unstimulated pea-T10 cells was subtracted to account for the antigen-specific response. The $CD69^+$ cells were

more abundant within the Tr1 cell subset than within the total memory population (mean percentage of $CD69^+$ cells in Tr1= 28.3±15.9%; in memory= 16.4±12.1%; p=0.0044; Fig. 5C; Table E1). Similarly, the percentage of $CD137^+$ cells was higher within the Tr1 cell subset than in the total memory population (26.0±20.8% and 8.3±5.4%, respectively, p=0.0044; Fig. 5D; Table E1). However, when the pea-T10 cells were re-stimulated with TT-mDC, there was no difference in the percentages of $CD69^+$ and of $CD137^+$ cells in the Tr1 cell subset compared to the memory population (Fig. E6). These data suggest that despite the lack of anergy, peanut-specific Tr1 cells are present in pea-T10 cells of PA individuals, and more abundant than in the total memory population.

As homing cues are essential for the migration of T cells to the tissues, we tested whether pea-Tr1 cells specifically express molecules that confer gut-homing properties. The expression of the gut-homing receptor GPR15 (42, 43) was significantly higher in the $CD49b^+LAG3^+$ Tr1 cell subset compared to that of the total memory population of pea-T10 from both HC and PA individuals (mean percentage of $GPR15^+$ cells in Tr1= 9.7±7.7%; in memory= 5.3±4.0%, p<0.0001; Fig. 6A; Table E1). The expression of CCR9, a chemokine receptor that supports the migration of T lymphocytes to the small intestine and colon (44), was also higher in the $CD49b^+LAG3^+$ Tr1 cell subset as compared to that of the total memory population of pea-T10 from HC and PA individuals (mean percentage of $CCR9^+$ cells in Tr1= 33.1±18.3%; in memory= 19.7±15.6%; p<0.0001; Fig. 6B; Table E1). Altogether, these data indicate that peanut-specific Tr1 cells induced *in vitro* express gut homing receptors, suggesting that they could have the capacity to migrate to the gut mucosa in both HC and PA individuals.

DISCUSSION

In the current study, we show that $CD4^+CD45RA^-CD49b^+LAG3^+$ Tr1 cells specific for the main peanut allergens Ara h 1 and 2 can be induced *in vitro* from $CD4^+$ T cells of both HC and PA individuals using tolerogenic DC-10 as antigen presenting cells. Pea-T10 cells from HC (containing 7.4±3.7% pea-Tr1 cells) are anergic upon antigen-specific rechallenge, whereas pea-T10 cells from PA individuals (containing 9.2±4.8% pea-Tr1 cells) are not anergic and they present high peanut-specific proliferative responses. In addition, we found that the lack of anergy of pea-T10 cells in PA individuals was not significantly modified by the presence or duration of OIT. Indeed, we only observed a trend towards induction of anergy in pea-T10 cells of LT-OIT PA. Pea-T10 cells of HC produce significantly less IL-4 and IL-5 vs. control pea-Tm cells, but pea-T10 cells of PA individuals secrete similar levels of these Th2 cytokines compared to pea-Tm cells. The absence of anergy observed for pea-T10 cells of PA individuals, was not due to lack of antigen specificity in the Tr1 population. In addition, Tr1 cells induced *in vitro* express higher levels of the gut-homing receptors GPR15 and CCR9 compared to the total memory population, suggesting preserved gut-homing capacity.

Our study is the first to show that peanut-specific $CD49b^+LAG3^+$ Tr1 cells can be induced *in vitro* from $CD4^+$ T cells of both HC and PA individuals. Downregulation of peanut-specific allergic responses have been achieved *in vivo* in an allergic mouse model using peanut-coated nanoparticles, which are known to favor the differentiation of Tr1 cells (45, 46). In addition, gliadin-specific Tr1 cells have been isolated from the gut of celiac individuals in remission (22). These studies support the role of Tr1 cells in maintenance of tolerance to food antigens and the rationale for inducing pea-Tr1 cells *in vitro* and test their inhibitory function.

The ability to induce allergen-specific anergy and downregulate Th2 cytokines has previously been demonstrated in PBMC isolated from individuals allergic to house dust mites (37). The authors showed that DC-10 are potent inducers of anergic cells that are specific for the aeroallergen Der p 2 *in vitro*. The level of anergy described towards Der p 2 was comparable to the level we observe in cultures of HC cells towards Ara h 1/2, which is also comparable to the minimum percentage of anergy of 67% that is required for the clinical use of IL-10 anergized T cells (39, 40). However, in the Der p 2 study, the presence of $CD49b^+LAG3^+$ Tr1 cells in the anergic cultures was not quantified since these biomarkers of Tr1 cells have been reported later (25). Using CD49b and LAG3 as *bona fide* markers of Tr1 cells, we clearly demonstrate that Tr1 cells can differentiate *in vitro* in an antigen-specific manner but, unlike in HC, their presence is not associated with anergy in T cells of PA individuals. Indeed, anergy above 67% was observed for only 2 patients in our cohort. As the percentage of Ara h 1/2-specific cells in peripheral blood is low for both HC and PA individuals (<1% of $CD4^+$ T cells (13, 47)), we were not able to test the suppression of primary proliferation or suppression of cytokine production by pea-T10 *in*

vitro. However, acquisition of anergy and induction of Tr1 cells have been consistently described as a consequence of antigen-specific priming in the presence of IL-10. Our results suggest that there is an underlying defect in PA individuals that prevents the induction of functional peanut-specific Tr1 cells in IL-10 dependent tolerogenic conditions.

Typically, $CD4^+$ T cells from allergic individuals present an abundant allergen-specific Th2 population that likely results in an imbalance in the Tr1/Th2 ratio skewed towards a Th2 response (30, 32, 48). We cannot exclude that functional peanut-specific Tr1 cells were induced in our culture system, but their numbers were not sufficient to dampen the proliferation and cytokine production of the pre-existing memory Th2 population. Indeed, one ST-OIT individual with severe peanut allergy for whom a very high percentage (21.6%) of Tr1 cells was induced in pea-T10 cells also presented very high anergy (80%), indicating that the Tr1 cells were functional, and that high numbers of Tr1 cells might be required to downregulate the response of a pre-existing peanut-specific Th2 memory population. Our data show that high IL-10 production does not correlate with induction on an anergic phenotype induced *in vitro* by allergen stimulation in tolerogenic conditions (IL-10 and DC-10), but is associated with high amounts of IL-4 and IL-5 Th2 cytokines in PA individuals, suggesting that IL-10 is produced by Th2 cells.

Whether chronic antigen exposure via peanut-OIT potentiates the function of Tr1 cells remains to be clarified. Although not statistically significant, we observed a trend towards induction of anergy for the pea-T10 cells of PA individuals who had been under OIT for several years. Once confirmed in a larger number of individuals, these data would be suggestive of the effect of LT-OITas we previously observed (33). Persistent antigen exposure favors Tr1 induction and antigen-specific hyporesponsiveness as broadly demonstrated *in vitro* and *in vivo* in both mouse and human studies (21). Repetitive intraperitoneal injection of anti-CD3 antibodies induces IL-10 producing Tr1-like cells localized in the small intestine (49). Similarly, non-allergic beekeepers and cat owners who are naturally exposed to continued antigenic stimulation develop high numbers of antigen-specific IL-10 secreting Tr1 cells which control undesired Th2 immune responses (23, 50, 51). These findings suggest the importance of OIT to control food allergen responses by induction of Tr1 cells in the intestine.

The development of food allergies is significantly influenced by the genetic background. Sicherer et al. estimated the heritability of peanut allergy at 81.6% studying a cohort of monozygotic twins (52). In addition, genetic defects affecting different components of the immune response and resulting in gain of function of the IL-4, IL-9, IL-13 and TGF-β pathways have been linked to atopic diseases (53-56). Mutations of the HLA-DQ/DR (57) and filaggrin (58) genes have been specifically associated with peanut allergy. A novel line of evidence suggests that epigenetic defects play a role in food allergies as a differential CpG methylation profile has been observed in children allergic to egg, peanut, milk and shrimp (59, 60). We

cannot exclude that the PA individuals in our study carry genetic or epigenetic defects in genes crucial for Tr1 cell generation and function

Determining why anergy could be induced for pea-T10 of only few PA individuals in our cohort is a crucial step in understanding peanut allergies and the mechanism of OIT. It should be considered that our experiments were performed starting with peripheral blood cells which may not represent what is occurring in the gut where the chronic antigen exposure is taking place and where Tr1 cells have preferential homing. However, our data indicate that pea-Tr1 cells induced in our system might possess gut-homing abilities as assessed by the expression of GPR15 and CCR9. Studies are ongoing to test whether the percentage and numbers of Tr1 cells increase over the course of OIT in the gut.

To conclude, our data show that peanut-specific Tr1 cells can be induced towards the main peanut allergens Ara h 1 and 2, and that the presence of functional Tr1 cells is associated with the inhibition of Th2 responses in HC but not in PA individuals. We can therefore hypothesize that providing functional peanut specific Tr1 cells to PA individuals could facilitate the regulation of allergic responses. For this achievement, autologous pea-Tr1 cells could be generated by enforced lentiviral-mediated expression of IL-10, which should overcome limitations dictated by genetic or epigenetic predisposition in allergic individuals (61).

412 REFERENCES
413
414 1. Gupta RS, Springston EE, Warrier MR, Smith B, Kumar R, Pongracic J, et al. The
415 prevalence, severity, and distribution of childhood food allergy in the United States. Pediatrics.
416 2011;128(1):e9-17.
417 2. Cox A, Sicherer SH. Peanut and tree nut allergy. Chemical immunology and allergy.
418 2015;101:131-44.
419 3. Bock SA, Munoz-Furlong A, Sampson HA. Further fatalities caused by anaphylactic
420 reactions to food, 2001-2006. The Journal of allergy and clinical immunology.
421 2007;119(4):1016-8.
422 4. Kulis M, Wright BL, Jones SM, Burks AW. Diagnosis, management, and investigational
423 therapies for food allergies. Gastroenterology. 2015;148(6):1132-42.
424 5. Kulis M, Wesley Burks A. Oral immunotherapy for food allergy: clinical and preclinical
425 studies. Advanced drug delivery reviews. 2013;65(6):774-81.
426 6. Begin P, Chinthrajah RS, Nadeau KC. Oral immunotherapy for the treatment of food
427 allergy. Human vaccines & immunotherapeutics. 2014;10(8):2295-302.
428 7. Thyagarajan A, Varshney P, Jones SM, Sicherer S, Wood R, Vickery BP, et al. Peanut
429 oral immunotherapy is not ready for clinical use. The Journal of allergy and clinical
430 immunology. 2010;126(1):31-2.
431 8. Yu GP, Weldon B, Neale-May S, Nadeau KC. The safety of peanut oral immunotherapy
432 in peanut-allergic subjects in a single-center trial. International archives of allergy and
433 immunology. 2012;159(2):179-82.
434 9. Delgoffe GM, Powell JD. Feeding an army: The metabolism of T cells in activation,
435 anergy, and exhaustion. Molecular immunology. 2015;68(2 Pt C):492-6.
436 10. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation
437 and function. Annual review of immunology. 2012;30:531-64.
438 11. Schwartz RH. T cell anergy. Annual review of immunology. 2003;21:305-34.
439 12. Valdor R, Macian F. Induction and stability of the anergic phenotype in T cells. Seminars
440 in immunology. 2013;25(4):313-20.
441 13. Ryan JF, Hovde R, Glanville J, Lyu SC, Ji X, Gupta S, et al. Successful immunotherapy
442 induces previously unidentified allergen-specific CD4+ T-cell subsets. Proceedings of the
443 National Academy of Sciences of the United States of America. 2016;113(9):E1286-95.
444 14. Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term
445 antigen-specific anergic state in human CD4+ T cells. The Journal of experimental medicine.
446 1996;184(1):19-29.
447 15. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, et al. A CD4+ T-
448 cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature.
449 1997;389(6652):737-42.
450 16. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nature reviews
451 Immunology. 2008;8(7):523-32.
452 17. Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T
453 cells. Immunity. 2013;38(3):414-23.
454 18. Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, et al. Regulatory T
455 cells: recommendations to simplify the nomenclature. Nature immunology. 2013;14(4):307-8.
456 19. Palomares O, Yaman G, Azkur AK, Akkoc T, Akdis M, Akdis CA. Role of Treg in
457 immune regulation of allergic diseases. European journal of immunology. 2010;40(5):1232-40.

458 20. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the
459 human immune system. Nature reviews Immunology. 2010;10(7):490-500.
460 21. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M. Tr1 cells and the counter-regulation
461 of immunity: natural mechanisms and therapeutic applications. Current topics in microbiology
462 and immunology. 2014;380:39-68.
463 22. Gianfrani C, Levings MK, Sartirana C, Mazzarella G, Barba G, Zanzi D, et al. Gliadin-
464 specific type 1 regulatory T cells from the intestinal mucosa of treated celiac patients inhibit
465 pathogenic T cells. Journal of immunology. 2006;177(6):4178-86.
466 23. Meiler F, Zumkehr J, Klunker S, Ruckert B, Akdis CA, Akdis M. In vivo switch to IL-
467 10-secreting T regulatory cells in high dose allergen exposure. The Journal of experimental
468 medicine. 2008;205(12):2887-98.
469 24. Passerini L, Di Nunzio S, Gregori S, Gambineri E, Cecconi M, Seidel MG, et al.
470 Functional type 1 regulatory T cells develop regardless of FOXP3 mutations in patients with
471 IPEX syndrome. European journal of immunology. 2011;41(4):1120-31.
472 25. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, et al.
473 Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells.
474 Nature medicine. 2013;19(6):739-46.
475 26. Bacchetta R, de Waal Malefijt R, Yssel H, Abrams J, de Vries JE, Spits H, et al. Host-
476 reactive CD4+ and CD8+ T cell clones isolated from a human chimera produce IL-5, IL-2, IFN-
477 gamma and granulocyte/macrophage-colony-stimulating factor but not IL-4. Journal of
478 immunology. 1990;144(3):902-8.
479 27. Levings MK, Bacchetta R, Schulz U, Roncarolo MG. The role of IL-10 and TGF-beta in
480 the differentiation and effector function of T regulatory cells. International archives of allergy
481 and immunology. 2002;129(4):263-76.
482 28. Cottrez F, Hurst SD, Coffman RL, Groux H. T regulatory cells 1 inhibit a Th2-specific
483 response in vivo. Journal of immunology. 2000;165(9):4848-53.
484 29. Hawrylowicz CM, O'Garra A. Potential role of interleukin-10-secreting regulatory T cells
485 in allergy and asthma. Nature reviews Immunology. 2005;5(4):271-83.
486 30. Chinthrajah RS, Hernandez JD, Boyd SD, Galli SJ, Nadeau KC. Molecular and cellular
487 mechanisms of food allergy and food tolerance. The Journal of allergy and clinical immunology.
488 2016;137(4):984-97.
489 31. Weiner HL, da Cunha AP, Quintana F, Wu H. Oral tolerance. Immunological reviews.
490 2011;241(1):241-59.
491 32. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, et al. Immune
492 responses in healthy and allergic individuals are characterized by a fine balance between
493 allergen-specific T regulatory 1 and T helper 2 cells. The Journal of experimental medicine.
494 2004;199(11):1567-75.
495 33. Syed A, Garcia MA, Lyu SC, Bucayu R, Kohli A, Ishida S, et al. Peanut oral
496 immunotherapy results in increased antigen-induced regulatory T-cell function and
497 hypomethylation of forkhead box protein 3 (FOXP3). The Journal of allergy and clinical
498 immunology. 2014;133(2):500-10.
499 34. Vickery BP, Scurlock AM, Jones SM, Burks AW. Mechanisms of immune tolerance
500 relevant to food allergy. The Journal of allergy and clinical immunology. 2011;127(3):576-84;
501 quiz 85-6.
502 35. Tsuji NM, Mizumachi K, Kurisaki J. Interleukin-10-secreting Peyer's patch cells are
503 responsible for active suppression in low-dose oral tolerance. Immunology. 2001;103(4):458-64.

36. Gregori S, Roncarolo MG, Bacchetta R. Methods for in vitro generation of human type 1 regulatory T cells. Methods in molecular biology. 2011;677:31-46.
37. Pacciani V, Gregori S, Chini L, Corrente S, Chianca M, Moschese V, et al. Induction of anergic allergen-specific suppressor T cells using tolerogenic dendritic cells derived from children with allergies to house dust mites. The Journal of allergy and clinical immunology. 2010;125(3):727-36.
38. Sampson HA, Munoz-Furlong A, Bock SA, Schmitt C, Bass R, Chowdhury BA, et al. Symposium on the definition and management of anaphylaxis: summary report. The Journal of allergy and clinical immunology. 2005;115(3):584-91.
39. Bacchetta R, Gregori S, Serafini G, Sartirana C, Schulz U, Zino E, et al. Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy. Haematologica. 2010;95(12):2134-43.
40. Bacchetta R, Lucarelli B, Sartirana C, Gregori S, Lupo Stanghellini MT, Miqueu P, et al. Immunological Outcome in Haploidentical-HSC Transplanted Patients Treated with IL-10-Anergized Donor T Cells. Frontiers in immunology. 2014;5:16.
41. Bacher P, Scheffold A. Flow-Cytometric Analysis of Rare Antigen-Specific T Cells. Cytom Part A. 2013;83A(8):692-701.
42. Kim SV, Xiang WV, Kwak C, Yang Y, Lin XW, Ota M, et al. GPR15-mediated homing controls immune homeostasis in the large intestine mucosa. Science. 2013;340(6139):1456-9.
43. Nguyen LP, Pan J, Dinh TT, Hadeiba H, O'Hara E, 3rd, Ebtikar A, et al. Role and species-specific expression of colon T cell homing receptor GPR15 in colitis. Nature immunology. 2015;16(2):207-13.
44. Agace WW. T-cell recruitment to the intestinal mucosa. Trends in immunology. 2008;29(11):514-22.
45. Srivastava KD, Siefert A, Fahmy TM, Caplan MJ, Li XM, Sampson HA. Investigation of peanut oral immunotherapy with CpG/peanut nanoparticles in a murine model of peanut allergy. The Journal of allergy and clinical immunology. 2016.
46. Clemente-Casares X, Blanco J, Ambalavanan P, Yamanouchi J, Singha S, Fandos C, et al. Expanding antigen-specific regulatory networks to treat autoimmunity. Nature. 2016;530(7591):434-40.
47. DeLong JH, Simpson KH, Wambre E, James EA, Robinson D, Kwok WW. Ara h 1-reactive T cells in individuals with peanut allergy. The Journal of allergy and clinical immunology. 2011;127(5):1211-8 e3.
48. Wisniewski JA, Commins SP, Agrawal R, Hulse KE, Yu MD, Cronin J, et al. Analysis of cytokine production by peanut-reactive T cells identifies residual Th2 effectors in highly allergic children who received peanut oral immunotherapy. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2015;45(7):1201-13.
49. Kamanaka M, Kim ST, Wan YY, Sutterwala FS, Lara-Tejero M, Galan JE, et al. Expression of interleukin-10 in intestinal lymphocytes detected by an interleukin-10 reporter knockin tiger mouse. Immunity. 2006;25(6):941-52.
50. Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. The Journal of clinical investigation. 1998;102(1):98-106.
51. Reefer AJ, Carneiro RM, Custis NJ, Platts-Mills TA, Sung SS, Hammer J, et al. A role for IL-10-mediated HLA-DR7-restricted T cell-dependent events in development of the modified Th2 response to cat allergen. Journal of immunology. 2004;172(5):2763-72.

52. Sicherer SH, Furlong TJ, Maes HH, Desnick RJ, Sampson HA, Gelb BD. Genetics of peanut allergy: a twin study. The Journal of allergy and clinical immunology. 2000;106(1 Pt 1):53-6.
53. Hershey GK, Friedrich MF, Esswein LA, Thomas ML, Chatila TA. The association of atopy with a gain-of-function mutation in the alpha subunit of the interleukin-4 receptor. The New England journal of medicine. 1997;337(24):1720-5.
54. Kayserova J, Sismova K, Zentsova-Jaresova I, Katina S, Vernerova E, Polouckova A, et al. A prospective study in children with a severe form of atopic dermatitis: clinical outcome in relation to cytokine gene polymorphisms. Journal of investigational allergology & clinical immunology. 2012;22(2):92-101.
55. Namkung JH, Lee JE, Kim E, Kim HJ, Seo EY, Jang HY, et al. Association of polymorphisms in genes encoding IL-4, IL-13 and their receptors with atopic dermatitis in a Korean population. Experimental dermatology. 2011;20(11):915-9.
56. Namkung JH, Lee JE, Kim E, Park GT, Yang HS, Jang HY, et al. An association between IL-9 and IL-9 receptor gene polymorphisms and atopic dermatitis in a Korean population. Journal of dermatological science. 2011;62(1):16-21.
57. Hemler JA, Phillips EJ, Mallal SA, Kendall PL. The evolving story of human leukocyte antigen and the immunogenetics of peanut allergy. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology. 2015;115(6):471-6.
58. Asai Y, Greenwood C, Hull PR, Alizadehfar R, Ben-Shoshan M, Brown SJ, et al. Filaggrin gene mutation associations with peanut allergy persist despite variations in peanut allergy diagnostic criteria or asthma status. The Journal of allergy and clinical immunology. 2013;132(1):239-42.
59. DeVries A, Vercelli D. Early predictors of asthma and allergy in children: the role of epigenetics. Current opinion in allergy and clinical immunology. 2015;15(5):435-9.
60. Martino D, Dang T, Sexton-Oates A, Prescott S, Tang ML, Dharmage S, et al. Blood DNA methylation biomarkers predict clinical reactivity in food-sensitized infants. The Journal of allergy and clinical immunology. 2015;135(5):1319-28 e1-12.
61. Andolfi G, Fousteri G, Rossetti M, Magnani CF, Jofra T, Locafaro G, et al. Enforced IL-10 expression confers type 1 regulatory T cell (Tr1) phenotype and function to human CD4(+) T cells. Molecular therapy : the journal of the American Society of Gene Therapy. 2012;20(9):1778-90.

Table 1: Demographic and clinical description of the study cohorts

Fig. 1: Pea-mDC and pea-DC-10 from HC and PA individuals present a mature phenotype.

Expression levels of CD11c, CD14 and CD86 in mature (mDC) and tolerogenic (DC-10) DC of healthy controls (HC) and peanut-allergic (PA) individuals. Representative data from one HC out of n=7 (upper panel) and one PA individual out of n=21 (lower panel) are shown. Red: mDC, unstained; blue: mDC; green: DC-10, unstained; orange: DC-10.

Fig 2: Tr1 cells are induced at similar frequencies from HC and PA individuals.

Frequency of $CD4^+CD45RA^-CD49b^+LAG3^+$ Tr1 cells in pea-Tm and pea-T10 of healthy controls (HC; **A**, **B** and **D**) and peanut allergic (PA) individuals (**A** and **C**-**E**). **A.** Dot plots for a representative HC out of 7 (upper panel) and PA individuals out of 21 (lower panel). Cumulative data of LAG3 and CD49b expression is represented for HC (**B**) and PA individuals (**C**). **D.** Comparison of the percentages of $CD49b^+LAG3^+$ cells in pea-T10 of HC and PA individuals **E.** Comparison of the percentages of $CD49b^+LAG3^+$ cells in pea-T10 of all 3 cohorts of PA individuals (Bas: baseline; ST-OIT: short-term oral immunotherapy; LT-OIT: long-term OIT). Mean values and standard deviation are shown.

Fig. 3: Pea-DC-10 induce antigen-specific $CD4^+$ T cell anergy in all HC but only in a minority of PA individuals.

Proliferation of pea-Tm and pea-T10 cell lines stimulated with autologous Ara h 1/2 (pea)-mDC for 5 days for healthy controls (HC) and peanut allergic (PA) individuals (Bas: baseline; ST-OIT: short-term oral immunotherapy; LT-OIT: long-term OIT) **A.** Dot plot for a representative HC (left panel), and 2 representative PA individuals (PA-1 presenting anergy and PA-2 not presenting anergy; middle and right panels, respectively) are shown. **B.** Proliferative responses of pea-Tm (black) and pea-T10 (grey) are shown. Percentage of anergy (calculated as percentage of proliferating (pea-Tm - pea-T10)/pea-Tm) is indicated for each experiment. ni: Anergy not induced. **C.** Cumulative data for HC (n=7) and PA individuals (n=21) and **D.** Comparison of PA cohorts, each dot represents a single experiment.

Fig. 4: Pea-T10 of HC but not PA individuals produce less Th2 cytokines than pea-Tm.

Detection of cytokines by ELISA (in pg/mL) in supernatants of pea-Tm and pea-T10 of healthy control (HC) and peanut-allergic (PA) individuals restimulated with pea-mDC for 48h. Results are shown for **A.** IL-4 and **B.** IL-5 in HC (left panels) and PA individuals (middle panels), each

dot representing a single experiment. Right panels represent a comparison of all HC and PA individuals; mean cytokine detection levels are shown.

Fig. 5: Peanut-specific T cells are more abundant in the Tr1 cells compared to total memory CD4$^+$ population of pea-T10 in PA individuals.

Expression of CD69 and CD137 after restimulation with pea-mDC was assessed for pea-T10 of peanut allergic individuals (PA). Dot plot for a representative donor shows expression of CD69 (**A**) and CD137 (**B**) in the CD45RA$^-$ and CD49b$^+$LAG3$^+$ populations for cells unstimulated (upper panels) or stimulated with pea-mDC (lower panels). The percentage of CD4$^+$ T cells specifically upregulating CD69 (**C**, n=14) and CD137 (**D**, n=12) upon activation (subtracting the background from the unstimulated cells) is represented for each donor within the CD45RA$^-$ and CD49b$^+$LAG3$^+$ populations.

Fig. 6: Expression of gut-homing molecules is higher in Tr1 cells than in total memory CD4$^+$ population of pea-T10.

Expression of the gut-homing molecules GPR15 (**A**) and CCR9 (**B**) in CD49b$^+$LAG3$^+$ Tr1 and total memory populations of pea-T10 cells of healthy controls (HC) and peanut allergic (PA) individuals. Cumulative data are represented for **A.** n=5 HC and 19 PA individuals and **B.** n=2 HC and 13 PA. Green: HC; red: PA individuals.

Fig. E1: Scheme of the induction of mDC, DC-10, peanut-specific T cell lines and readouts.

A. DC and T cell line induction and **B.** Readouts for the T cell lines. DC-10: tolerogenic dendritic cells; mDC: mature dendritic cells; pea-: peanut; pea-T10: CD4$^+$ T cells co-incubated with tolerogenic dendritic cells pulsed with the main peanut allergens; pea-Tm: CD4$^+$ T cells co-incubated with mature dendritic cells pulsed with the main peanut allergens; TT: tetanus toxoid.

Fig. E2: mDC and DC-10 from HC and PA individuals present a mature phenotype.

Expression levels of **A.** CD11c, **B.** CD14 and **C.** CD86 in mature (mDC) and tolerogenic (DC-10) DC of healthy controls (HC, n=7) and peanut-allergic (PA, n=21) individuals. Comparison of the percentages of CD11c, CD14 and CD86 cells in mDC and DC-10 of all 3 cohorts of PA individuals (Bas: baseline; ST-OIT, n=7: short-term oral immunotherapy; LT-OIT, n=7: long-term OIT, n=7).

Fig. E3: Pea-Tm and pea-T10 of HC and PA individuals present a partially activated phenotype.

Frequency of $CD4^+CD45RA^-CD25^+$ cells in pea-Tm and pea-T10 of healthy controls (HC) and peanut allergic (PA) individuals. **A.** Dot plot for a representative HC (out of 7 tested; upper panel) and PA individual (out of 21 tested; lower panel) donors show expression of CD25 in pea-Tm (left panel) and pea-T10 (right panel). **B.** Histogram representing the cumulative data of CD25 expression for HC and PA individuals and **C.** Comparison of PA cohorts (Bas: baseline; ST-OIT, n=7: short-term oral immunotherapy; LT-OIT, n=7: long-term OIT, n=7).. Mean and standard deviation are shown.

Fig. E4: Pea-DC-10 induce antigen-specific $CD4^+$ T cell anergy in all HC but only in a minority of PA individuals.

Proliferation of pea-Tm and pea-T10 cell lines stimulated with autologous Ara h 1/2 (pea)-mDC for 4 days or tetanus (TT)-mDC for 5 days for healthy controls (HC) and peanut allergic (PA) individuals (Bas: baseline; ST-OIT: short-term oral immunotherapy; LT-OIT: long-term OIT) **A.** Proliferative responses of pea-Tm (black) and pea-T10 (grey) in response to Ara h 1/2. **B.** Cumulative data for HC and PA individuals and **C.** comparison of PA cohorts, each dot represents a single experiment. **D.** Dot plots of proliferation in response to TT restimulation are shown for representative HC (left panel), and PA individuals. **E.** Proliferative responses of pea-Tm (black) and pea-T10 (grey) to TT are shown. Percentage of anergy (calculated as percentage of proliferating (pea-Tm - pea-T10)/pea-Tm) is indicated for each experiment. ni: Anergy not induced.

Figure E5: Levels of IL-10 and IFN-γ are comparable in pea-Tm and pea-T10 of HC and PA individuals, and levels of IL-4 and IL-5 in pea-T10 are negatively correlated with the percentage of anergy.

Detection of cytokines by ELISA (in pg/mL) in supernatants of pea-Tm and pea-T10 of healthy controls (HC) and peanut-allergic individuals (PA) restimulated with pea-mDC for 48h. Results are shown for **A.** IL-10 and **B.** IFN-γ of HC (left panels) and PA individuals (middle panels), each dot representing a single experiment. Right panels represent a comparison of all HC and PA individuals; mean cytokine detection levels are shown. Scatter plots comparing the levels of **C.** IL-4 (in pg/mL), **D.** IL-5 (in pg/mL) and **E.** IL-10 (in pg/mL) detected in pea-T10 cultures restimulated with pea-mDC for 48h and the percentage of anergy (calculated as percentage of proliferating (pea-Tm - pea-T10)/pea-Tm) after restimulation with pea-mDC for 5 days). Trendline is shown, correlation coefficient and statistical significance are indicated.

<u>Fig. E6:</u> TT-specific T cells are present in the same frequencies in the Tr1 cells compared to total memory CD4$^+$ population of pea-T10 in PA individuals.

Expression of CD69 and CD137 after restimulation with TT-mDC was assessed for pea-T10 of peanut allergic individuals (PA). Dot plot for a representative donor shows expression of CD69 (**A**) and CD137 (**B**) in the CD45RA$^-$ and CD49b$^+$LAG3$^+$ populations for cells unstimulated (upper panels) or stimulated with TT-mDC (lower panels). The percentage of CD4$^+$ T cells specifically upregulating CD69 (**C**, n=13) and CD137 (**D**, n=9) upon activation (subtracting the background from the unstimulated cells) is represented for each donor within the CD45RA$^-$ and CD49b$^+$LAG3$^+$ populations.

<u>Table S1:</u> Summary table of the data obtained on pea-Tm and pea-T10 cells

For each experiment, the number of samples tested is indicated. Anergy is indicated only when present.

Characteristics	Baseline	Short-term OIT (3 to 12months)	Long-term OIT (>3years)
n	7	7	7
Sex (F/M)	4/3	2/5	4/3
Age (years)	11.9+/-3.1	18.9+/-15.3	14.6+/-6.9
Range	8-15	8-51	10-30
Concurrent symptoms of asthma	5/7	5/7	3/7
Concurrent symptoms of allergic rhinitis	6/7	6/7	4/7
Concurrent symptoms of atopic dermatitis	3/7	5/7	3/7
Family history of atopic diseases	3/7	6/7	4/7

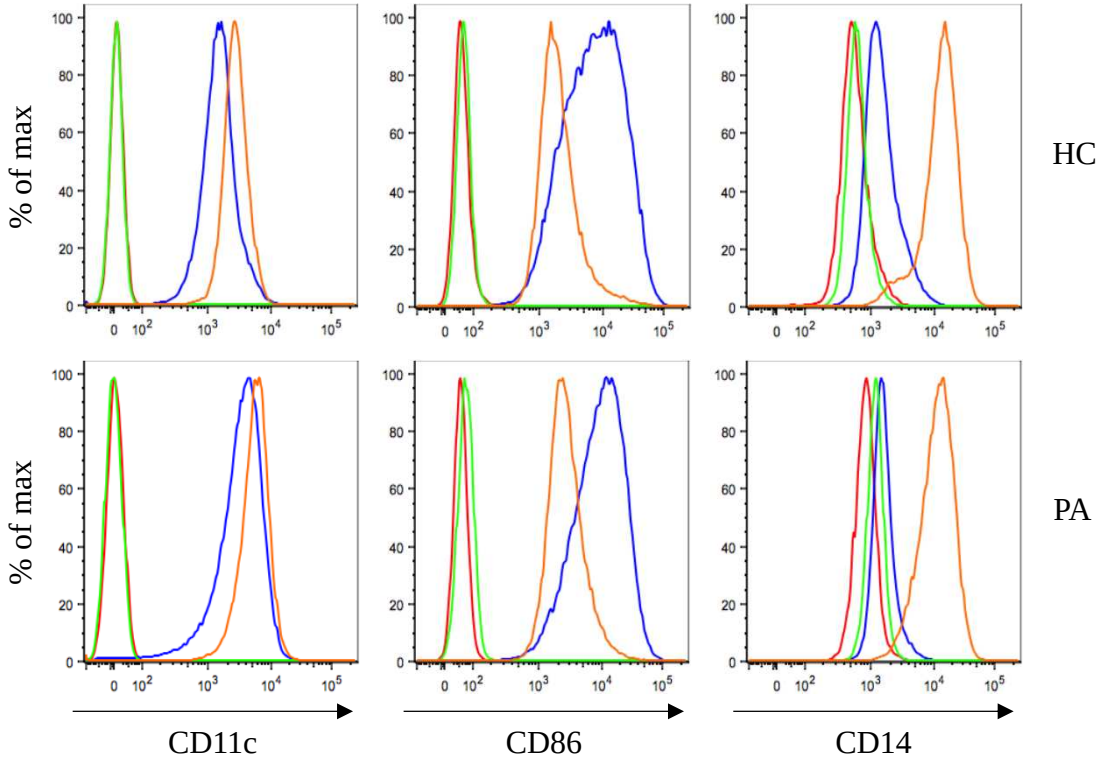


Figure 1

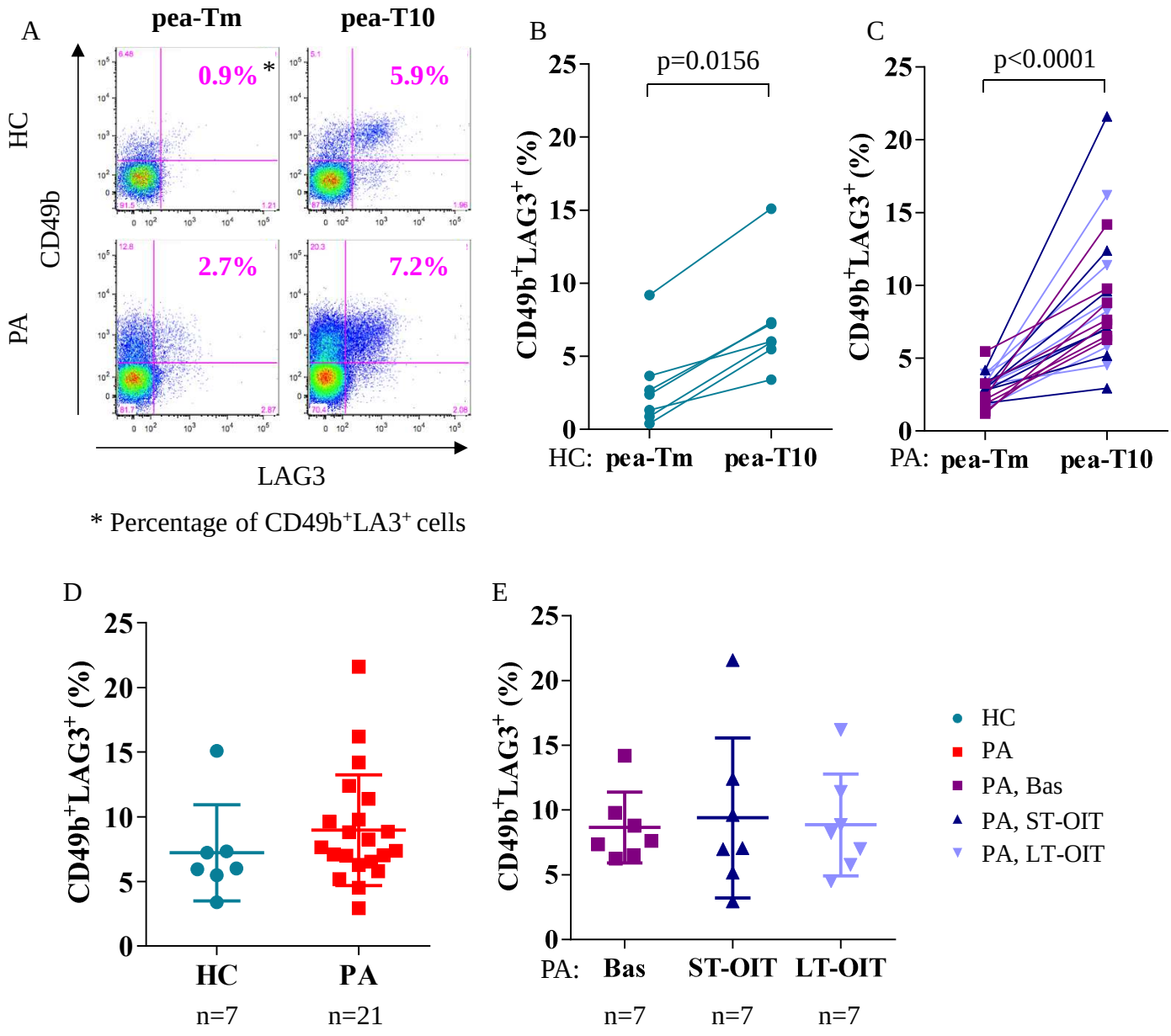

A
pea-Tm
pea-T10
HC
PA
CD49b
LAG3
0.9% *
5.9%
2.7%
7.2%
B
p=0.0156
CD49b+LAG3+ (%)
HC: pea-Tm pea-T10
C
p<0.0001
PA: pea-Tm pea-T10
* Percentage of CD49b+LA3+ cells
D
CD49b+LAG3+ (%)
HC
PA
n=7
n=21
E
PA: Bas ST-OIT LT-OIT
n=7
n=7
n=7
HC
PA
PA, Bas
PA, ST-OIT
PA, LT-OIT

Figure 2

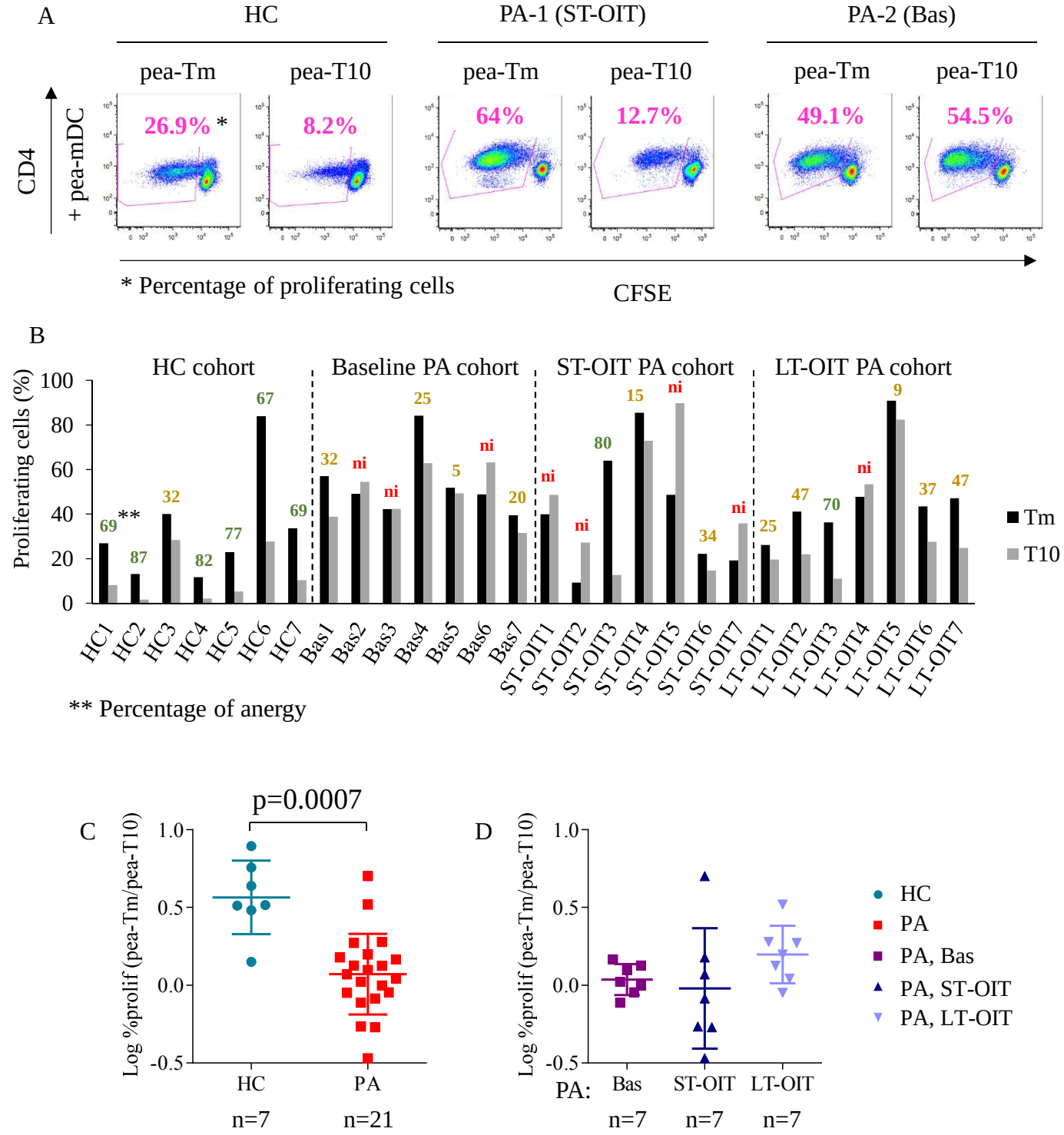

A
HC
PA-1 (ST-OIT)
PA-2 (Bas)
pea-Tm
pea-T10
pea-Tm
pea-T10
pea-Tm
pea-T10
26.9% *
8.2%
64%
12.7%
49.1%
54.5%
CD4
+ pea-mDC
* Percentage of proliferating cells
CFSE
B
HC cohort
Baseline PA cohort
ST-OIT PA cohort
LT-OIT PA cohort
Proliferating cells (%)
100
80
60
40
20
0
Tm
T10
HC1
HC2
HC3
HC4
HC5
HC6
HC7
Bas1
Bas2
Bas3
Bas4
Bas5
Bas6
Bas7
ST-OIT1
ST-OIT2
ST-OIT3
ST-OIT4
ST-OIT5
ST-OIT6
ST-OIT7
LT-OIT1
LT-OIT2
LT-OIT3
LT-OIT4
LT-OIT5
LT-OIT6
LT-OIT7
** Percentage of anergy
C
p=0.0007
Log %prolif (pea-Tm/pea-T10)
1.0
0.5
0.0
-0.5
HC
PA
n=7
n=21
D
Log %prolif (pea-Tm/pea-T10)
1.0
0.5
0.0
-0.5
PA:
Bas
ST-OIT
LT-OIT
n=7
n=7
n=7
HC
PA
PA, Bas
PA, ST-OIT
PA, LT-OIT

Figure 3

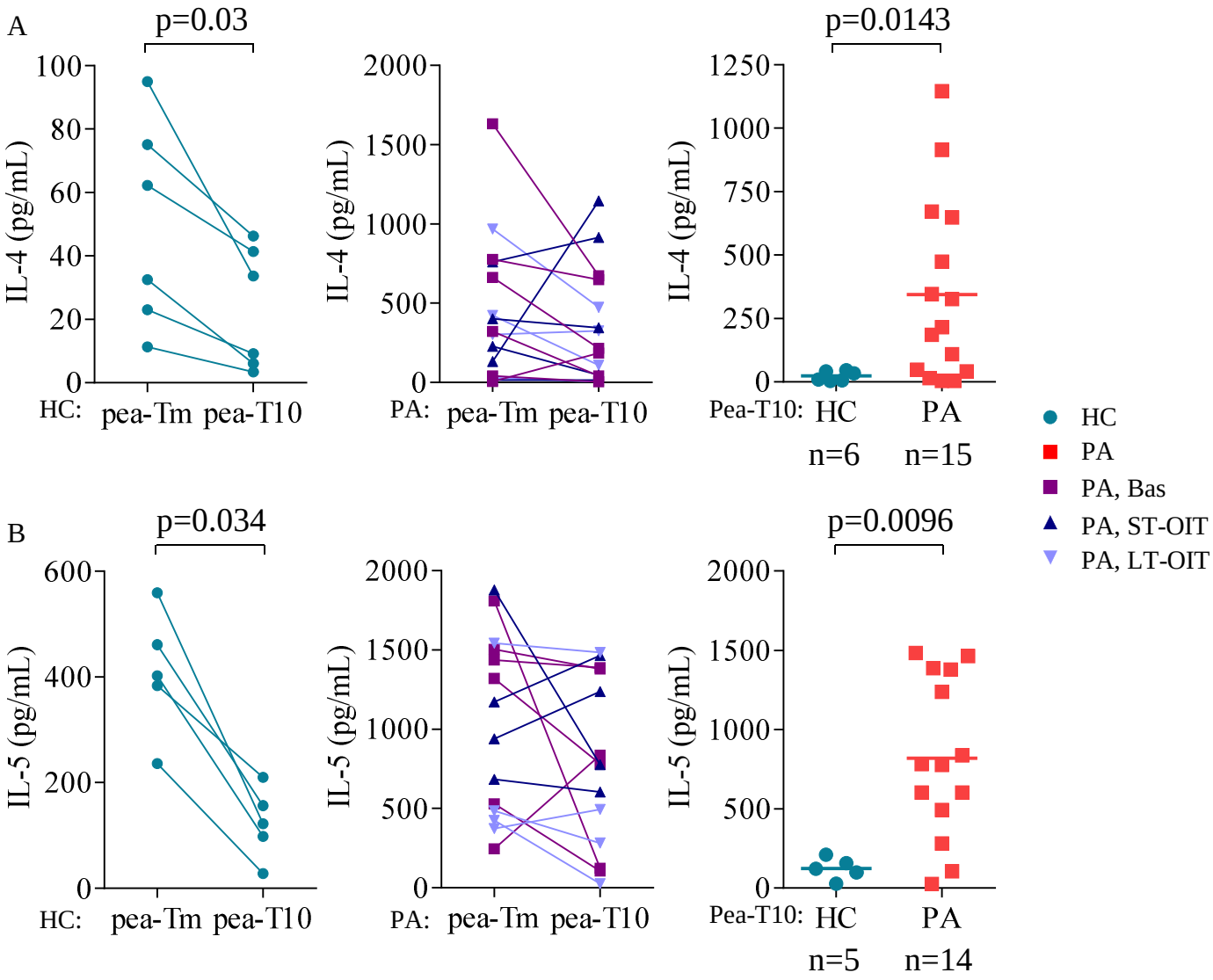

A
p=0.03
IL-4 (pg/mL)
100
80
60
40
20
0
HC:
pea-Tm
pea-T10
2000
1500
1000
500
0
PA:
pea-Tm
pea-T10
p=0.0143
1250
1000
750
500
250
0
Pea-T10:
HC
PA
n=6
n=15
HC
PA
PA, Bas
PA, ST-OIT
PA, LT-OIT
B
p=0.034
IL-5 (pg/mL)
600
400
200
0
HC:
pea-Tm
pea-T10
2000
1500
1000
500
0
PA:
pea-Tm
pea-T10
p=0.0096
2000
1500
1000
500
0
Pea-T10:
HC
PA
n=5
n=14

Figure 4

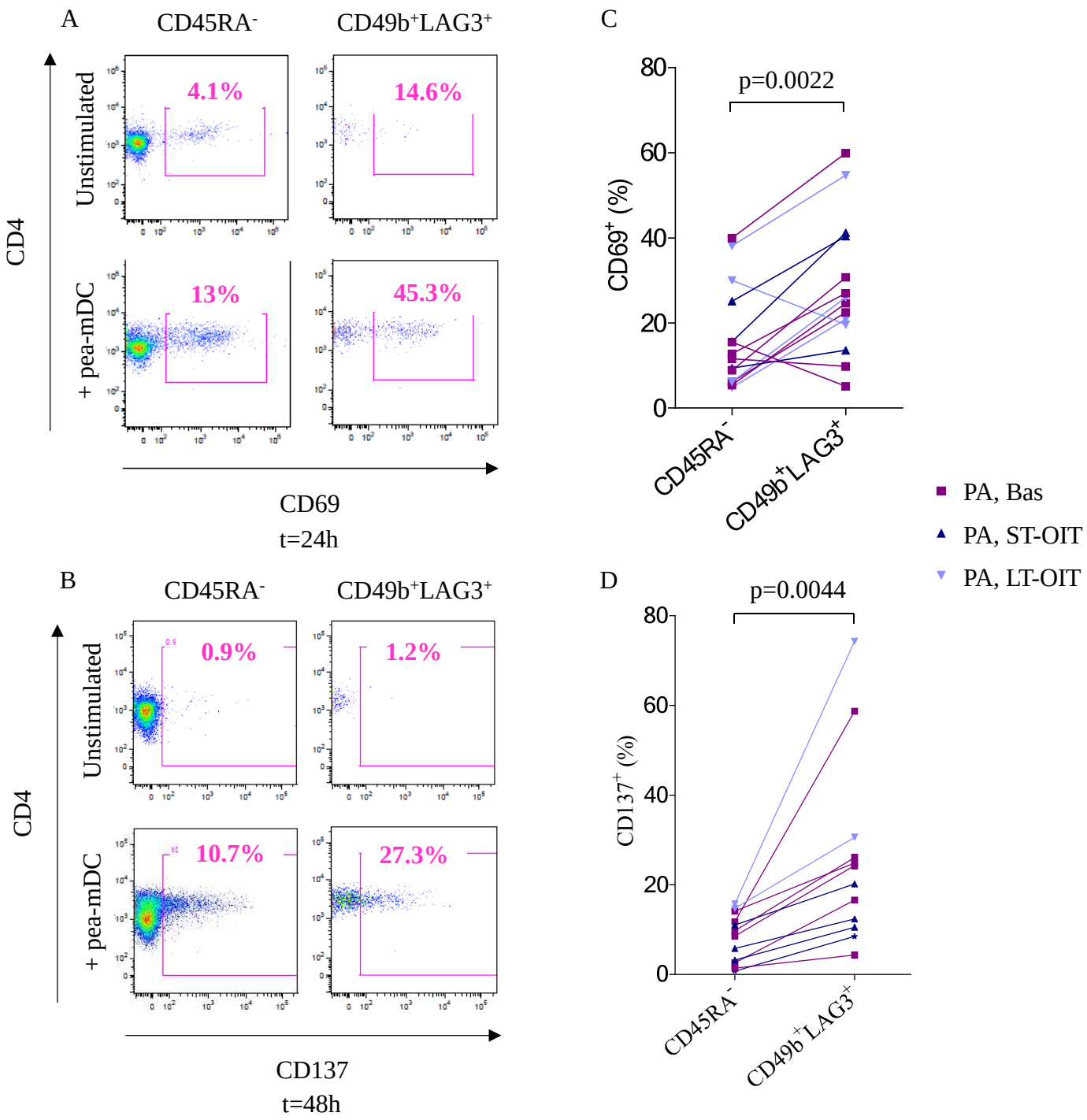

A
CD45RA-
CD49b+LAG3+
Unstimulated
+ pea-mDC
4.1%
14.6%
13%
45.3%
CD4
CD69
t=24h
B
CD45RA-
CD49b+LAG3+
Unstimulated
+ pea-mDC
0.9%
1.2%
10.7%
27.3%
CD4
CD137
t=48h
C
p=0.0022
CD69+ (%)
CD45RA-
CD49b+LAG3+
PA, Bas
PA, ST-OIT
PA, LT-OIT
D
p=0.0044
CD137+ (%)
CD45RA-
CD49b+LAG3+

Figure 5

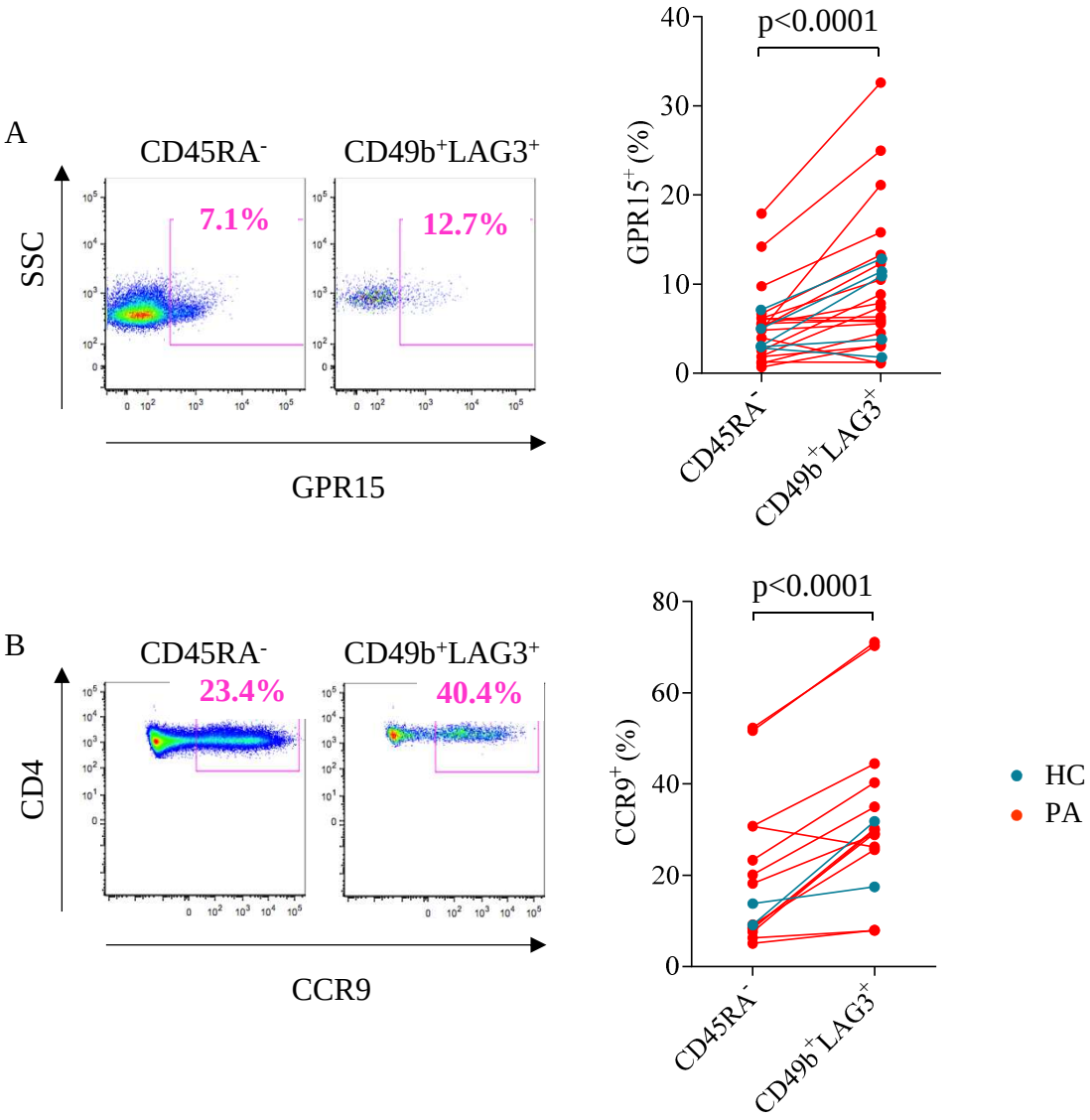

A
CD45RA-
CD49b+LAG3+
7.1%
12.7%
SSC
GPR15
p<0.0001
GPR15+ (%)
CD45RA-
CD49b+LAG3+
B
CD45RA-
CD49b+LAG3+
23.4%
40.4%
CD4
CCR9
p<0.0001
CCR9+ (%)
CD45RA-
CD49b+LAG3+
HC
PA

Figure 6

		% CD25+ (mean±SD)	% LAG3+CD49b+ (mean±SD)	% anergy (mean, [range])	IL-4 concentration (pg/mL)	IL-5 concentration (pg/mL)	IL-10 concentration (pg/mL)	IFN-γ concentration (pg/mL)
HC	Pea-Tm	28.2±6.7 n=7	2.6±3 n=7		49.9±32.7 n=6	408.5±118.2 n=5	208.5±50.77 n=6	787±438.1 n=6
	Pea-T10	34.3±9.9 n=7	7.4±3.7 n=7	69 [32-87] n=7	23.4±19.3 n=6	123.1±67.6 n=5	175±131.9 n=6	488±422.5 n=6
PA, total	Pea-Tm	38.1±12.7 n=21	2.9±1 n=21		447±449.6 n=15	1024.2±566.4 n=14	873.3±602.8 n=15	461.3±458.1 n=15
	Pea-T10	38±13.3 n=21	9±4.3 n=21	34.3 [5-80] n=6	343.5±358.7 n=15	784.2±534.8 n=14	450.3±268.9 n=15	488.24±425.3 n=15

		% CD69+ (Memory, mean±SD)	% CD69+ (Tr1, mean±SD)	% CD137+ (Memory, mean±SD)	% CD137+ (Tr1, mean±SD)	% GPR15+ (Memory, mean±SD)	% GPR15+ (Tr1, mean±SD)	%CCR9+ (Memory, mean±SD)	%CCR9+ (Tr1, mean±SD)
HC	Pea-Tm								
	Pea-T10					4.2±1.8 n=5	8.1±4.9 n=5	11.7±3.3 n=2	24.9±10.1 n=2
PA, total	Pea-Tm								
	Pea-T10	15.5±10.8 n=12	26.7±15.1 n=12	9.2±5.1 n=13	26.9±16.3 n=13	5.6±4.4 n=19	10.1±8.4 n=19	20.93±16.4 n=13	34.4±19.2 n=13

OR FIGURE LEGENDS

Fig. E1: Scheme of the induction of mDC, DC-10, peanut-specific T cell lines and readouts.

A. DC and T cell line induction and **B.** Readouts for the T cell lines. DC-10: tolerogenic dendritic cells; mDC: mature dendritic cells; pea-: peanut; pea-T10: $CD4^+$ T cells co-incubated with tolerogenic dendritic cells pulsed with the main peanut allergens; pea-Tm: $CD4^+$ T cells co-incubated with mature dendritic cells pulsed with the main peanut allergens; TT: tetanus toxoid.

Fig. E2: mDC and DC-10 from HC and PA individuals present a mature phenotype.

Expression levels of **A.** CD11c, **B.** CD14 and **C.** CD86 in mature (mDC) and tolerogenic (DC-10) DC of healthy controls (HC, n=7) and peanut-allergic (PA, n=21) individuals. Comparison of the percentages of CD11c, CD14 and CD86 cells in mDC and DC-10 of all 3 cohorts of PA individuals (Bas: baseline; ST-OIT, n=7: short-term oral immunotherapy; LT-OIT, n=7: long-term OIT, n=7).

Fig. E3: Pea-Tm and pea-T10 of HC and PA individuals present a partially activated phenotype.

Frequency of $CD4^+CD45RA^-CD25^+$ cells in pea-Tm and pea-T10 of healthy controls (HC) and peanut allergic (PA) individuals. **A.** Dot plot for a representative HC (out of 7 tested; upper panel) and PA individual (out of 21 tested; lower panel) donors show expression of CD25 in pea-Tm (left panel) and pea-T10 (right panel). **B.** Histogram representing the cumulative data of CD25 expression for HC and PA individuals and **C.** Comparison of PA cohorts (Bas: baseline; ST-OIT, n=7: short-term oral immunotherapy; LT-OIT, n=7: long-term OIT, n=7).. Mean and standard deviation are shown.

Fig. E4: Pea-DC-10 induce antigen-specific $CD4^+$ T cell anergy in all HC but only in a minority of PA individuals.

Proliferation of pea-Tm and pea-T10 cell lines stimulated with autologous Ara h 1/2 (pea)-mDC for 4 days or tetanus (TT)-mDC for 5 days for healthy controls (HC) and peanut allergic (PA) individuals (Bas: baseline; ST-OIT: short-term oral immunotherapy; LT-OIT: long-term OIT) **A.** Proliferative responses of pea-Tm (black) and pea-T10 (grey) in response to Ara h 1/2. **B.** Cumulative data for HC and PA individuals and **C.** comparison of PA cohorts, each dot represents a single experiment. **D.** Dot plots of proliferation in response to TT restimulation are shown for representative HC (left panel), and PA individuals. **E.** Proliferative responses of pea-

Tm (black) and pea-T10 (grey) to TT are shown. Percentage of anergy (calculated as percentage of proliferating (pea-Tm - pea-T10)/pea-Tm) is indicated for each experiment. ni: Anergy not induced.

Figure E5: Levels of IL-10 and IFN-γ are comparable in pea-Tm and pea-T10 of HC and PA individuals, and levels of IL-4 and IL-5 in pea-T10 are negatively correlated with the percentage of anergy.

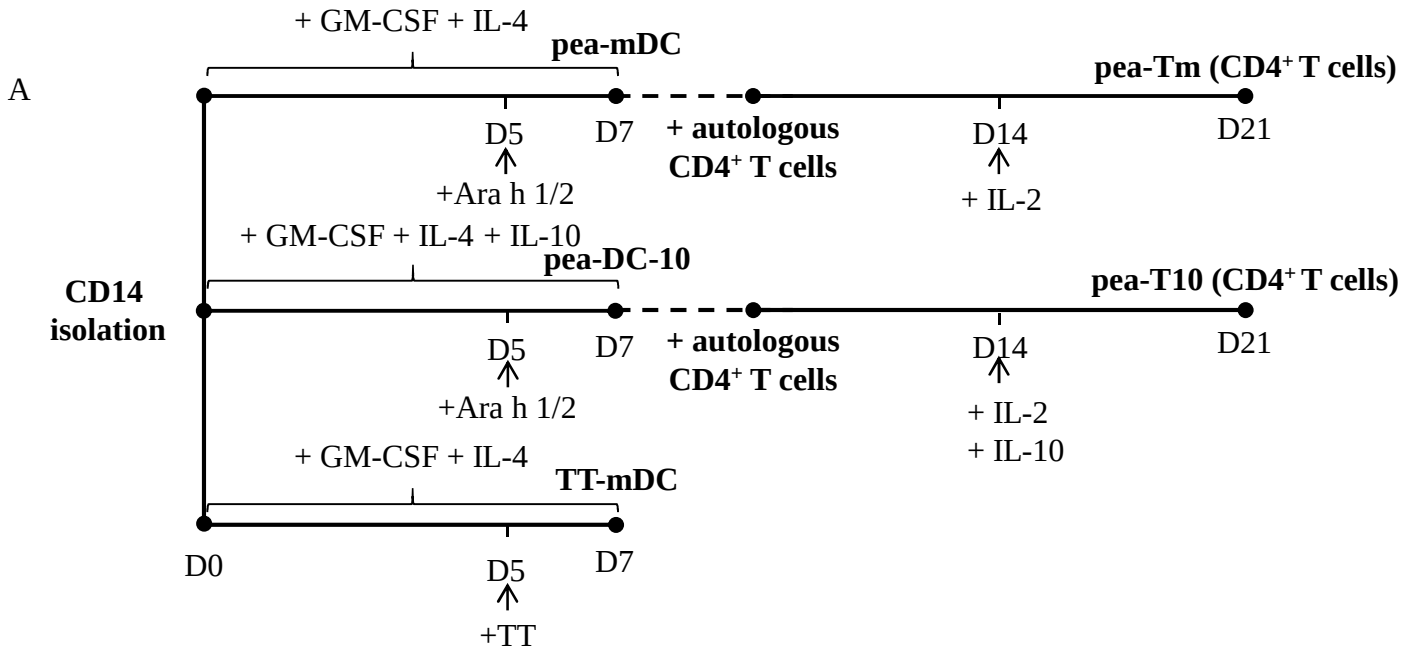
Detection of cytokines by ELISA (in pg/mL) in supernatants of pea-Tm and pea-T10 of healthy controls (HC) and peanut-allergic individuals (PA) restimulated with pea-mDC for 48h. Results are shown for **A.** IL-10 and **B.** IFN-γ of HC (left panels) and PA individuals (middle panels), each dot representing a single experiment. Right panels represent a comparison of all HC and PA individuals; mean cytokine detection levels are shown. Scatter plots comparing the levels of **C.** IL-4 (in pg/mL), **D.** IL-5 (in pg/mL) and **E.** IL-10 (in pg/mL) detected in pea-T10 cultures restimulated with pea-mDC for 48h and the percentage of anergy (calculated as percentage of proliferating (pea-Tm - pea-T10)/pea-Tm) after restimulation with pea-mDC for 5 days). Trendline is shown, correlation coefficient and statistical significance are indicated.

Fig. E6: TT-specific T cells are present in the same frequencies in the Tr1 cells compared to total memory $CD4^+$ population of pea-T10 in PA individuals.

Expression of CD69 and CD137 after restimulation with TT-mDC was assessed for pea-T10 of peanut allergic individuals (PA). Dot plot for a representative donor shows expression of CD69 (**A**) and CD137 (**B**) in the $CD45RA^-$ and $CD49b^+LAG3^+$ populations for cells unstimulated (upper panels) or stimulated with TT-mDC (lower panels). The percentage of $CD4^+$ T cells specifically upregulating CD69 (**C**, n=13) and CD137 (**D**, n=9) upon activation (subtracting the background from the unstimulated cells) is represented for each donor within the $CD45RA^-$ and $CD49b^+LAG3^+$ populations.

Table S1: Summary table of the data obtained on pea-Tm and pea-T10 cells

For each experiment, the number of samples tested is indicated. Anergy is indicated only when present.


A
+ GM-CSF + IL-4
pea-mDC
pea-Tm (CD4+ T cells)
D5
D7
+ autologous
CD4+ T cells
D14
D21
+Ara h 1/2
+ IL-2
+ GM-CSF + IL-4 + IL-10
pea-DC-10
CD14
isolation
pea-T10 (CD4+ T cells)
D5
D7
+ autologous
CD4+ T cells
D14
D21
+Ara h 1/2
+ IL-2
+ IL-10
+ GM-CSF + IL-4
TT-mDC
D0
D5
D7
+TT

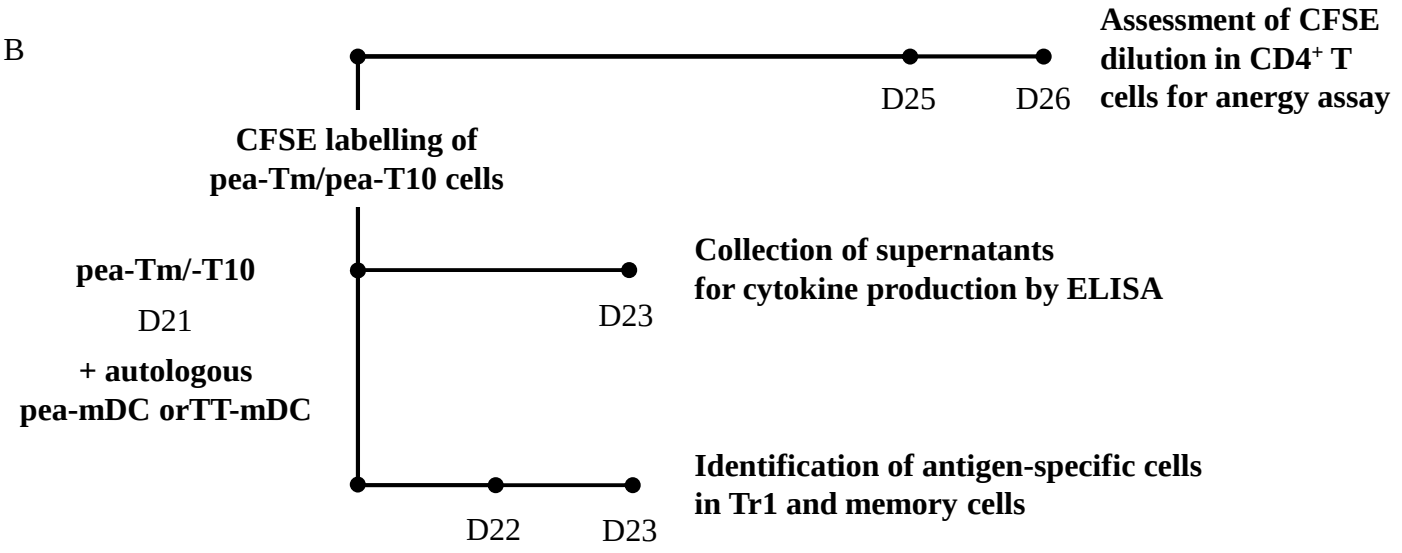

B
Assessment of CFSE
dilution in CD4+ T
cells for anergy assay
D25
D26
CFSE labelling of
pea-Tm/pea-T10 cells
pea-Tm/-T10
D21
+ autologous
pea-mDC orTT-mDC
Collection of supernatants
for cytokine production by ELISA
D23
Identification of antigen-specific cells
in Tr1 and memory cells
D22
D23

Figure E1

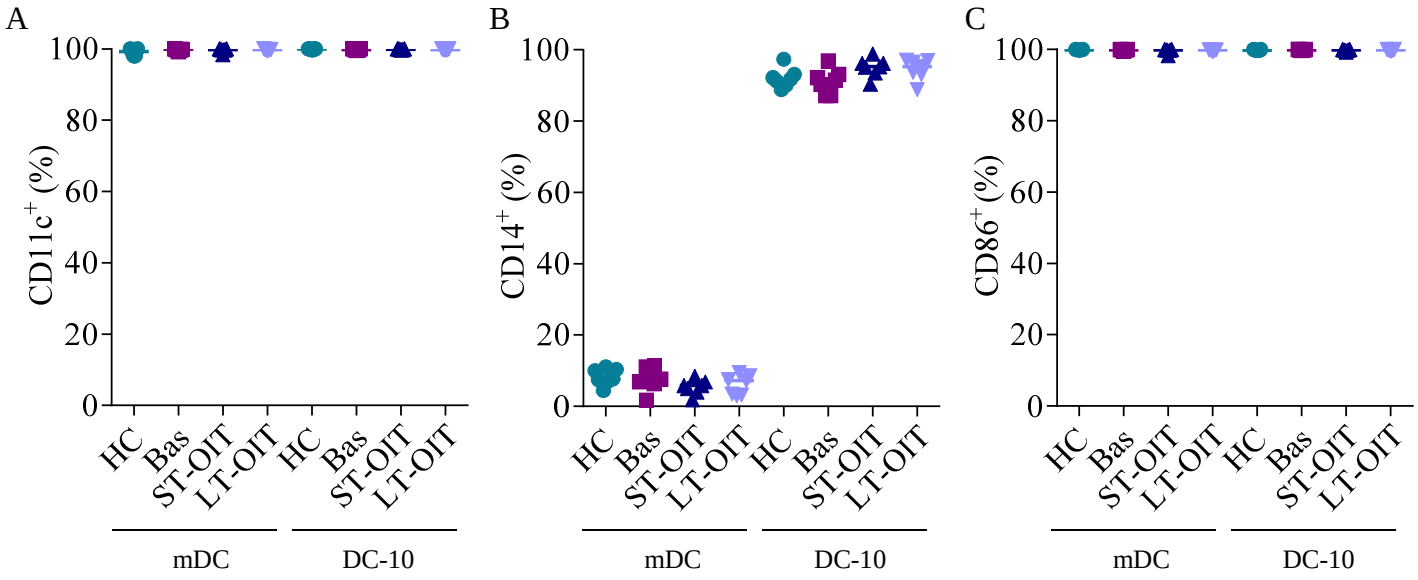

A
B
C
CD11c+ (%)
CD14+ (%)
CD86+ (%)
100
80
60
40
20
0
HC
Bas
ST-OIT
LT-OIT
mDC
DC-10

Figure E2

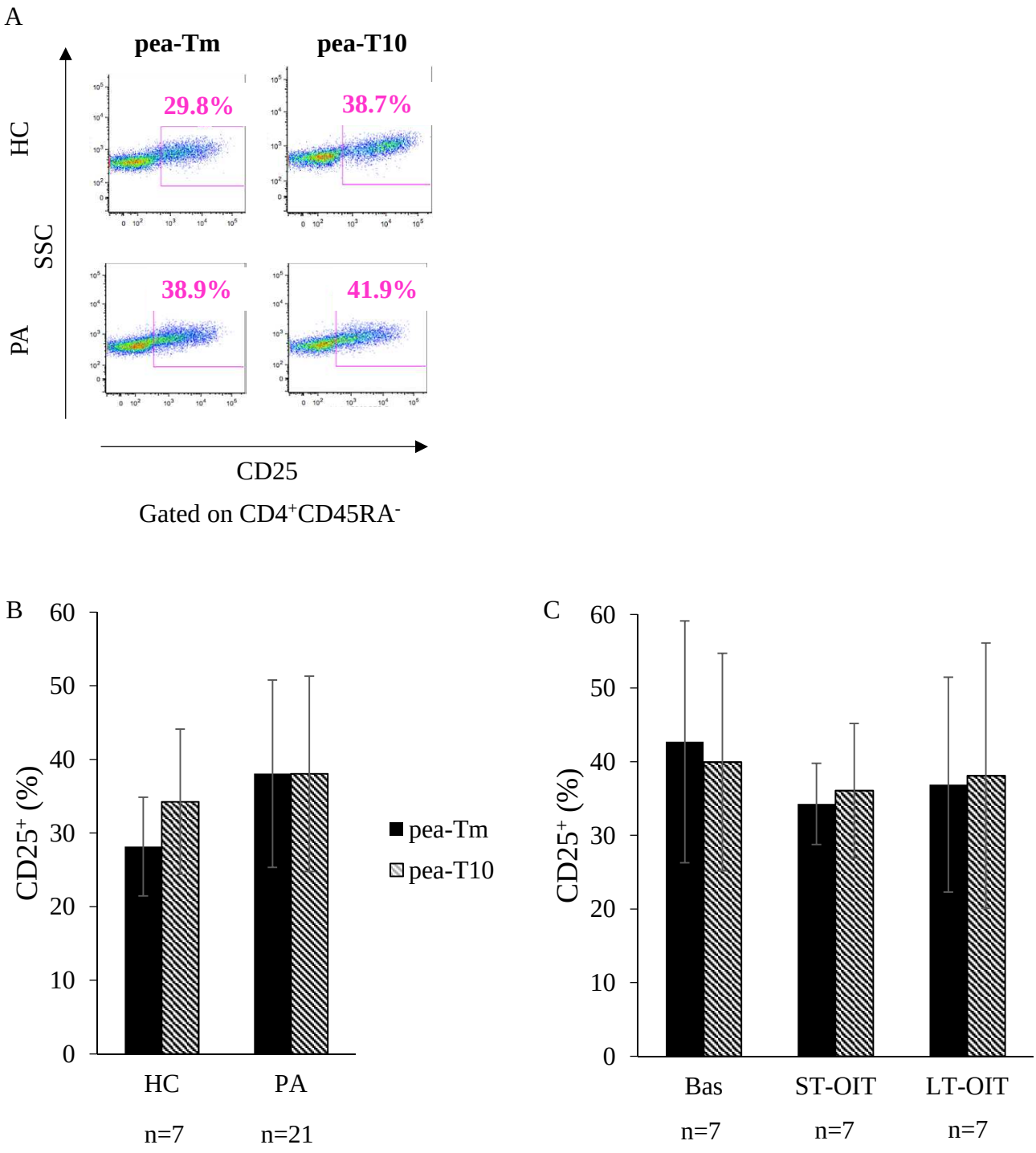

A
pea-Tm
pea-T10
29.8%
38.7%
38.9%
41.9%
HC
PA
SSC
CD25
Gated on CD4+CD45RA-
B
CD25+ (%)
60
50
40
30
20
10
0
pea-Tm
pea-T10
HC
PA
n=7
n=21
C
CD25+ (%)
Bas
ST-OIT
LT-OIT
n=7
n=7
n=7

Figure E3

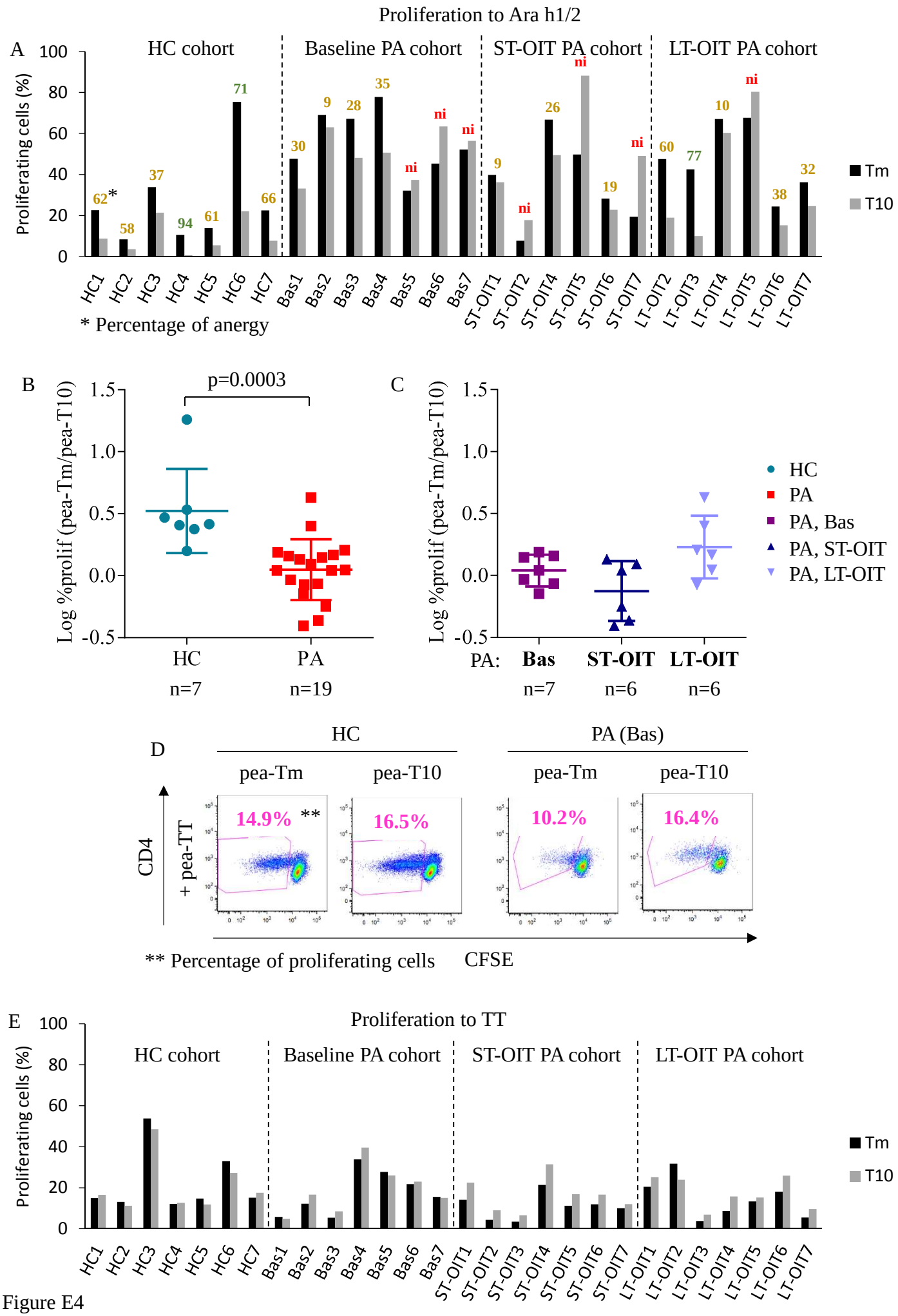

A
Proliferation to Ara h1/2
HC cohort
Baseline PA cohort
ST-OIT PA cohort
LT-OIT PA cohort
Proliferating cells (%)
Tm
T10
* Percentage of anergy
B
p=0.0003
Log %prolif (pea-Tm/pea-T10)
HC
PA
n=7
n=19
C
PA:
Bas
ST-OIT
LT-OIT
n=7
n=6
n=6
HC
PA
PA, Bas
PA, ST-OIT
PA, LT-OIT
D
HC
PA (Bas)
pea-Tm
pea-T10
14.9%
16.5%
10.2%
16.4%
CD4
+ pea-TT
** Percentage of proliferating cells
CFSE
E
Proliferation to TT
HC cohort
Baseline PA cohort
ST-OIT PA cohort
LT-OIT PA cohort
Proliferating cells (%)
Tm
T10

Figure E4

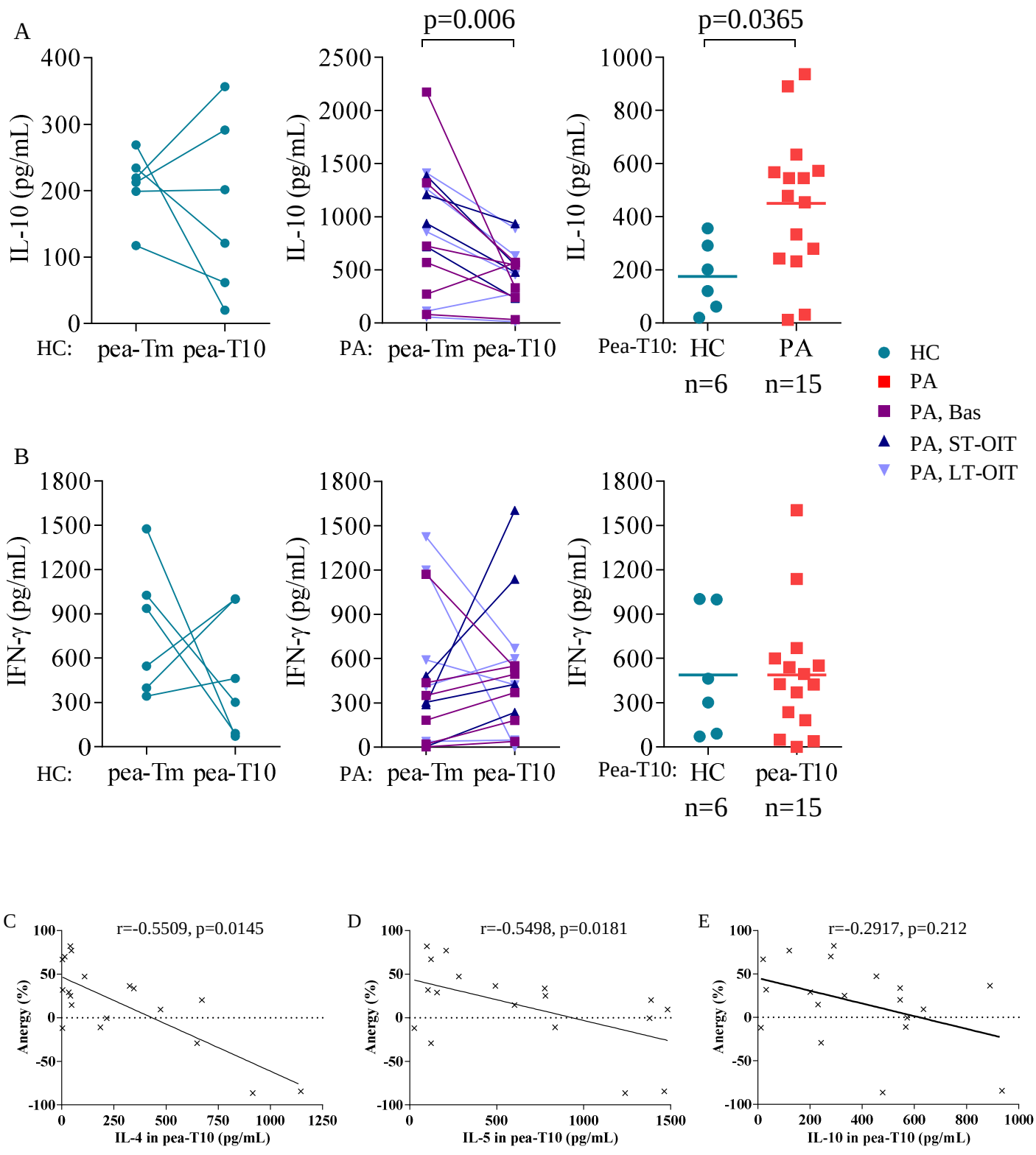

A
IL-10 (pg/mL)
HC: pea-Tm pea-T10
p=0.006
PA: pea-Tm pea-T10
p=0.0365
Pea-T10: HC PA
n=6 n=15
HC
PA
PA, Bas
PA, ST-OIT
PA, LT-OIT
B
IFN-γ (pg/mL)
HC: pea-Tm pea-T10
PA: pea-Tm pea-T10
Pea-T10: HC pea-T10
n=6 n=15
C
r=-0.5509, p=0.0145
Anergy (%)
IL-4 in pea-T10 (pg/mL)
D
r=-0.5498, p=0.0181
IL-5 in pea-T10 (pg/mL)
E
r=-0.2917, p=0.212
IL-10 in pea-T10 (pg/mL)

Figure E5

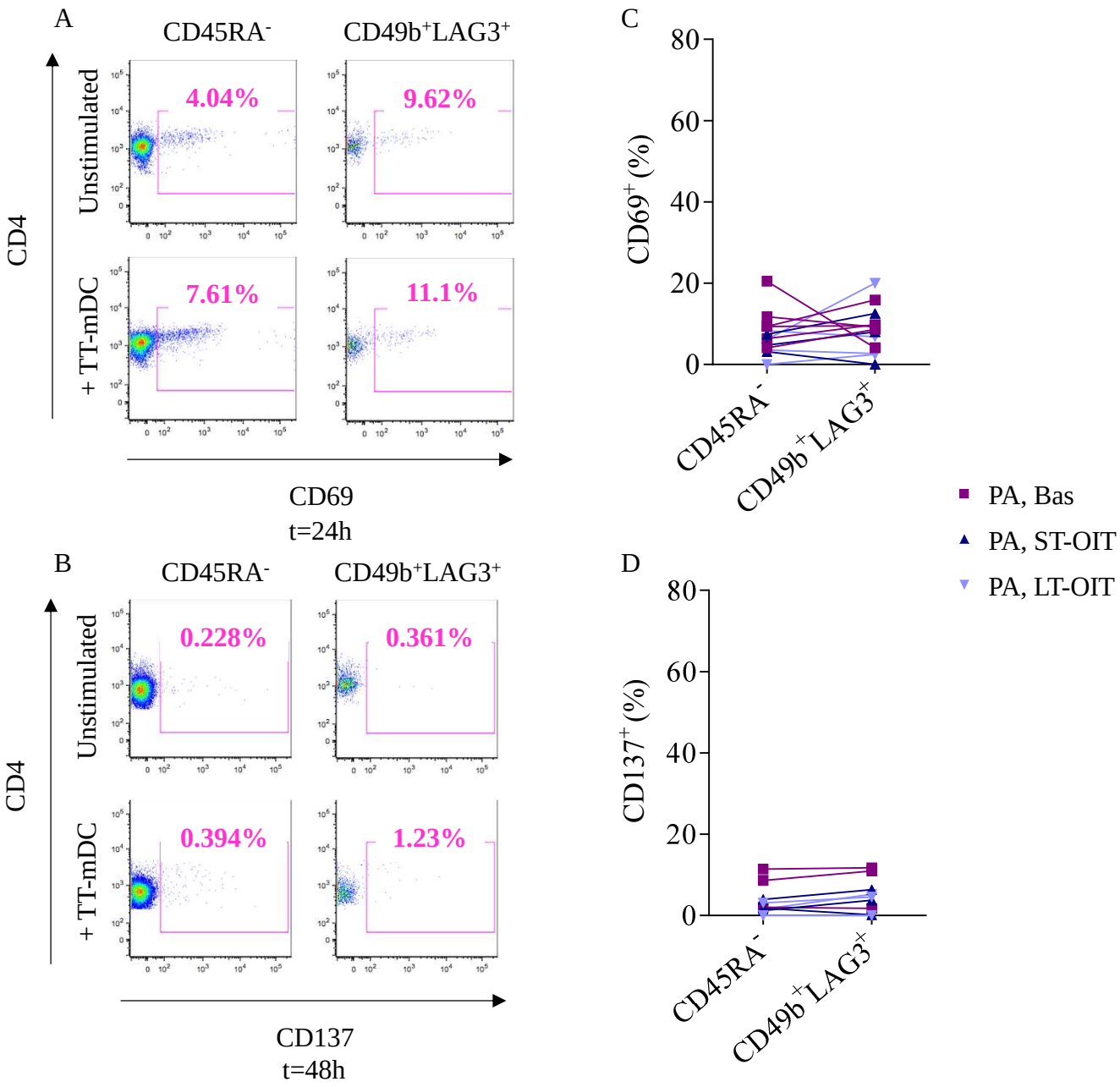

A
CD45RA-
CD49b+LAG3+
Unstimulated
+ TT-mDC
4.04%
9.62%
7.61%
11.1%
CD4
CD69
t=24h
B
CD45RA-
CD49b+LAG3+
Unstimulated
+ TT-mDC
0.228%
0.361%
0.394%
1.23%
CD4
CD137
t=48h
C
CD69+ (%)
80
60
40
20
0
CD45RA-
CD49b+LAG3+
D
CD137+ (%)
80
60
40
20
0
CD45RA-
CD49b+LAG3+
PA, Bas
PA, ST-OIT
PA, LT-OIT

Figure E6

SUPPLEMENTAL DATA

DC differentiation

$CD14^+$ monocytes were isolated from peripheral blood mononuclear cells (PBMC) using a positive selection kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions. $CD14^+$ cells were differentiated into mature dendritic cells (mDCs) and tolerogenic DCs (DC-10) as previously described (37). Briefly, $CD14^+$ were incubated for 7 days in RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 5% pooled AB human serum (Sigma-Aldrich, Saint Louis, MO) and 100 U/mL penicillin/streptomycin (Life Technologies, Carlsbad, CA) in the presence of 10ng/mL of recombinant human (rh) IL-4 (R&D Systems, Minneapolis, MN) and of 100ng/mL of rhGM-CSF, with the addition of 10ng/mL of IL-10 (BD Biosciences,San Jose, CA) to differentiate DC-10. After 5 days, purified endotoxin-free Ara h 1 and 2 (Indoor Biotechnologies, Charlottesville, VA) or inactivated Tetanus Toxoid (TT; EMD Millipore, Billerica, MA) proteins were added for 2 days to the DCs (pea-mDC, pea-DC-10 and TT-mDC, respectively). The optimal concentration of the peptides was determined from a dose-response curve (0.1 to 20µg/mL) testing the proliferation of autologous $CD4^+$T cells. The optimal concentration of TT was 1µg/mL, and 10 µg/mL for Ara h 1 and 2. For each experiment, the maturation of the DCs was confirmed by flow cytometry to determine the expression of CD11c, CD86 (BioLegend Inc., San Diego, CA), and CD14 (eBioscience, San Diego, CA). DC established from a single blood donation were either used for primary stimulation, or frozen and used later to test for antigen-specificity or cytokine production.

T cell differentiation

$CD4^+$ T cells were isolated using the EasySep Human $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies, Vancouver, Canada) from the PBMC $CD14^-$ fraction. 1×10^5 pea-mDC or pea-DC-10 were co-incubated with 1×10^6 autologous $CD4^+$ T cells in X-VIVO 15 medium supplemented with gentamycin (Lonza, Switzerland) and 5% pooled AB human serum (complete medium). IL-10 was added at 10ng/mL to the $CD4^+$ - DC-10 cocultures. After 7 days of culture, IL-2 (Peprotech, Rocky Hill, NJ) was added at 20 units/mL and the cells were expanded for an additional 7 days. The phenotype of the $CD4^+$ T cells cocultured with the pea-mDC (pea-Tm) or with the pea-DC-10 (pea-T10) was assessed by flow cytometry testing the expression of LAG3, CD49b (Miltenyi Biotech, Germany), CD3, CD4, CD45RA, and CD25 (BioLegend Inc., San Diego, CA), GPR15 (R&D Systems, Minneapolis, MN) and CCR9 (BD Biosciences, San Jose, CA).

Ara h 1/2 specific T cell proliferation assay

To assess the proliferative response to a secondary antigen challenge, pea-Tm and pea-T10 cells were stained with carboxyfluorescein succinimidyl ester (CFSE; Life Technologies, Carlsbad, CA) and plated alone (5×10^4) or with autologous pea-mDC or TT-mDC as a control (5×10^3) in complete medium in 96 well, round bottom plates in a final volume of 200µL. The percentage of divided cells within the $CD3^+CD4^+$ T cell population was assessed after 4 and 5 days.

Ara h 1/2 antigen specificity assay and cytokine detection

To assess the percentage of antigen-specific Tr1 cells, pea-T10 cells were plated alone (1×10^5) or with autologous pea-mDC or TT-mDC (1×10^4) in complete medium in 96 well, round bottom plates in a final volume of 200µL. The expression of the activation markers CD69 and CD137 (BD Biosciences, San Jose, CA) was assessed by flow cytometry on gated $CD3^+CD4^+CD45RA^-$ T cells after 24 and 48h, respectively. The optimal stimulation time to assess the expression of these markers was determined by a time course experiment (12h to 72h). At 48h, supernatants were collected and frozen for analysis of cytokine detection. Levels of IL-4, IL-10, IFN-γ and GM-CSF in the supernatants of pea-Tm/T10 cells and pea-mDC cocultures were assessed by ELISA (BD Biosciences, San Jose, CA).

Flow cytometry

For phenotyping of the dendritic cells, the samples were acquired using a Dxp10 FACScan™ (Cytek Dev, Fremont, CA). For all the other analyses, the samples were acquired using a FACS Aria (BD Biosciences, San Jose, CA). The staining of pea-Tm and pea-T10 cells was performed at 37ºC for 15min (Protocol based on (25)). All other stainings were performed according to the manufacturer's recommendations.

Statistical analyses

GraphPad Prism 6.07 (GraphPad Software, Inc., La Jolla, CA) software was used for statistical analyses. Results are presented as mean ± SD, unless stated otherwise. We used Mann-Whitney test, Wilcoxon test, Spearman correlation test and paired student t test to determine the statistical significance of the data. P values of less than 0.05 were considered significant. For computing summary statistics, we considered the log value of pea-Tm/pea-T10 cell proliferation.