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MONOMERIC ALLERGOID INTRAGASTRIC ADMINISTRATION INDUCES LOCAL AND SYSTEMIC TOLEROGENIC RESPONSE INVOLVING IL-10-PRODUCING CD4$^+$CD25$^+$ T REGULATORY CELLS IN MICE

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The efficacy of sublingual immunotherapy, at present one of the treatments of choice for respiratory allergy, relies on the tolerance induced by oral mucosa-associated immune system; however, the gut-associated lymphoid tissue (GALT: Peyer's patches and isolated lymphoid follicles) and mesenteric lymph nodes could also be involved, being stimulated by the ingested part of the allergen extract. The aim of the present study is to assess whether the exposure of the allergen exclusively to the GALT induces a tolerogenic response. To this purpose, mice were sensitized with ovalbumin or Par j 1 allergens. The corresponding gastric-resistant monomeric allergoids were then administered via orogastric gavage. After treatment, all mice were tested for: serum IgE, *in vitro* Th1 and Th2 cytokine release by allergen-stimulated peripheral blood lymphocytes, CD4$^+$CD25$^+$ and CD4$^+$CD25$^+$IL-10$^+$ T cells in Peyer's patches, mesenteric lymph nodes and spleen. Compared to the control, sensitized groups showed higher levels of serum IgE, lower frequency of CD4$^+$CD25$^+$IL-10$^+$ T cells, at all sites, and higher amounts of *in vitro*-released IL-4, IL-6 and TNF-α. Compared to the sensitized groups, higher frequency of CD4$^+$CD25$^+$IL-10$^+$ T cells was observed in the spleen of both Par-j 1 and OVA sensitized/treated groups and, only for ovalbumin-treated mice, in the Peyer's patches and mesenteric lymph nodes, IgE and *in vitro* cytokines were significantly lower and equivalent to the control group. The results give the first evidence that the intragastric-restricted administration of gastric-resistant allergens restores local and peripheral tolerance in allergen-sensitized mice.

Immune responses in allergy are characterized by impaired inhibitory function of allergen-specific T regulatory (Treg) cells and aberrant activity of Th2 cells (1). Recent studies on mice and humans indicate that Tregs, able to control and/or inhibit the Th1 and Th2 lymphocyte function, play a central role in the effective intervention (2) made by specific immunotherapy. Sublingual immunotherapy (SLIT), recently extensively studied (3-4), has been demonstrated to restore systemic tolerance by re-enabling Treg function, inducing peripheral ("oral") tolerance (5). In

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SLIT, the vaccine is applied sublingually, where is kept for at least 2 minutes. The allergen extract is then swallowed and reaches the intestine. Recent studies on the mechanism of immunological tolerance clearly demonstrated that the recognition of fed antigens (of food and microbial origin) is confined to the intestinal immune system, where oral tolerance originates (5-6). However, it is not known whether the ingested portion of the allergen could induce immunological tolerance through the stimulation gut-associated lymphoid tissue (GALT). In humans, oral allergen immunotherapy gave contrasting and often discouraging results (7-8), presumably because allergen is digested by gastric acid and intestinal enzymes and loses its immune potential. On the other hand, some studies in mice show that the feeding with the specific allergen restores the immune tolerance towards the sensitizing antigen (9). However, it cannot be excluded that this tolerogenic effect may be due to the contact that the antigen has with the oral mucosa during feeding.

To verify the possible immune activity of ingested allergens, the present work was designed to obtain a direct stimulation of GALT, avoiding the contact between the allergen and the oral mucosa, and choosing an allergen for therapeutic use that maintains its biochemical and immunological characteristics during the passage through the gastrointestinal tract. The monomeric allergoid, a chemically modified allergen (10), was chosen for this purpose because, differently from the natural allergen, it resists gastrointestinal enzyme attack (11).

For our study, we chose the monomeric allergoids of Par-j1 protein, the major allergen of *Parietaria judaica,* and of ovalbumin (OVA), that were administered via orogastric gavage to avoid contact with oral mucosa.

Two groups of mice were sensitized with Par-j1 or OVA and subsequently treated by the intragastric administration of the corresponding monomeric allergoids.

IgE levels, the distribution of systemic and local (GALT) Tregs and the pattern of cytokines secreted by *in vitro* allergen-stimulated peripheral blood mononuclear cells were measured as indicators of sensitization and tolerance.

MATERIALS AND METHODS

Animals

Thirty pathogen-free Balb/c mice (Charles River, Milan, Italy) were housed in a laminar flow cabinet, with care and use performed in accordance with the ETS No. 123, article 5. Balb/c strain was suitable for our studies being characterized by high Treg level and a Th2-polarized immunological profile (12).

Sensitization and treatment protocols

Two groups of 12 mice were sensitized by two intraperitoneal (i.p.) injections of Par-j1, 12 kDa, (Lofarma S.p.A., Milan, Italy) and OVA, 43 kDa, (grade V, Sigma-Aldrich, St. Louis, MO, USA), pre-adsorbed to $Al(OH)_3$. After three weeks, subsets of six mice of each sensitized group were given intragastrically (i.g.), through gavage, either 15 µg Par-j1 or 40 µg of OVA allergoids (Lofarma S.p.A., Milan, Italy) daily for 18 days. The concentrations of Par-j1 and OVA for sensitization and treatment were based on literature data (13-14). A group of 6 mice (group A) were studied as controls, 3 of them i.p. injected with saline+alumen and the other 3 left untreated. A set of blood analyses was performed on day 25. Four days after the last i.g. administration, all mice were sacrificed and lymphoid tissues, namely Peyer's patches (PP), mesenteric lymph nodes (MLN) and spleen (SP), and blood were taken for analysis (Fig. 1). Each animal of the specific groups were used and evaluated for each parameter individually.

Total IgE levels

Sets of 200 µl blood samples were collected at day 25 from the tail vein, after sensitization, and at day 62 by cardiac puncture bleeding. Total serum IgE were measured with anti-mouse IgE capture and biotinylated anti-mouse IgE detection (BD Biosciences, San Jose, CA, USA).

Flow cytometry of $CD4^+CD25^+$ T cells and IL-$10^+CD4^+CD25^+$ Treg cells

PP, MLN and SP were immediately processed to obtain single cell suspensions. After erythrocyte lysis, $1x10^6$ lymphoid cells were incubated with Cy7-labelled mAb to mouse CD4 and APC-labeled mAb to mouse CD25 or fluorochrome-conjugated isotype control antibodies. To assess intracellular IL-10 accumulation, cell suspensions were first incubated with brefeldin A (10 µg/mL) for 6 h. $CD4^+CD25^+$ stained cells were fixed and permeabilized with 8% paraformaldehyde and 1% BSA/0.1% saponin PBS and incubated with phycoerythrin-labelled anti-IL-10 (5 µg/ml) or PE-conjugated control isotype (5 µg/ml). Cells were analyzed by flow cytometry using a BD

FACSCalibur™ cytometer and BD CellQuest™ software (BD Biosciences, San Jose, CA, USA).

For each group of treatment *in vivo*, the frequency of CD4 CD25$^+$ T cells and the frequency of the IL-10-expressing CD4$^+$CD25$^+$ T cells in total tissue cells were assessed.

In vitro allergen-driven cytokine production by PBMCs

PBMCs from individual mice were cultured (2.0 x 10^5 cells/well) for 72 h at 37°C in the presence of 5 μg/ml Par-j1 or 100 μg/ml OVA, according to the allergen used for sensitization, or without allergens as control.

Cytokine levels in supernatants were determined using a customized multiplex anti-mouse cytokine sandwich chemiluminescent ELISA (Pierce, Rockford, IL, USA), including IL-1β, IL-4, IL-5, IL-6, TNF-α and IFN-γ. Sensitivity of the ELISA was 12.5 pg/ml for IL-1β, 3.1 pg/ml for IL-4, 6.3 pg/ml for IL-5, 21.9 pg/ml for IL-6, 12.5 pg/ml for TNF-α, and 31.3 pg/ml for IFN-γ. Statistical analysis was performed on normalized values obtained subtracting spontaneous from allergen/stimulated release.

Statistical analysis

Data are represented by descriptive statistics. Distribution of values of considered parameters in the various experimental groups were analyzed by the Mann-Whitney rank sum test and the Wilcoxon signed-ranks test. Both inter- and intra-group comparisons were performed.

RESULTS

No statistically significant difference between the different experimental groups was observed in the mouse weight (an average of 26.42 ± 1.27 g) and in the total cell number of the SP, MLN and PP. Spleen average weight was 96 mg. Total cell numbers in the spleen were 13-15x10^7 cells. The weight of MLN was 12-14 mg and total number of lymphoid cells 42-57x10^5 per mg tissue. Immunohistochemical analysis of all studied lymphoid tissues showed no structural modifications between test and the control groups (data not shown).

Serum immunoglobulin E levels

Mice sensitized with either Par-j1 or OVA, showed a 250-300% higher total serum IgE level, compared with control mice, at day 25 evaluation (Table I).

At day 62, no significant spontaneous changes in IgE levels were measured in control sera from group A mice compared to basal values, similarly, groups B and C showed no changes in IgE values at the end of the study compared to the values detected after sensitization (Table I). On the contrary, the groups D and E, who received the i.g. treatments, were characterized by significantly lower IgE levels ($p<0.01$) as compared to the values detected at day 25, and those measured in groups B and C ($p<0.01$) (Table I).

CD4$^+$CD25$^+$ T cells and CD4$^+$CD25$^+$ IL-10$^+$ T cell frequency

Group A: control mice. The mean frequency of CD4$^+$CD25$^+$ T cells in the PP, MLN and SP was 2.02%, 5.15%, and 3.98% of the total cells, respectively; the proportion of those expressing IL-10 was 1.92% in the PP, 3.03% in the ML and 2.72% in the SP (Table II). Therefore, IL-10$^+$ cells constituted approximately 95% in PP, 60% in ML and 70% in SP of total CD4$^+$CD25$^+$ T cells. Such parameters were equivalent to those reported for healthy adult mice of the same strain (Balb/c) (12).

Group B and D: Par-j1 sensitized and sensitized/treated mice. Significant higher frequency of total CD4$^+$CD25$^+$ T cells was found in PP, ML and SP of both group B and D, compared to the control group ($p<0.05$), with no significant differences between the two groups (Table II). On the contrary, IL-10-expressing CD4$^+$CD25$^+$ T cell frequency was significantly lower in group B (Par-j1 sensitized) compared to both control groups ($p<0.05$ when expressed as % of the total cells and $p<0.02$ when expressed as % of the CD4$^+$CD25$^+$ T cells) and Group D ($p<0.05$ when expressed as % of total cells and $p<0.02$ when expressed as % of CD4$^+$CD25$^+$ T cells) in SP (Table II). No statistical differences were found in CD4$^+$CD25$^+$IL-10$^+$ T cell levels between group A and group D (Table II). Also in the PP, the frequency of these cells was lower in group B in respect group A ($p<0.05$), whereas no significant changes were found in their frequency between group B and group D (Table II). Finally, a significantly lower number of IL-10 expressing CD4$^+$CD25$^+$ T cells ($p<0.05$) were found in the ML of group D as compared to both control and group B mice (Table II). Fig. 2 shows a representative dot plots of the cytofluorimetric analysis of splenic cell suspensions from individual mice ex vivo showing the frequency of CD4$^+$CD25$^+$ regulatory T cells and the frequency of the IL-10$^+$ cells subset in the

different experimental groups.

Group C and E: OVA sensitized and sensitized/treated mice: Total $CD4^+CD25^+$ T cells were significantly more frequent in group C than in group E ($p<0.05$) and in both these groups as compared to the group A ($p<0.05$) in SP (Table II). In contrast, these cells were significantly lower in group C compared to both the control ($p<0.05$) and group E ($p<0.02$) in PP (Table II). Finally, both groups C and E showed a similar significantly higher frequency of these cells as compared to group A ($p<0.05$) in ML (Table II).

Similarly to the Par-j1 groups, the frequency of the $CD4^+CD25^+IL\text{-}10^+$ T cells was significantly lower in group C as compared to the control ($p<0.05$) and to group E ($p<0.02$) in the SP (Table II); the values of these Tregs were similar in groups A and E; a similar pattern was found in the PP, with IL-10 expressing $CD4^+CD25^+$ T cells significantly lower in group C as compared to group A ($p<0.05$) and group E ($p<0.05$), these last two groups having similar numbers of these T cells (Table II). In the ML, a significant difference in the proportion of $CD4^+CD25^+IL\text{-}10^+$ T cells was detected in group C as compared to group E ($p=0.05$) (Table II).

In vitro allergen-driven cytokine production by PBMCs

No significant differences between spontaneous and allergen induced release of all studied cytokines were found in group A (Figs. 3, 4).

The spontaneous release of IL-4 and IL-6 was significantly higher in group B (Par-j1 sensitized) compared to that observed in group A ($p<0.05$). Moreover, in the same group B, allergen-driven release of IL-4, IL-6 and TNF-α was significantly higher ($p<0.01$) as compared to unstimulated cultures (from 34.5±9 pg/ml to 117.3±15 pg/ml; 34.1±4.1 pg/ml to 142.3±16.7 pg/ml; 12.5±4.4 pg/ml to 87.4±10.4 pg/ml, respectively) and to allergen-driven release of the same cytokines in groups A and D (Fig. 3). Similar results were found in OVA-sensitized mice, group C, with a significant increase of IL-4, IL-6 and TNF-α in allergen stimulated cultures as compared to the unstimulated ones and to groups A and E (Fig. 4).

Similarly to group A, groups D and E showed no significant increase of allergen-driven as compared to spontaneous release of all cytokines except for IL-4 of group E, the values of which were significantly higher ($p<0.05$) in supernatants of allergen-stimulated cultures in respect to non-stimulated ones (Fig. 4). Furthermore, the absolute value of the allergen-driven release of IL-6 in group D and IL-4, IL-6 and TNF-α in group E were significantly higher compared to those found in control mice (Fig. 4). However, when considering the normalized values, only the release of IL-4 in group E was found significantly higher as compared to that detected in group A, from 1.05±1 pg/ml to 18.3±4.1 pg/ml.

Finally, no significant difference in the concentration of spontaneous or allergen-driven release of IFN-γ, IL-1β and IL-5 was found amongst the various experimental groups (data not shown).

DISCUSSION

The present study demonstrated that monomeric allergoid intragastric administration induces in mice a systemic tolerogenic response involving IL-10-producing $CD4^+CD25^+$ Tregs in spleen and cytokine release in peripheral blood, with different induction of immune changes in the GALT, mainly PP and MLN, depending on the sensitizing allergen.

Allergen sensitization in groups B and C was demonstrated by a) the increase of total IgE in blood, b) a concomitant reduction of IL-10-expressing $CD25^+CD4^+$ T cell frequency in all analyzed lymphoid tissues, compared to the control group, suggesting that local and peripheral loss of tolerance towards the sensitizing allergen had taken place, and c) an allergen-driven increase of IL-4 release by PBMCs *in vivo*, suggesting the occurrence of a peripheral Th2-dominant T helper cell activation.

Subsequently, mice were treated by administering the monomeric allergoid of the sensitizing allergen by gavage for 18 days. Allergy vaccines are usually administered by oral route, favoring a long contact of the allergen with the oral mucosa - at least 2 minutes. In the oral mucosa, the allergen is taken up and processed by DCs that favor the maturation of resident $CD4^+$ T cells into antigen-specific $CD4^+CD25^+$ Treg (3, 15) and IFN-γ/IL10-producing T cells in draining lymph nodes (16) that in turn suppress the function of effector Th2 cells by secreting the inhibitory cytokines IL-10 and TGF-β,

Table I. *IgE in control, sensitized and allergen treated mice.*

	Controls	Par j1 sensitized	Par j1 sensitized untreated	Par j1 sensitized treated	OVA sensitized	OVA sensitized untreated	OVA sensitized treated
day 25	68 ± 22	200 ± 40 *	-	-	180 ± 35 *	-	-
day 62	70 ± 12	-	210 ± 19 *	80 ± 15 †	-	228 ± 25 *	90 ± 30 †

** $p < 0.01$, compared to untreated group; † $p < 0.01$, compared to the sensitized group*

Table II. *Frequency of $CD4^+CD25^+$ T cells and IL-10 expressing $CD4^+CD25^+$ T regulatory cells in lymphoid organs.*

		Group A	Group B	Group D	Group C	Group E
$CD4^+CD25^+$ (% of total cells)	PP	2.02 ± 0.23	3.20 ± 0.19*	3.82 ± 0.15*	1.03 ± 0.12*	6.51 ± 0.34*§
	ML	5.15 ± 0.23	9.12 ± 0.30*	8.03 ± 0.20*	7.32 ± 0.27*	7.81 ± 0.31*
	SP	3.98 ± 0.42	5.16 ± 0.26 *	6.02 ± 0.27*	6.10 ± 0.17*	4.97 ± 0.23 *
$CD4^+CD25^+IL\text{-}10^+$ (% of total cells)	PP	1.92 ± 0.34	1.01 ± 0.20*	0.52 ± 0.13	1.03 ± 0.12*	1.64 ± 0.24†
	ML	3.03 ± 0.32	2.44 ± 0.18	0.21 ± 0.10*	1.69 ± 0.21*	2.02 ± 0.17†
	SP	2.72 ± 0.25	1.07 ± 0.15 *	3.49 ± 0.35 §	0.57 ± 0.18*	2.81 ± 0.22 §

Group A: control group; group B: Parj1-sensitized; group C: OVA-sensitized; group D: Parj1-sensitized/treated; group E: OVA-sensitized/treated

PP= Peyer's Patches; ML: Mesenteric Lymph nodes; SP: Spleen

*Results are shown as mean % of total tissue cells ± SEMs (n=6). *$p < 0.05$, compared to the untreated group; †$p < 0.05$, compared to the sensitized group; § $p < 0.02$, compared to the sensitized group.*

as well as through direct cell-cell contact (17).

Subsequently, the allergen extract is swallowed, but in literature there are no demonstrations on the immune potential of the ingested portion of the allergen. As mentioned above, in humans, oral allergen immunotherapy, relying on the stimulation of the GALT, gave contrasting, often discouraging, results (7-8), and one of the possible explanation of its failure seems due to the fact that ingested allergen is digested by gastric acid and intestinal enzymes and loses its immune potential. Therefore, in the present study monomeric allergoids instead of the native allergens were administered, since they are virtually unmodified when adsorbed (11), as described for Par-j1 (10). The monomeric allergoids are chemically modified allergens generated to obtain improved molecules for immunotherapy (10) with a high efficacy and a safety profile in preventive and therapeutic SLIT (18-19).

In our study, both Par-j1 and OVA allergoids induced systemic effects with a significant decrease in serum IgE, a reduction of IL-4 production and an increase of IL-10 expressing $CD4^+CD25^+$ T lymphocytes, demonstrating the immunogenic activity of the ingested allergoids. An increase of IL-10 expressing $CD4^+CD25^+$ T lymphocytes was also observed in the PP and ML of sensitized mice treated by OVA allergoid, but not in the Par-j1 allergoid

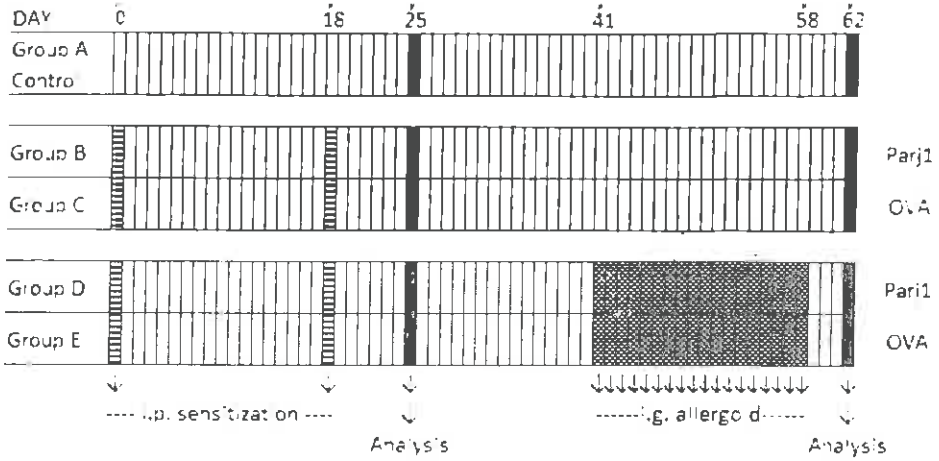

Fig. 1. *Experimental design for treatments and analysis. Mice were divided into the following 5 groups according to the treatment: Group A: control mice neither sensitized nor treated; Group B and Group C were sensitized to Par j1 and Ovalbumin (OVA,) respectively, by two intraperitoneal (i.p.) injections of the specific allergen (Day 0 and 18); Group D and Group E were firstly sensitized to Parj1 and OVA according to protocol used in Group B and C on days 0 and 18, then were treated by Parj1 and OVA monomeric allergoids, respectively, daily from day 41 to 58, by intragastric (i.g.) administration of the specific allergen. Analyses were performed on day 25 (serum IgE) and on day 62 (serum IgE, CD4/25/IL10 T cells on Peyer's patches, mesenteric lymph nodes and spleen and cytokine release from peripheral blood mononuclear cells).*

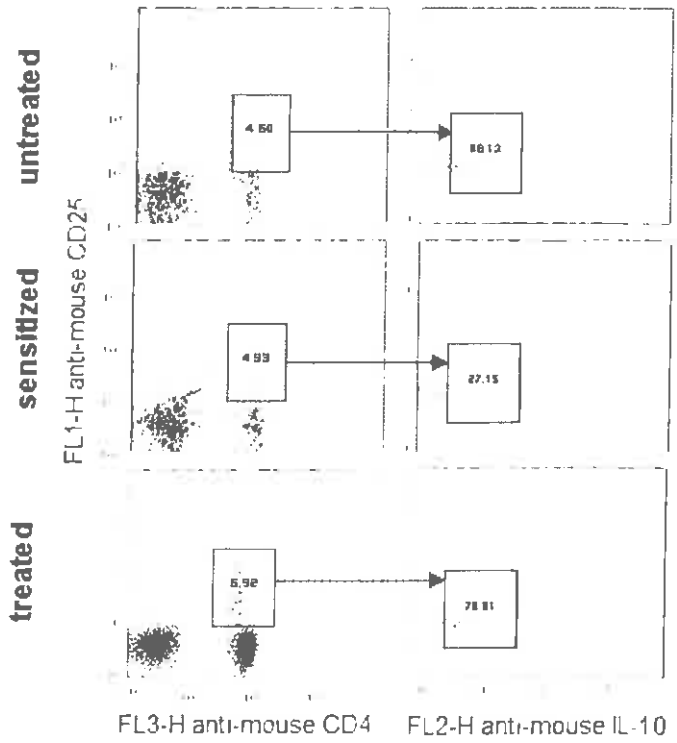

Fig. 2. *Representative dot plots of the cytofluorimetric analysis of splenic cell suspensions from individual mice ex vivo showing the frequency of CD4$^+$CD25$^+$ regulatory T cells and the frequency of the IL-10$^+$ cell subset in the different experimental groups of Parj-1.*

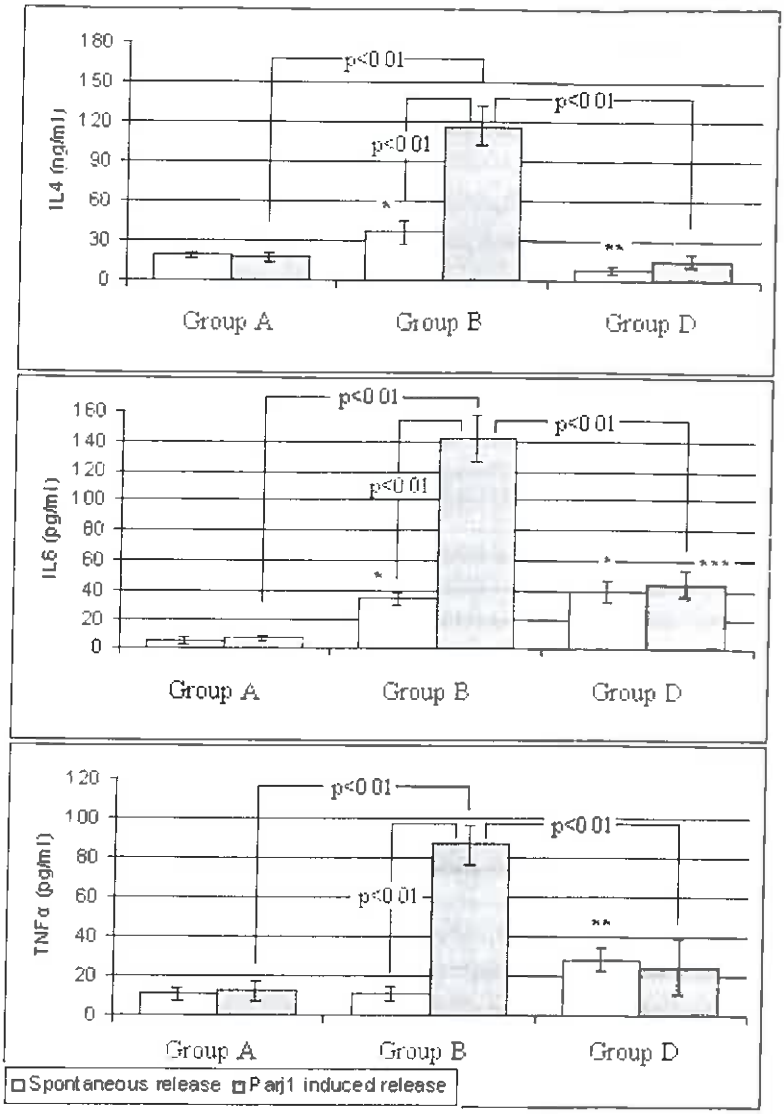

Fig. 3. *IL-4, IL-6 and TNF-α allergen-driven in vitro release by PBMCs from OVA-sensitized/treated mice. PBMCs Par-j 1 of individual mice were cultured for 72 hours in parallel wells with or without OVA allergen used for sensitization. Spontaneous and allergen-induced release of cytokine was determined by multiplex ELISA in supernatants. Spontaneous release of IL-4 and IL-6 was significantly higher in sensitized mice in respect to controls and sensitized/treated group, with significantly lower levels in sensitized/treated group in respect to controls. Spontaneous release of IL-6 was significantly higher in sensitized and sensitised/treated groups in respect to controls and, finally, TNF-α spontaneous release was significantly higher in sensitized/treated in respect to both controls and sensitized group. Allergen-driven release of all cytokines was significantly greater than the spontaneous one in sensitized mice, whereas no significant differences were found in control and sensitized/treated mice. Allergen-driven releases of all cytokines were significantly higher in sensitized mice in respect to the corresponding value of controls and sensitized/treated mice, with similar levels between these last groups except for IL-6 that was significantly higher in sensitized/treated mice. Results were expressed as mean ± SD of cytokine levels in single cell cultures from same group of mice (n=6).*

**: $p<0.05$ in respect to spontaneous release of control group; **: $p<0.05$ in respect to spontaneous release of control and sensitized groups; ***: $p<0.05$ in respect to allergen driven release of control group.*

Group A: control mice; Group B: Par-j 1-sensitized mice; Group D: Par-j 1-sensitized and allergoid-treated mice

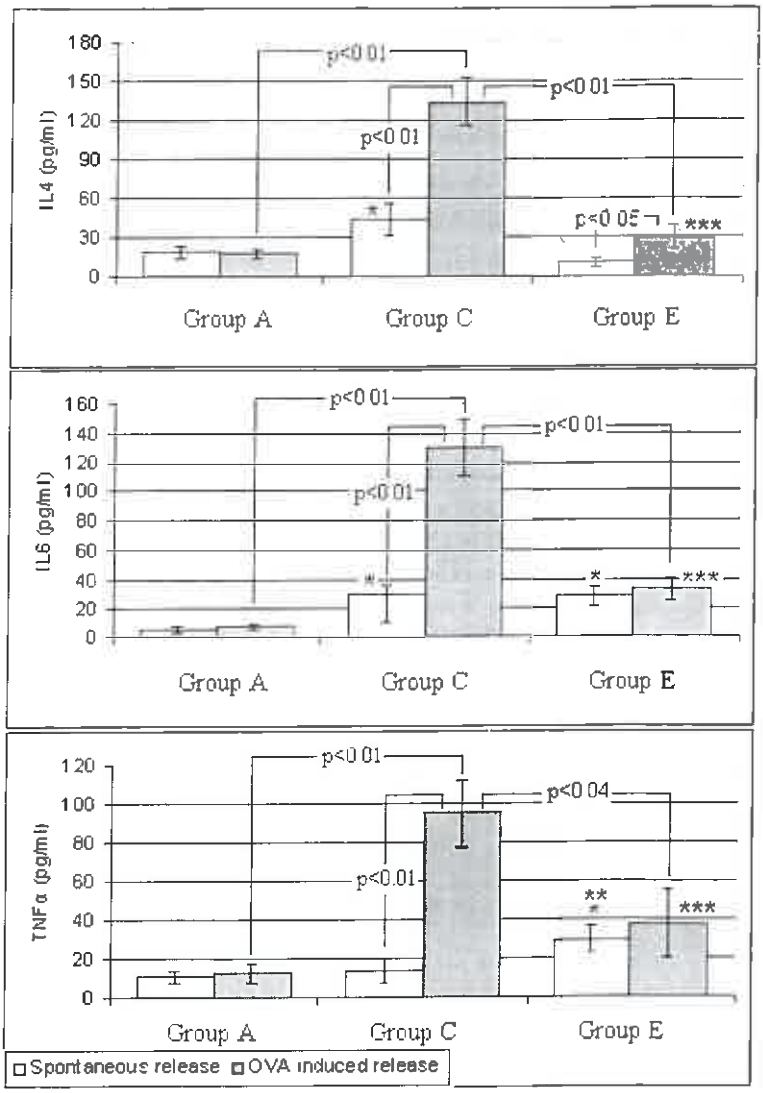

Fig. 4. *IL-4, IL-6 and TNF-α allergen-driven release in vitro by PBMCs from OVA sensitized/treated mice. PBMCs of individual mice were cultured for 72 hours in parallel wells with or without OVA allergen used for sensitization. Allergen-induced and the spontaneous cytokine levels were determined by multiplex ELISA. Spontaneous release of IL-4 and IL-6 was significantly higher in sensitized mice in respect to controls and sensitized/treated group, with similar levels in sensitized/treated group and controls. Spontaneous release of IL-6 was significantly higher in sensitized and sensitised/ treated groups in respect to controls and, finally, TNF-α* spontaneous release was significantly higher in sensitized/treated *group in respect to both controls and sensitized group. Allergen-driven release of all cytokines was significantly greater than the spontaneous one in sensitized mice and only for IL-4 in sensitized/treated group. Allergen-driven releases of all cytokines was significantly higher in sensitized mice in respect to the corresponding value of controls and sensitized/ treated mice, with a significantly higher release in sensitized/treated mice in respect to controls.*
Results were expressed as mean ± SD of cytokine levels in single cell cultures from same group of mice (n=6).
**: $p<0.05$ in respect to spontaneous release of control group; **: $p<0.05$ in respect to spontaneous release of control and sensitized groups; ***: $p<0.05$ in respect to allergen driven release of control group.*
Group A: control mice; Group C: Ova-sensitized mice; Group E: OVA-sensitized and allergoid-treated mice

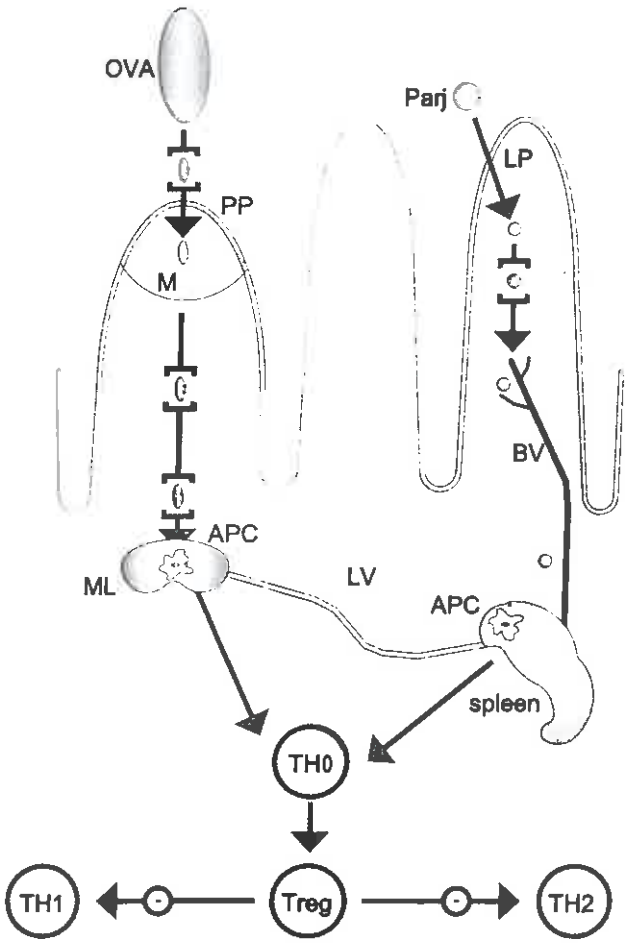

Fig. 5. *The path to oral tolerance for OVA and Parj allergens. OVA antigen can cross the intestinal epithelial-cell barrier through Peyer's patches captured by APC and carried to local mesenteric lymph nodes; Parj1, with a lower molecular weight (12 kDa respect to 69 kDa of OVA) can be processed and presented on MHC molecules by intestinal epithelial cells or cross through the epithelium, where it is absorbed into capillaries that drain directly into the general circulation. Each of these pathways ultimately converges at the spleen. APC cells stimulate the proliferation and differentiation on Th0 into T regulatory cells, which inhibit both Th1 and Th2 cells (1).*
ML = mesenteric lymph node; BV = Blood Vessel; LV= Lymphatic Vessel; APC= Antigen Presenting Cell; OVA= Ovalbumin; PP = Peyer's Patches; LP = Lamina Propria; M = M cell

treated mice. This difference can be explained by the fact that Par-j1 and OVA could have followed different uptake and processing pathways (20) due to both differences in biochemical and immunogenic features. They can induce Treg cell subsets not expressing IL-10 (not evaluated in this study) and/or Treg expressing alternative patterns of chemokine receptors and pursuing different migratory paths (21-22). The spleen exclusive involvement in Par-j1 treated mice could be explained by the fact that, due to the low size, Par-j1 (12 kDa) could have permeated the intestinal epithelium and moved straight into the blood stream, overcoming the M cells (5), going directly to general circulation at the spleen (Fig. 5): the epithelial-cell route described by Mayer in Nature Review Immunology (23).

Fed antigens can cross the intestinal epithelial-cell barrier through the epithelium. From the intestinal mucosa they are absorbed into capillaries that drain into the portal vein and the liver (essential in inducing tolerance) and carried through the general circulation to the spleen (23). Alternatively, another important port of entry for antigens are the PP. However, although early studies supported a role for PP in tolerance induction (24), more recent studies have cast doubt on this concept. M cells are specialized to take up particulate antigens, whereas peptide presentation of orally administered soluble antigens occurs in the absence of PP (25). In this case the tolerance can be obtained in MLN, where antigens are carried to.

The pathways by which antigen either passes through the epithelium into blood capillaries, or is carried by phagocytic cells to the MLN through lymphatics (taken up through M cells or captured by dendritic cells) ultimately converge at the spleen and would implicate the spleen as a potential site for tolerance induction.

The analysis of *in vitro* allergen-driven release of cytokines by PBMCs supports the notion that Th2 related cytokines, such as IL-4, are up-regulated in sensitized mice and down-modulated following allergoid treatment reflecting the restoration of the tolerance against sensitizing allergens.

Allergen-induced IL-6 release significantly varied in mice with various treatments with an increase of this cytokine in association with a low frequency of Tregs. In fact, IL-6 trans-signaling into T cells emerged as a key pathway for blockade of the development of adaptive Tregs in shifting the balance between effector and regulatory T cell

numbers (26).

The significance of the changes in TNF-α in our experiments is not easily explained, in fact, TNF-α is a pleiotropic cytokine which can have proinflammatory or immunosuppressive effects, depending on the context, duration of exposure and disease state. Some authors found this cytokine as a potent activator of Tregs (27), whereas others described the absence of Treg expression and function depending on the presence of TNF-α and other soluble mediators of inflammation (28).

In our study, the concentration of IFN-γ does not vary among the various groups of mice. This confirms that IFN-γ changes could be relevant in late phases during allergen-SIT, being in the first phase of treatment lowered by the action of Tregs, active on both Th1 and Th2 cytokines (29-30).

This study provides, for the first time, evidence that the ingested part of allergoid used for SLIT could abrogate allergen-specific cytokine production and T-cell responses *in vitro*, accompanied by suppressed IgE production. These findings imply that the immunogenicity of the monomeric allergoids is due to the activation either from sublingual immune system either from the GALT.

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