

ORIGINAL ARTICLE

Oral immunotherapy in hen's egg-allergic children increases a hypo-proliferative subset of CD4⁺ T cells that could constitute a marker of tolerance achievement

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Abstract

Background: Food allergy affects a significant number of children and its prevalence, and persistence is undergoing an important increase in the last years. Specific oral tolerance induction (SOTI) is a promising therapy for food allergy. However, little is known about the immune mechanisms implicated in the desensitization to allergens. Our purpose was to study which immune parameters are modified during the process of tolerance achievement with the goal of identifying markers of tolerance induction.

Methods: We performed an extensive immune analysis in 19 allergic children following SOTI with hen's egg before and after the immunotherapy. Changes in lymphocyte subpopulations and serum cytokines were identified in children with desensitization achievement.

Results: Sixteen children achieved complete tolerance to egg, and the immune analysis reveals that desensitization was accompanied in all the cases by a significant decrease in the percentage and absolute counts of effector-memory CD4⁺ T cells (T_{EM}) and a marked increase in the absolute counts of a subset of CD4⁺CD38⁺CD45RO⁻ cells. Additionally, we also observed a marked reduction in the plasma levels of different Th1 and Th2 cytokines after tolerance achievement.

Conclusions: Acquisition of tolerance in children after oral immunotherapy is accompanied by a decrease in the T_{EM} population and the increase in a particular subset of CD4⁺ T cells with a hypo-proliferative and non-reactive phenotype. This hypo-proliferative subset of cells could constitute a marker of the development of oral tolerance, and the study of this subset could contribute to the better understanding of the immune responses in allergic subjects.

Food allergy affects a significant number of children, with an estimated prevalence of 2–8%, and is the most frequent reason for anaphylactic reactions at this age (1). Recent studies suggest that both hen's egg and milk allergies are now more commonly persisting into late childhood and adolescence (2). However, in the last years desensitization protocols as oral immunotherapy have proven to be safe and effective as treatment for food allergy (3) including egg allergy (3). Specific oral tolerance induction (SOTI) consists of oral administration of increasing amounts of a food allergen to achieve clinical tolerance against the allergen that persists even after

therapy is stopped. Although studies suggest that a majority of patients with food allergy can be desensitized with SOTI, little is known about the immune mechanism that mediates the desensitization. Several studies described decreased levels of specific IgE and increased IgG4 levels in children following desensitization protocols (3, 4). Nevertheless, little is known about the underlying mechanism responsible for these changes in antibody production. In fact, there are not data about the immune subsets implicated in the process of tolerance achievement, and there are not markers that reliably predict the potential for spontaneous or induced development

of oral tolerance. We hypothesize that tolerance induction would produce profound changes in the immune system allowing the desensitization against the allergen, and probably this changes would be reflected in peripheral blood by the presence of modified values of immune subsets. Our objective is to analyze what immune parameters are modified after SOTI and to identify markers of tolerance achievement.

Methods

We performed an extensive analysis of immune cells in a cohort of 19 allergic children following SOTI with hen's egg [mean (range) age = 9.2 (4–14) yrs] and 22 age-matched healthy children as control group [mean (range) age = 7.2 (3–13) yrs]. The study was conducted according to the principles expressed in the Declaration of Helsinki. It was approved by the clinical ethics committee of the *Hospital Gregorio Marañón* of Madrid (Spain), and written informed consent was obtained from all legal guardians.

Prior to being included to the desensitization protocol, an open oral challenge test with egg was carried out on those who had not had clinical episodes within the last 3 months. The oral challenge tests were preceded in some cases by cutaneous and/or labial contact tests. Before and after the procedure and in the follow-up at 6 and 12 months, prick tests with powdered egg at various dilutions (10, 5 & 1 mg/ml) were carried out.

Desensitization protocol

A SOTI protocol with powdered pasteurized egg mixed with juice or milkshakes was established enabling patients to continue treatment at home, with periodic weekly visits to the outpatient allergy department. On the first day, fractionated doses were administered until reaching 31 mg of egg, beginning with 1 mg, and continuing with 3, 9, and 18 mg at 30 min intervals. On the second day, 30 mg in one single dose was administered, with the treatment continuing at home at this same dosage. Subsequently, weekly increases were made in the clinic until 10 g of powdered egg, the equivalent of one egg, was reached. Lastly, tolerance of the natural food was checked. The procedure was initially programmed to last for 13 wks. The patients had follow-ups at the clinic 6 and 12 months after achieving tolerance. A normal diet was recommended to patients with tolerance achievement.

Analysis of immune subsets, cytokines, and immunoglobulins

Peripheral blood samples were taken before SOTI (T0) and when egg desensitization was achieved (Tend). All the samples were processed immediately after the extraction, and plasma samples were stored for the determination of serum cytokines. We determine by flow cytometry the frequency and absolute counts of B cells, NK cells, CD4⁺ T cells, and CD8⁺ T cells including markers for the following subsets: naïve (CD45RA⁺CD27⁺), activated (HLA-DR⁺), memory (CD45RA⁺CD45RO⁺), and effector memory (CD45RA⁺CD27⁻) as previously described (5, 6). IgE total

and specific to egg white, yolk, ovalbumin (OVA) and ovomucoid (OVM) and total IgG specific to white were measured using commercial kits (CAP-Phadia, Uppsala, Sweden). Specific IgG4 was not determined. Plasma concentration of different cytokines was determined using the *Human Th1/Th2/Th9/Th17/Th22 13plex kit* (eBioscience, San Diego, CA, USA) as previously described (5).

Statistical analysis

Statistical analysis was performed using SPSS software (IBM, New York, NY, USA). Nonparametric Mann–Whitney test was used for comparison between groups. Wilcoxon test for paired samples was used for the analysis of cytokines. *p* value < 0.05 by 2-sided test was considered significant.

Results

Desensitization achievement

A total of 19 hen's egg-allergic children started the SOTI protocol, and blood samples were obtained before the first administration of allergen (T0). At the end of SOTI, 16 of the 19 allergic children (84.2%) achieved complete tolerance to egg and a new blood sample from each child was extracted (Tend). A child was considered desensitized when he was capable of ingesting 10 g of powdered egg without any adverse reactions. The mean $\pm$ s.e.m. time from T0 until Tend was 9.7 ± 0.9 wks. SOTI did not induce tolerance in two children, who interrupted the protocol because of anaphylactic reactions, and one child became partially tolerant to cooked egg but remained intolerant to uncooked egg.

SOTI modifies values of T-cell subsets

We analyzed the immune parameters that were modified between T0 and Tend in the 16 children who achieved tolerance. Statistical analysis revealed that percentages of the majority of immune subsets analyzed and absolute counts of B cells, NK cells, NKT cells, and CD8⁺ T cells remained unchanged after SOTI (Table 1). However, SOTI induced a significant decrease in the percentage and absolute counts of effector-memory CD4⁺ T cells (T_{EM}). In addition, absolute counts of CD4⁺ recent thymic emigrants (RTEs) significantly increased at Tend (Table 1). RTE is a subset of CD4⁺ T cells (identified as CD3⁺CD4⁺CD45RA⁺CD31⁺) recently produced by the thymus, which constitutes a marker of the thymical production of new T cells (7). SOTI also induced a marked increase (*p* = 0.003) in the absolute counts of a particular subset of cells defined as CD4⁺CD38⁺CD45RO⁻, which we called CD38/ROneg (Table 1). The CD38 molecule has been considered as an activation marker, but further analyses of this increased population revealed that these CD38/ROneg cells did not express HLA-DR neither CD45RO (Fig. S1). Moreover, absolute counts of CD38/ROneg cells showed a significant positive correlation with absolute counts of CD4⁺ RTEs (*p* = 0.003) (Fig. 1).

Table 1 Immune parameters in 16 allergic children with tolerance achievement after SOTI. Percentages and absolute counts (cells/ μ l of total blood) for immune subsets are showed. Values are given as median (25–75th percentiles)

	Percentage			Cells/ μ l		
	T0	Tend	p	T0	Tend	p
Total Lymphocytes	40.1 (30.9–45.4) [†]	41.1 (35.5–43.3) [†]	0.653	2217 (1750–2818)	2533 (2030–2892)	0.356
CD4+ T cells	39.9 (34.7–42.7)	41.2 (37.6–42.4)	0.297	804 (697–1090)	942 (881–1220)	0.200
CD8+ T cells	24.4 (21.5–26.7)	22.2 (20.4–25.4)	0.300	548 (390–695)	600 (453–703)	0.621
NK cells	9.39 (6.9–10.2)	8.07 (7.2–10.8)	0.710	185 (153–270)	204 (152–260)	0.621
NKT cells	1.22 (0.9–2.1)	1.44 (1.0–1.8)	0.891	33 (20–48)	38 (22–44)	0.837
B cells	15.2 (12.5–18.6)	15.8 (13.4–18.1)	0.984	353 (203–507)	375 (287–493)	0.653
CD4+ T cells						
Naive	57.1 (52.9–62.6)	58.1 (50.4–68.8)	0.751	477 (382–625)	621 (478–758)	0.130
Activated	3.92 (3.0–4.8)	3.92 (2.3–5.8)	0.860	36 (26–48)	41 (27–73)	0.279
Memory	34.2 (29.2–36.4)	30.6 (25.6–36.8)	0.468	287 (242–410)	329 (253–418)	0.421
Effector memory	0.56 (0.45–1.5)	0.29 (0.16–0.58)	0.017*	5 (3–12)	3 (1.2–4.7)	0.032*
RTEs	42.4 (39.2–49.2)	43.5 (40–56.1)	0.685	368 (277–489)	514 (404–650)	0.041*
CD38 ⁺ CD45RO ⁻	42.6 (31.4–51.7)	50.4 (42.6–57.6)	0.109	353 (263–596)	638 (415–718)	0.003*
CD8+ T cells						
Naive	57.5 (51.7–63.6)	57.6 (51.3–65.6)	0.846	303 (254–372)	327 (286–365)	0.397
Activated	4.18 (2.0–6.7)	6.68 (2.4–8.3)	0.173	22 (11–44)	51 (13–63)	0.059
Memory	24.8 (16.6–25.6)	22.6 (16.1–28.4)	0.922	140 (79–193)	150 (115–231)	0.468
Effector memory	14.5 (3.9–22.1)	15.5 (10.8–20.3)	0.983	76 (22–125)	95 (59–134)	0.450
RTEs	55.9 (46.1–58.6)	51.5 (48.2–61.4)	0.786	270 (227–316)	322 (263–355)	0.139
Immunoglobulins						
IgE (total) (kU/l)	738 (282–1578)	772 (329–1550)	0.896			
IgE white	8.18 (3.6–14.2)	9.51 (4.1–15.1)	0.779			
IgE ovalbumin	4.63 (3.0–12.4)	4.61 (1.7–7.9)	0.464			
IgE ovomucoid	6.08 (2.2–12.8)	8.30 (2.6–17.8)	0.488			
Total IgG white (mg/l)	5.02 (3.2–7.0)	20.8 (8.2–44.5)	0.002*			

*p < 0.05 in Student's *t*-test comparing T0 and Tend values.[†]Percentage of total lymphocytes into the leukocytes population.

Values of immune subsets observed in allergic children at T0 and Tend were compared with immune values in age-matched healthy children. Allergic children at T0 had a higher percentage and absolute counts of T_{EM} than healthy controls ($p < 0.05$), and after SOTI (Tend), these values returned to levels similar to those of healthy children (Fig. 2 c,d). In the same way, percentage and absolute counts of CD38/ROneg cells were decreased in allergic children when compared to healthy controls ($p < 0.01$), and at the end of SOTI, these values became comparable to the values observed in the control group (Fig. 2a,b). Moreover, the three children who did not achieve egg-tolerance during SOTI showed a reduction in both the frequency (mean $\pm$ s.e.m.: T0 = $40.4 \pm 6.3\%$ $\rightarrow$ Tend = $33.4 \pm 4.1\%$) and absolute counts of CD38/ROneg (T0 = 442 ± 125 cells/ μ l $\rightarrow$ Tend = 394 ± 68 cells/ μ l) between T0 and the last sample obtained.

SOTI increases specific IgG values in plasma

We determined the levels of total and specific IgE and white-specific IgG total in allergic children at T0 and Tend time-points. The analysis indicated that total and specific IgE

(white, ovalbumin, and ovomucoid specific IgE) did not change after SOTI, but white IgG levels significantly increased between T0 and Tend ($p = 0.002$) (Table 1). In addition, the analysis of correlation between variables in allergic children (taking in consideration both T0 and Tend values) indicated that white IgG levels correlates positively and uniquely with the absolute counts of CD38/ROneg cells ($p = 0.007$).

SOTI modifies the serum concentration of cytokines

Concentration of serum cytokines at T0 and Tend was determined in each patient (Table 2). Plasma concentration of IL-6 was undetectable in any of the children, and we did not find significant differences for IL-12, IL-4, IL-13, and IL-1 β ($p > 0.05$). However, children at Tend showed a marked decrease in the concentration of some Th1 cytokines such as IL-2, TNF- α , and IFN-. We also observe a decrease in Th2 cytokines such as IL-5 and IL-10; Th9 cytokines such as IL-9; Th22 cytokines (IL-22); and Th17 cytokines (IL-17A) (Table 2). Although there are no significant differences in IL-13 between T0 and Tend, it was the only cytokine with a tendency to increase after SOTI (Table 2).

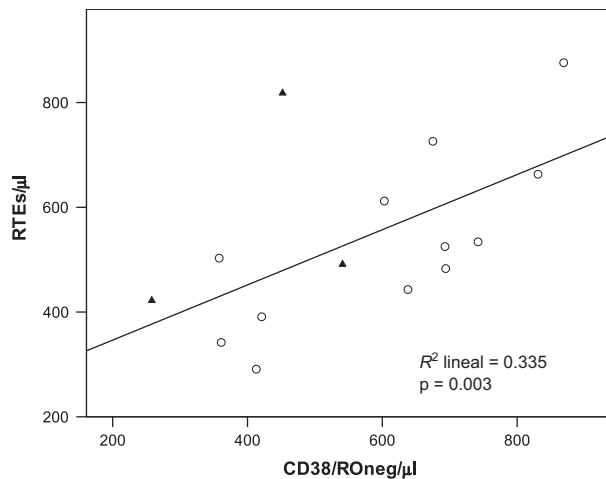


Figure 1 Correlation between RTEs and CD38/RO neg cells. Correlation between the absolute counts (cells/ μ l) of CD4⁺ RTEs and CD38/ROneg cells in egg-allergic children at Tend. $\circ$: patients with tolerance achievement after SOTI. $\triangle$: patients without tolerance achievement.

Discussion

In this study, we showed that a SOTI protocol with incremental doses of egg allergen induces tolerance in the majority of the children enrolled in the study. This desensitization protocol must induce changes in the allergen-specific immune subsets, which would be responsible for the disappearance of

adverse immune responses to the allergen, but also could modify the immune homeostasis and the changes would be reflected in systemic immune population. Effectively, our results reveal that children with tolerance achievement after SOTI undergo a marked decrease in the percentage and absolute counts of CD4⁺ T_{EM} cells. T_{EM} cells have immediate effector function and can rapidly produce inflammatory mediators, such as Th2-associated cytokines IL-4 and IL-5 (8), playing a key role in food allergy (9). Thus, a decrease in the frequency of T_{EM} could be a sign of desensitization to the allergen. These results are in agreement with other studies where a decrease in CD4⁺ T_{EM} cells has been also observed in a murine model of oral tolerance to OVA (10).

In addition, we observed in all the desensitized children a marked increase in the absolute counts of a subset defined as CD38/ROneg. The CD38 molecule has been commonly regarded as an activation marker for human CD4⁺ T cells, and increased values of CD4⁺HLA-DR⁺CD38⁺ T cells have been associated with hyperimmune activation (11). However, this CD38/ROneg subset was negative for CD45RO and HLA-DR. Scalzo-Inguanti et al. (12) recently described a new T-cell subpopulation that does not fit into existing naive and memory cell paradigms and that would corresponds to the CD38/ROneg subset that we found. These cells are hypo-proliferative and do not acquire full/classical effector capacity after TCR stimulation, remaining in a 'static' state. Additionally, CD4⁺CD38⁺ cells had a bias toward IL-13 secretion, but not other Th2 cytokines such as IL-4 or IL-5 (12). Which is the origin of these cells? The increase observed in the absolute counts of RTEs after SOTI [considered a marker of the thymical production of new T cells (7)],

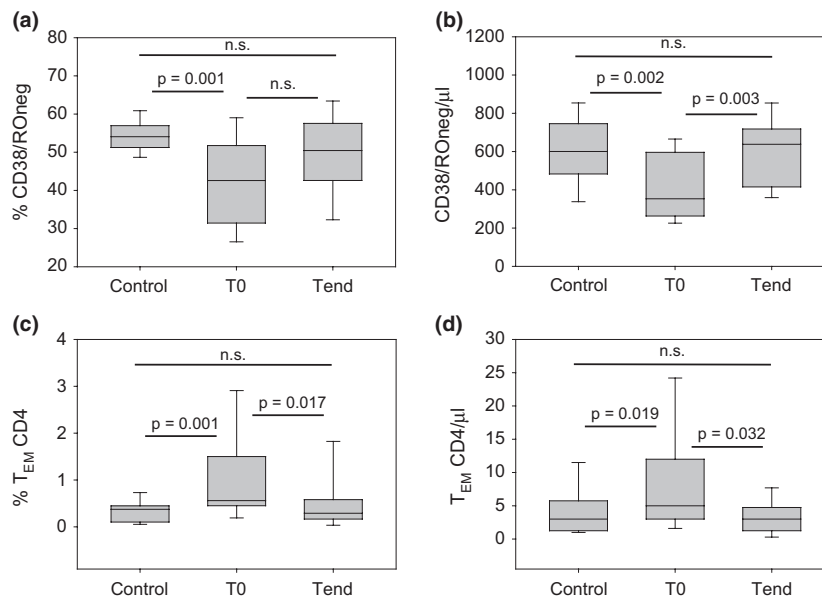


Figure 2 Immune changes in allergic children with tolerance achievement after SOTI. Percentage (a) and absolute counts (b) of CD38/ROneg cells and percentage (c) and absolute counts (d) of effector-memory CD4⁺ T cells (T_{EM}) are represented by box plot graphics (25–75th percentiles). Error bars indicate the 90th and 10th percentiles, and median is represented by a line within the box. p values for Mann–Whitney statistical analysis are showed. n.s.: non-significant differences.

Table 2 Plasma concentration of cytokines in 16 allergic children with tolerance achievement after SOTI. Mean values ($\pm$ s.e.m.) of plasma concentration (pg/ml) of cytokines at T0 and Tend

	Plasma concentration (pg/ml)		p
	T0	Tend	
Th1 cytokines			
IL-2	125.05 (36.4)	27.53 (14.2)	0.041
IFN-gamma	174.69 (57.4)	42.15 (27.2)	0.036
IL-12	42.43 (25.2)	<1.5*	0.068
TNF-alpha	30.73 (12)	<3.2*	0.046
Th2 cytokines			
IL-4	88.49 (29.8)	25.32 (12.1)	0.097
IL-5	75.95 (27.9)	3.31 (3.2)	0.028
IL-10	36.94 (12)	12.9 (4.4)	0.025
IL-13	119.76 (33.2)	158.86 (17.9)	0.657
Others			
IL-9	76.15 (29.8)	51.2 (40.9)	0.021
IL-17A	279.96 (68.6)	197.94 (103.1)	0.006
IL-22	467.47 (104)	275.73 (68.2)	0.028
IL-1 beta	140.33 (38.8)	67.07 (43.8)	0.059

*Cytokine concentration was undetectable in more than 40% of the samples, and value of the limit of detection was used in these cases. p value was determined using the Wilcoxon test for paired samples.

and the positive correlation between the absolute counts of CD38/ROneg cells and counts of RTE CD4⁺ T cells indicates a probable new generation of these cells in the thymus. Therefore, we propose that SOTI induces changes in the immune homeostasis that increases the replenishment of the CD4 pool by the thymus, whose function is preserved in children being capable of reconstituting the CD4 population (13). In the presence of egg antigens, the new T cells would experiment the thymic negative selection, which ensures the selection of non-reactive clones, acquiring this 'non-reactive' phenotype and reducing the immune responses against the allergen.

The appearance of this hypo-proliferative phenotype could be associated with the acquisition of tolerance because of the static or non-reactive characteristics of this subset. In fact, we observed that the frequency and absolute counts of CD38/ROneg cells in allergic children (T0) were lower than in the control group; however, these values recovered after SOTI and were comparable to those of healthy controls, indicating a possible association between the presence of this phenotype and the tolerance to the allergen. We also observed that allergic children have a higher percentage and absolute counts of T_{EM} than healthy controls, which play a key role in the allergy (9) and after SOTI these values returned to levels similar to those of healthy children, which could be related to a decrease in the allergic responses. As mentioned in results, the three children who did not achieve egg tolerance during SOTI showed a reduction in both the frequency and absolute counts of CD38/ROneg (between T0 and the last sample obtained), indicating that this subset of

cells could be a reliable marker of desensitization. However, the finding of incomplete tolerance in only 3 children does not allow drawing definitive conclusions. Further studies in larger cohorts of patients including placebo groups will be necessary to determine whether the absence of CD38/ROneg cells recovery could constitute a predictive marker of unsuccessful induction of tolerance.

Achievement of tolerance with desensitization protocols is frequently accompanied of decreased levels of specific IgE and increased IgG4 levels (3, 4). Moreover, CD38/ROneg cells have shown to be the main IL-13 producing cell type following *in vitro* stimulation (12), and IL-13 is known to induce IgG4 and direct IgE isotype switching in human B cells (14). We did not measure specific IgG4 levels, but we observed a significant increase in white IgG total levels between T0 and Tend. Interestingly, we also found a direct correlation between white IgG levels and the absolute counts of CD38/ROneg cells, which could be mediated by the bias for IL-13 production described for this subset (12). In fact, IL-13 was the only cytokine with a tendency to increase after SOTI. The majority of the other cytokines analyzed showed a decreased production after SOTI. This decrease could be associated with the reduction in T_{EM} and the replenishment of the CD4⁺ pool with CD38/ROneg cells, which have shown a reduced capacity to produce IFN-, TNF- α , IL-4, and IL-5 (12). The reduced production of cytokines such as IL-10 and IL-5, with a demonstrated key role in food allergy (9, 15), and other cytokines implicated in allergic processes (16) would also contribute to the achievement of tolerance.

We cannot rule out the possibility that other immune cells, such as granulocytes, could be implicated in both achievement of tolerance and the observed decrease in cytokine plasma levels. Further studies would be necessary to determine the exact role of CD38/ROneg cells in allergy and how this subset of cells is generated after SOTI. Another limitation of the study is the fact that immune subsets analyzed are not allergen specific. The frequency of allergen-specific T cells is typically 0.05–0.005%, and changes in these allergen-specific subsets would not explain the changes observed in the mentioned subsets. However, the significant increase in plasma levels of allergen-specific IgG in desensitized children indicates that SOTI has affected the systemic immune responses, and probably there are widespread changes, beyond those that are allergen specific, which could be detectable in blood. In this way, SOTI would induce changes in immune homeostasis or cytokine levels that can modify the phenotype of allergen-specific T cells, but their effects would be also observed in the rest of T cells.

Summing up, the replacement of T_{EM} by a subset of CD38/ROneg hypo-proliferative cells would be an indicator of reduced immune responses and a sign of the acquisition of tolerance. We hypothesize that the prevalence of this hypo-proliferative phenotype (CD38/ROneg) in CD4⁺ T cells could be implicated in tolerance to allergens in healthy children. This subset of cells is markedly decreased in children with egg allergy, although a desensitization protocol such as SOTI can return CD38/ROneg cell values to levels

comparable to those of healthy children. In light of these results, we propose that absolute counts of CD38/ROneg and T_{EM} cells could be a reliable marker of the development of oral tolerance. The study of these populations could contribute to better understanding of immune responses in allergic patients and could provide an interesting tool for the clinical management of the food allergic children.

Authorship contributions

V.F-A, E.A-L, L.Z., and S.I were responsible of the administration of SOTI protocol, the clinical follow-up of the patients and the extraction of blood samples. They also participate in the discussion and analysis of data; R.L. processed the samples and performed cytometry analysis; M.A.M-F contributed to the design of the project and the

preparation of the manuscript; R.C-R. was responsible for the overall study, designed the project, and wrote the manuscript.

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Conflict of interest

All the authors declare that they have no competing financial interests.

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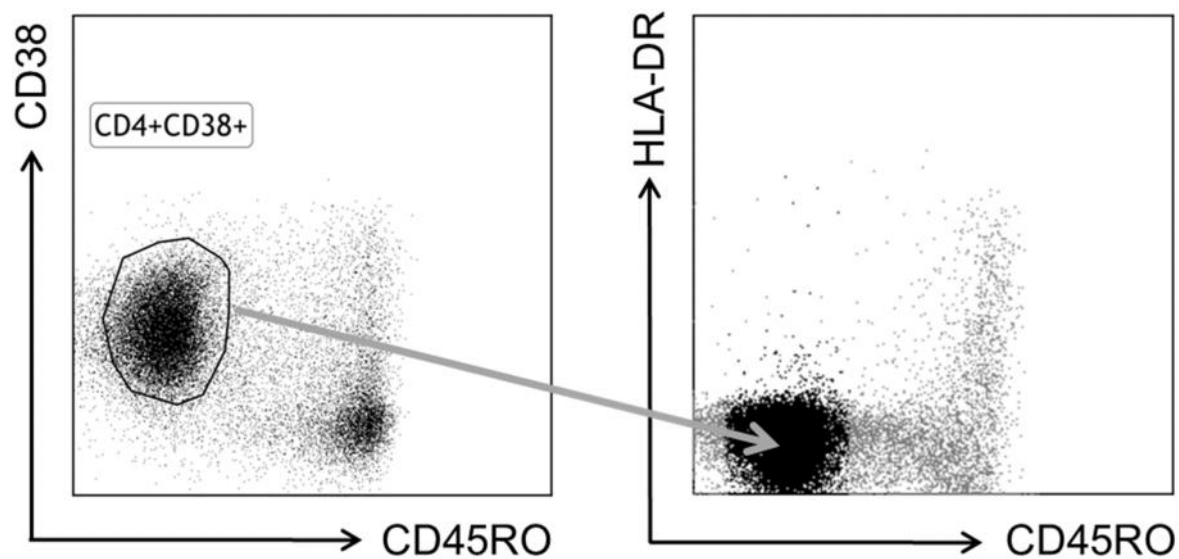
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Dot-plot gated in CD4⁺ T cells from a representative patient showing the CD38/ROneg subset defined as CD4⁺CD38⁺CD45RO[−]HLA-DR[−].

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Supplemental Figure 1. (A) Dot-plot gated in CD4+ T cells from a representative patient showing the CD38/Roneg subset defined as CD4⁺CD38⁺CD45RO⁻HLA-DR⁻.