# *LACTOBACILLUS PARACASEI* LP6 FAVORS IMMUNE MODULATION INDUCED BY ALLERGOID TREATMENT IN RAGWEED SENSITIZED MICE

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**It has been hypothesized that lactic acid bacteria (LAB) could be used as adjuvant for specific immunotherapy (SIT), as various studies conducted on humans and animals converge to define LAB as anti-Th2 modulators and Treg inducers. In the present study we evaluated the effects of LAB, in particular *Lactobacillus paracasei* Lp6 (Lp6), in a mouse model of ragweed (RW) allergy. Groups of Balb/c mice, experimentally sensitized towards ragweed, were treated by viable Lp6 or by RW-allergoid with or without co-administration of Lp6. A control group was sham-sensitized with PBS and sham-treated with water and a group was sensitized with RW and treated with water. Serum IgE, RW-induced release of IFN-γ, IL-4 and IL-10 from splenocytes and the frequency of CD4CD25 regulatory T cells (Tregs) expressing Foxp3 or IL-10 were evaluated in various groups. RW-allergoid treatment induced a reduction of serum IgE, with a decrease in RW-induced release of IL-4, and an increase in IL-10 and IFN-γ, along with a significant change in the frequency of Tregs, both CD25+ and -. The joint RW-allergoid+Lp6 treatment induced the highest degree of suppression of allergen-driven IL-4, the greatest reduction of IL-4/IFN-γ and IL-4/IL-10 ratios and the most significant increase of Foxp3 and IL-10 expressing Tregs: The study shows that Lp6 strengthens the immune modulation induced by allergoid-SIT in RW-sensitized mice, essentially characterized by a differential induction of Tregs associated to a reduction of IL-4; data converge to define a role of SIT adjuvant for Lp6.**

Lactic acid bacteria (LAB) have been proposed as adjuvant for SIT, as various studies conducted on humans and animals converge to define the effect of LAB as anti-Th2 modulators (1) and Treg inducers (2). LABs have been shown to be effective in the prevention and control of allergy in children with atopic dermatitis (3-4), although some studies have shown the association of atopic disorders with specific strains of intestinal microflora at birth (5). As a mechanism of action, LAB can bind to toll-like receptors (TRL) leading to dendritic cell (DCs) activation and production of IL-12 or IL-10

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(6), which in turn might drive the maturation of naïve $CD4^+$ T cells into Th1 or Treg, respectively (7-8). LABs have been mainly studied for their potential role in preventing allergy onset (9) in mice; moreover, allergen specificity and functionality of the LAB-induced Tregs as well as the strain-dependency of these effects on Tregs have been limitedly investigated.

In the present study we evaluated the effects of LAB, in particular *Lactobacillus paracasei* Lp6 (Lp6), in a mouse model of allergy. In the first part of the protocol, Balb/c mice, experimentally sensitized towards ragweed (RW), were treated by viable Lp6 *in vivo,* to evaluate the possible immune effects of lactobacilli in sensitized animals. In the second part of the study, sensitized groups of mice were submitted to a mucosal desensitizing protocol by RW-allergoid with or without co-administration of Lp6, in order to evaluate the possible role of Lp6 as adjuvant for SIT.

## MATERIALS AND METHODS

Thirty pathogen-free Balb/c mice (Charles River, Milan, Italy), aged 8-10 weeks, were housed in a laminar flow cabinet and cared for in accordance with the guidelines of the European Convention (ETS No. 123). Experiments were approved by the Animal Experiments Committee of Chieti University.

Lp6, cultured in DeMan-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose, was supplied by Dr M. G. Fortina (University of Milan, Italy) This strain was chosen on the basis of its capacity to induce activation *in vitro* of bone marrow-derived dendritic cells and T regulatory cells (Granucci MG, unpublished data). Amb a1 (Lofarma, Milan, Italy), the major allergen of ragweed, was used to induce sensitization by intraperitoneal injections. SIT was performed in sensitized mice using a RW allergoid generated as described by Mistrello (10).

*Sensitization and treatment protocols*

Groups of six Balb/c mice were treated as described in Fig. 1. Briefly, three groups of mice were immunized by three intraperitoneal (i.p.) injections, 7 days apart, of 100 µg native allergen pre-adsorbed to 2 mg $Al(OH)_3$ in 200 µl saline solution. Seven days after the last injection, the mice were administered intragastrically, through gavage, 5 days a week, for three weeks, with water (group B), RW-allergoid (group C) or RW-allergoid+Lp6 (group D). The RW-allergoid dose was 15 µg per mouse per day, in 200 µl pirogen-free water, that of Lp6 was 200 µl of bacteria suspension containing $10^9$ cfu. The control group (group A) consisted of animals which were sham-sensitized with PBS and sham-treated with corresponding volumes of water. At the end of the study the spleens were harvested and disaggregated to single cell suspensions for Treg frequency determination; splenocytes were cultured and assessed for cytokine production *in vitro.*

In a preliminary experiment, a group of 6 mice (sens/Lp6 group) were sensitized by RW as described and then treated by 200 µl of viable Lp6 through gavage, 5 days a week, for three weeks, to verify the potential of Lp6 to modulate the immune system, particularly serum IgE, cytokine release, and T regulatory cell; concurrently, another group of 6 mice (sens/sham group) was only sensitized with RW and sham treated with water to be used as control.

*Cell cultures and cytokine assays*

Single cell suspensions of spleen were prepared by passing the cells through 70-µm cell strainers (BD Labware, San Jose, CA). Erythrocytes were lysed by incubation with red cell lysis buffer (Sigma Aldrich srl, Milan, Italy). For cytokine measurements, $5x10^6$ splenocytes, isolated soon after sacrifice, were incubated in triplicate 48-well plates in 500 µL complete medium for each *in vitro* condition. Cells were stimulated with 50 µg/mL RW purified protein extract or with 10 µg/mL phytohemagglutinin (PHA), for 48 hours; parallel splenocyte cultures were kept without any stimulus to assess the spontaneous cytokines release. Cell culture supernatants were harvested and stored at -80°C until analyzed. Cytokine responses were determined on diluted supernatants (1:3 for IFN-γ; 1:2 for IL-4 and IL-10) using the commercial kit for mouse IFN-γ (limit of detection: 15 pg/ml), IL-4 (limit of detection: 4 pg/ml) and IL-10 (limit of detection: 30 pg/ml) (eBiosciences, San Diego, CA). Spontaneous cytokine release was subtracted to the allergen-induced or PHA-induced release to obtain the net release value that was used to evaluate the treatment effect.

*ELISA for measurement of total IgE*

Approximately 50-200 µl of blood was collected from the tail vein from each mouse at day 20. At day 45, 500-800 µl blood was collected by cardiac puncture in anesthetized mice. Blood samples were stored at 4°C to allow clotting then centrifuged for 10 minutes at 1,000x g. Serum layers were removed and individually stored at -70° C until analyzed. Total serum IgE were quantified in serum using ELISA MAX™ Set Deluxe Mouse IgE (BioLegend, San Diego, CA), following the manufacturer's instructions.

*Flow cytometry*

Spleens were rapidly processed to obtain single cell suspensions for cytofluorimetric analysis. After erythrocytes lysis, cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse CD4 (clone GK1.5) and allophycocyanin-conjugated anti-mouse CD25 (clone PC61.5), for 1 h (both by eBioscience) in 100 µl FACS buffer (0.002% sodium azide, and 0.2% BSA in PBS). For intracellular Foxp3 staining, CD4CD25 double stained samples were fixed and permeabilized with BD Perm/ Wash™ buffer; then 0.5 µg phycoerythrin-conjugated anti-mouse Foxp3 (FJK-16s) were added and samples were incubated for 1 h at 4°C in the dark. To assess intracellular IL-10, freshly isolated splenocytes were firstly incubated (2 h, 37°C, 5% $CO_2$) with 5 ng/ml phorbol-myristate acetate and ionomicyn (500 ng/ml) in complete 10% fetal calf serum RPMI medium; then, brefeldin A (10 µg/ml) was added for further 4 h in the same conditions to block IL-10 transport and secretion. At the end of the stimulation, cells were stained for CD4 and CD25 surface antigens and finally fixed and permeabilized as described above, before the last incubation with 5 µg/ml phycoerythrin-labelled anti-IL-10. To assess non-specific fluorescence, defining positive events, we used Fluorescence Minus One (FMO) controls. Samples were acquired within 18 hours using a BD FACSCalibur™ flow cytometer with CellQuest 3.2.1.f1 software and data were analysed using FlowJo™ software (TreeStar, Ashland, OR, USA). In order to obtain clear and interpretable results, we initially set up a gating strategy: lymphocytes were first selected in a Forward Scatter-Area *vs* Side Scatter-Area gate. Next, CD4+ cells were gated and subsequently CD25+ and Foxp3+ (or IL-10) cells were isolated and then combined using a Boolean gate (11).

For each group of *in vivo* treatment, the frequency of $CD4^+Foxp3^+$ T cells and the frequency of the IL-10-expressing $CD4^+$ T cells in total splenocytes were assessed.

*Statistical analysis*

Experimental data were reported as mean±SD. Statistical analysis of the data and significance assessment for all control/treated group pairs were performed by 1-way ANOVA with Newman-Keuls Multiple Comparison post-test using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, US). $P<0.05$ was considered statistically significant.

## RESULTS

*Lp6-induced immune modulation in mice sensitized towards RW*

The intragastric administration of Lp6 to RW-sensitized mice was able to significantly reduce total IgE production. In fact, serum IgE level was 2466.0±196.4 ng/ml in RW-sensitized and water-treated mice (group sens/sham), whereas the Lp6-treated mice showed a lower level (464.8±456.7 ng/ml, $p<0.001$) (Table I). Moreover, *in vitro* cultured splenocytes from Lp6-treated mice released significantly less allergen-induced IL-4 (20.0±6.5 pg/ml) and produced far more PHA-induced IFN-γ (2345.0±415.2 pg/ml) than cultures derived from the spleens of mice which received the water-treatment (118.5±20.34 pg/ml for IL-4, $p<0.01$, and 511.4±193.9 pg/ml for IFN-γ, $p<0.001$; Table I). Lp6 was able to induce a significant increase in the frequency of Tregs, expressing Foxp3 and IL-10, the latter showing the most significant changes, either CD25 positive or negative (Table I).

*Lp6 in RW Allergoid-treated Sensitized Mice*

*Total IgE levels*

RW-allergen-sensitized mice (group B) were characterized by a very high level of total IgE (2465.8±196.4 ng/ml), which was significantly higher compared with the negative control group A (61.2±5.2 ng/ml) ($p<0.001$). The intragastric administration of RW-allergoid (group D) was associated with a marked reduction of total IgE levels (578.9±421.6 ng/ml), compared with those measured in sensitized mice ($p=0.001$). Also the treatment with the combination RW-allergoid+Lp6 (group D) determined the reduction of total serum IgE (1456.0±526.8 ng/ml) ($p=0.01$), although to a minor extent than in group B.

*Allergen-driven IL-4 production from spleen cells in vitro*

Spleen cells of group A were able to secrete a barely detectable amount of IL-4 4.16 ±3.6 pg/ml) upon stimulation with the RW-allergen, while splenocytes of RW-allergen-sensitized mice (group B) produced a significantly higher amount of IL-4 (118.5±20.34 ng/ml) ($p=0.001$). In the group C, who received the treatment with RW-allergoid, *in vitro* IL-4 release was significantly lower than in group B (80.47±2.9 ng/ml) ($p<0.01$); however, the strongest reduction was observed for the group D that was treated with RW-allergoid+Lp6 (45.32±18.9 ng/ml) ($p=0.001$) (Table II).

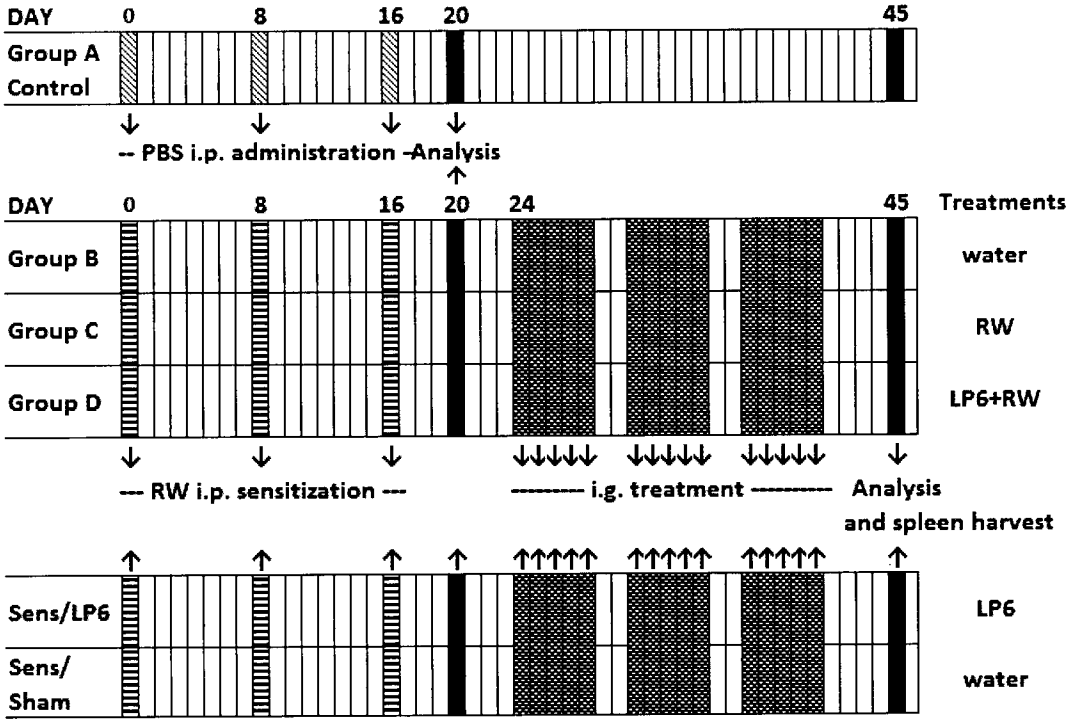


**Fig. 1.** *Experimental design for treatments and analysis. Mice were divided into four groups of six animals according to the treatment carried out. Group A: control mice were sham-sensitized with PBS and sham-treated with water; Groups B, C, D were sensitized to Ragweed (RW), by three intraperitoneal (i.p.) injections of the Amb a1 (Day 0, 8 and 16); after 5 days from the sensitization, mice received 5 days a week, for three weeks, either water (Group B), or RW-allergoid (Group C), or RW-allergoid+Lp6 (Group D). Analysis for the detection of serum IgE were performed on day 20 and on day 45. Treg frequency and in vitro cytokine release from spleen cells were measured on day 45. A preliminary experiment was carried out on RW-sensitized mice for the effect of Lp6 treatment alone on cytokine release and T regulatory cell expression.*

*Allergen-induced in vitro release of* IFN-γ *from splenocytes*

RW-induced IFN-γ release by splenocytes *in vitro* was similar in group A (not sensitized, not treated) and in group B mice (640.3±364.0 and 511.4±193.9 pg/ml, respectively), irrespective of the allergen sensitization. On the contrary, splenocytes from mice treated with RW-allergoid (group C) or RW-allergoid+Lp6 (group D) secreted higher levels of IFN-γ (1106.5±134.3 and 753.8±156.2 pg/ml, respectively) compared to groups A and B which received no treatment at all; however, such difference reached statistical significance only for group D in respect to group B ($p=0.01$) (Table II). The ratio IL-4/IFN-γ was significantly lower in group D in respect to group C ($p<0.05$) and group C and in group D in respect to group B ($p<0.01$ for both) (Fig. 2A).

*Allergen-driven IL-10 production from spleen cells in vitro*

IL-10 was undetectable in supernatants of cultures from the control group A, while RW sensitization was associated with a release of 248.4±75.6 pg/ml IL-10. The amount of this cytokine was significantly higher in the supernatants of the RW-allergen-stimulated cultures set up from spleens of group C and D mice treated with RW-allergoid (611.4±89 pg/ml; $p<0.01$) and RW-allergoid+Lp6 (531.9±74.4 pg/ml; $p<0.001$), respectively (Table II).

Both IL-4/IFN-γ ratio (Fig. 2A) and IL-4/IL-10 ratio (Fig. 2B) were significantly lower in

**Table I.** *In vivo immunomodulatory effects of Lactobacillus paracasei Lp6 in RW-sensitized mice (n=6) on total serum IgE ($IgE_{tot}$) level, in vitro release of IL-4 and IFN-γ by spleen (SP) cells and frequency ex-vivo of T regulatory cells (both $CD25^+$ and $CD25^-$) among spleen cells. Results are compared with values obtained in the group of mice which received the sham treatment (n=6).*

| | | *In vivo* treatment | | |
|---|---|---|---|---|
| Parameter | | water (n=6) | Lp6 (n=6) | *p-value* |
| IgE total | ng/ml | 2466.0±196.40 | 464.8±456.7 | *<0.001* |
| IL-4 RW | pg/ml | 118.5±21.30 | 20.0±6.5 | *<0.01* |
| IFN-γ PHA | pg/ml | 776.9±667.90 | 2345.0±415.2 | *<0.001* |
| $CD4^+25^+Foxp3^+$ | % of total spleen cells | 2.80±0.38 | 4.51±1.34 | *<0.05* |
| $CD4^+25^+IL10^+$ | | 0.28±0.13 | 4.92±1.30 | *<0.001* |
| $CD4^+25^-Foxp3^+$ | | 8.54±1.49 | 25.57±5.09 | *<0.01* |
| $CD4^+25^-IL10^+$ | | 7.20±3.07 | 25.57±5.09 | *<0.01* |

**Table II.** *IL-4, IFN-γ and IL-10 release from spleen cells in vitro in various mice groups.*

| | Group A **control** | Group B **water** | Group C **RW-allergoid** | Group D **RW-allergoid+Lp6** |
|---|---|---|---|---|
| **IL-4** (pg/ml) | 4.16 ±3.6 | **118.5 ± 20.34*** | **80.47 ± 2.9#** | **45.32 ±18.9#§** |
| IFN-γ (pg/ml) | 640.3 ± 364.0 | 511.4 ±193.9 | **1106.5 ± 134.3#** | 753.8 ±156.2 |
| **IL-10** (pg/ml) | *undetectable* | **248.4 ±75.6*** | **611.4 ± 89#** | **531.9 ±74.4#** |

** statistically significant difference vs group A; # statistically significant difference vs group B; § statistically significant difference vs group C. Group A: control mice (n=6); Group B: RW-sensitized mice (n=6); Group C: RW-sensitized and RW-treated mice (n=6); Group D: RW-sensitized and RW+Lp6-treated mice (n=6). RW: ragweed; Lp6: Lactobacillus paracasei 6.*

group C, RW-allergoid-treated, and group D, RW-allergoid+Lp6-treated, in respect to the sensitized group B. Besides, both ratios were significantly lower in group D, which was treated with the Lp6 supplement, in respect to group C, which did not received Lp6 ($p<0.05$).

*Regulatory T cells*

A comparable frequency of regulatory T cells, characterized by the immunophenotype CD4+CD25+Foxp3+, was found in negative control mice (group A, 2.43±0.36%) and RW-sensitized mice (group B, 2.80±0.38%) (Fig. 3A). Compared to these groups, a significantly higher occurrence of these cells was found in groups C and D, that were treated respectively with RW-allergoid (4.44±0.77%; $p<0.05$) and RW-allergoid+Lp6 (4.83±1.22%; $p<0.01$) (Fig. 3A). The abundance of another subpopulation of regulatory T cells, expressing the immunosuppressive cytokine IL-10, was found to be significantly increased only in the group D (0.73±0.22%) treated with RW-allergoid+Lp6, compared to the other groups (0.25±0.10% in group A, 0.28±0.13% in group B and 0.34±0.23% in group C; $p<0.05$, group D *vs* each group) (Fig. 4A).

Besides, a high frequency of the CD4+Foxp3+ T cells lacking the CD25 marker was detected in groups C and D treated with RW-allergoid (19.80±3.73%) and RW-allergoid+Lp6 (24.00±5.60%), with significant differences ($p<0.01$) compared to groups A (8.53±1.48%) and B (7.15±0.69%) (Fig. 3B). Similarly, the frequency of CD4+ T cells

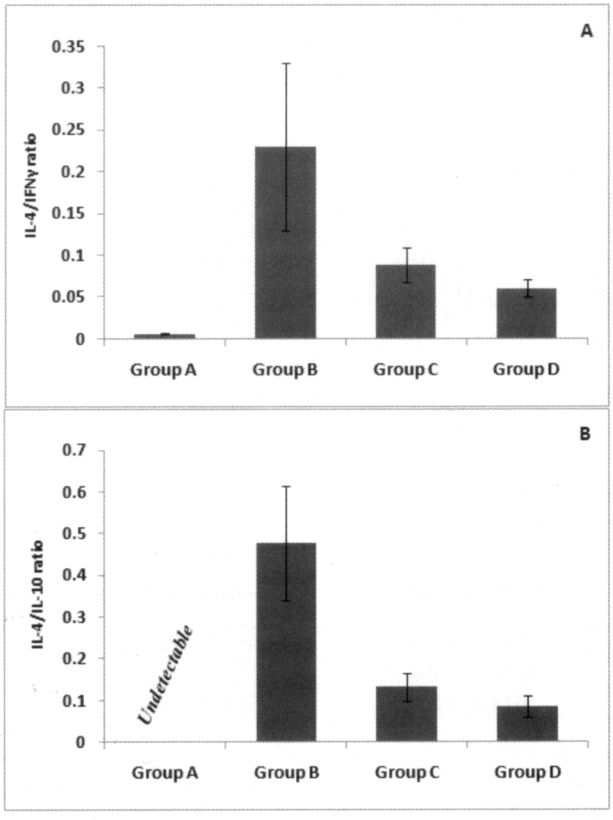


**Fig. 2.** *IL-4/IFN-γ and IL-4/IL-10 ratios among the various mice groups. In group C and D mice, treated by RW-allergoid and RW-allergoid+Lp6, respectively, the ratio IL-4/INF-γ (**A**) and IL-4/IL-10 (**B**) were significantly lower in respect to group B, RW-sensitized and not treated. Besides, the same ratio was significantly lower in group D, RW-allergoid+Lp6 treated, in respect to group C, RW-allergoid treated. * p<0.01 in respect to groups A and B; # p<0.05 in respect to group C. Group A: control mice (n=6) ; Group B: RW-sensitized mice (n=6); Group C: RW-sensitized and RW-treated mice (n=6); Group D: RW-sensitized and RW+Lp6-treated mice (n=6).*
*RW: ragweed; Lp6: Lactobacillus paracasei 6*

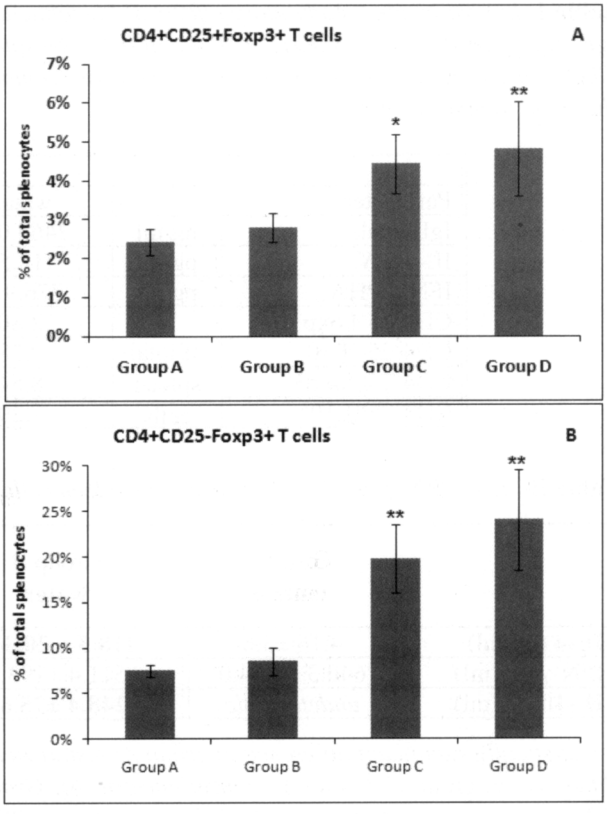


**Fig. 3.** *Percentage of regulatory T cells expressing Foxp3. A significantly higher occurrence of CD4+Foxp3+ cells expressing (**A**) or lacking (**B**) the CD25 antigen was found in groups C and D mice which underwent the treatments with RW-allergoid and RW-allergoid+Lp6. * p<0.05 in respect to Groups A and B; ** p<0.01 in respect to Groups A and B. Group A: control mice (n=6); Group B: RW-sensitized mice (n=6); Group C: RW-sensitized and RW-treated mice (n=6); Group D: RW-sensitized and RW+Lp6-treated mice (n=6). RW: ragweed; Lp6: L. paracasei 6*

expressing intracellular IL-10, but not CD25, were significantly increased in groups C (22.7±3.9%) and D (19.8±6.4%) in respect to groups A (7.2±3.1%; p<0.01) and B (5.5±1.0%; p<0.01) (Figs. 4B and 6). Representative cytofluorimetric dot plots describing the gating strategy and results for the evaluation of the subpopulations of regulatory T cells described above are shown in Figs. 5-8.

## DISCUSSION

Our results demonstrate that SIT was effective in inducing peripheral Tregs in RW-sensitized animals. Consistent with these findings, two molecular traits of allergic sensitization were inhibited, including total IgE and allergen-driven IL-4 release; IL-10, an immunosuppressive cytokine, was detected in high

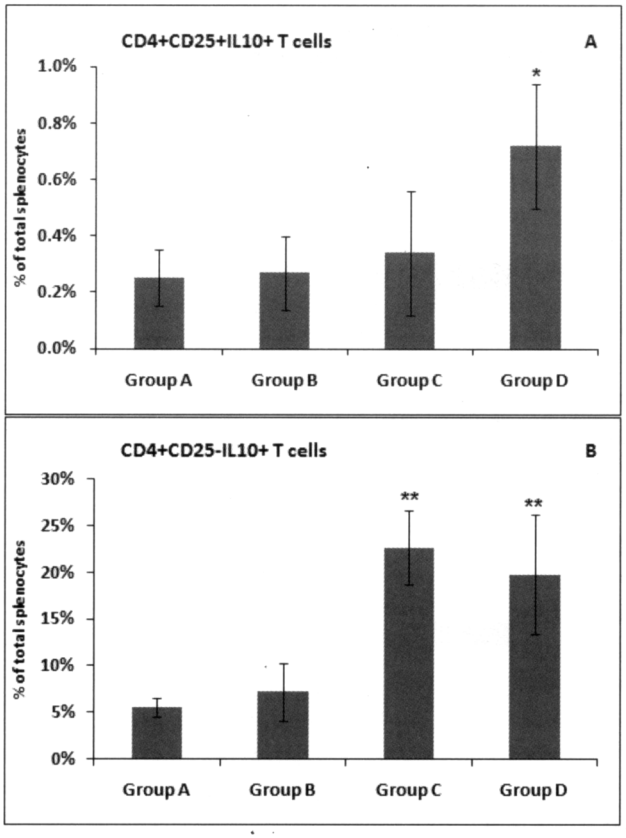


**Fig. 4.** *Percent of T regulatory cells expressing IL-10. The percentage of IL-10-expressing CD4+CD25+ cells (A) was significantly higher in group D, that received the treatment with RW-allergoid+Lp6 in respect to any other group. Besides, the frequency of CD4+ T cells expressing intracellular IL-10 and lacking CD25 was significantly higher in groups C and D in respect to control groups A and B. * $p<0.01$in respect to all groups; ** $p<0.01$ in respect to Groups A and B. Group A: control mice (n=6); Group B: RW-sensitized mice (n=6); Group C: RW-sensitized and RW-treated mice (n=6); Group D: RW-sensitized and RW+Lp6-treated mice (n=6). RW: ragweed; Lp6: Lactobacillus paracasei 6.*

quantities in the culture medium of splenocytes of SIT-treated mice after re-exposure to allergen. This treatment was also associated with high *in vitro* allergen-driven IFN-γ production, an immunological feature of a therapeutic effect (12), with an increased ratio of allergen-induced Th1/Th2 cytokines.

In mice receiving Lp6 in addition to RW-allergoid SIT, the most significant increase of Foxp3 and IL-10 expressing Tregs were observed (Figs. 3-8).

Besides the conventional $CD4^+CD25^+Foxp3^+$ Tregs, we observed a high and significant increase of $CD4^+Foxp3^+$ T cells, lacking the CD25 antigen, both in mice treated with the RW-allergoid, and in those treated with the RW-allergoid+Lp6 (Figs. 3B, 4B, 6 and 7). It has been proposed that $CD4^+CD25^-$ $Foxp3^+$ T cells constitute a reservoir of committed regulatory cells that can regain CD25 upon antigen stimulation and are able to confer tolerance when transferred into allergic recipient mice (13). On the other hand, mice lacking CD25 can develop functional CD4+Foxp3+ T cells in the thymus, with normal suppressive function *in vitro* (13); however, these cells might have a survival defect (14). Moreover, bacterial strain-specific induction of $Foxp3^+$ Tregs is protective in murine models of allergy (2), and a mixture of five probiotics could upregulate Foxp3 levels more than 2-fold in the $CD4^+CD25^-$ population (15).

Furthermore, we observed a significant increase of $CD4^+IL\text{-}10^+$ T lymphocytes, both $CD25^+$ and $CD25^-$, after the combined RW-allergoid and Lp6 treatment. Indeed, allergen immunotherapy has been found to induce a population of $CD4^+CD25^-$ T cells producing IL-10 able to suppress allergen-driven proliferation of $CD4^+CD25^+$ effector T cells *in vitro* (16). Such IL-10 producing $CD4^+$ T cells could be analogous to the adaptive Tr1 cells $CD25^-$ (also not expressing Foxp3, IFN-γ, IL-4 and IL-17) associated with down-regulation of Th1 pro-inflammatory responses during chronic parasite infections (17) or involved in colitis mediated by *Helicobacter* infection (18).

In our study we chose not to perform Foxp3/IL-10 co-staining because it was evident that several types of antigen-induced natural and adaptive regulatory T cells characterized by the capacity to secrete IL-10 exist, but Foxp3 was found not to be a requirement for the activity of Treg expressing IL-10 activity *in vitro* or *in vivo* (19) and a recent clinical study showed that allergen-specific IL-10-secreting type I Tregs, but not $CD4^+CD25^+Foxp3^+$ T cells, are decreased in the peripheral blood of patients with persistent allergic rhinitis (20), suggesting that IL-10 expression is not always subordinated to that of the Foxp3 transcription factor. A few examples of Foxp3/IL-10 co-staining protocols for FACS

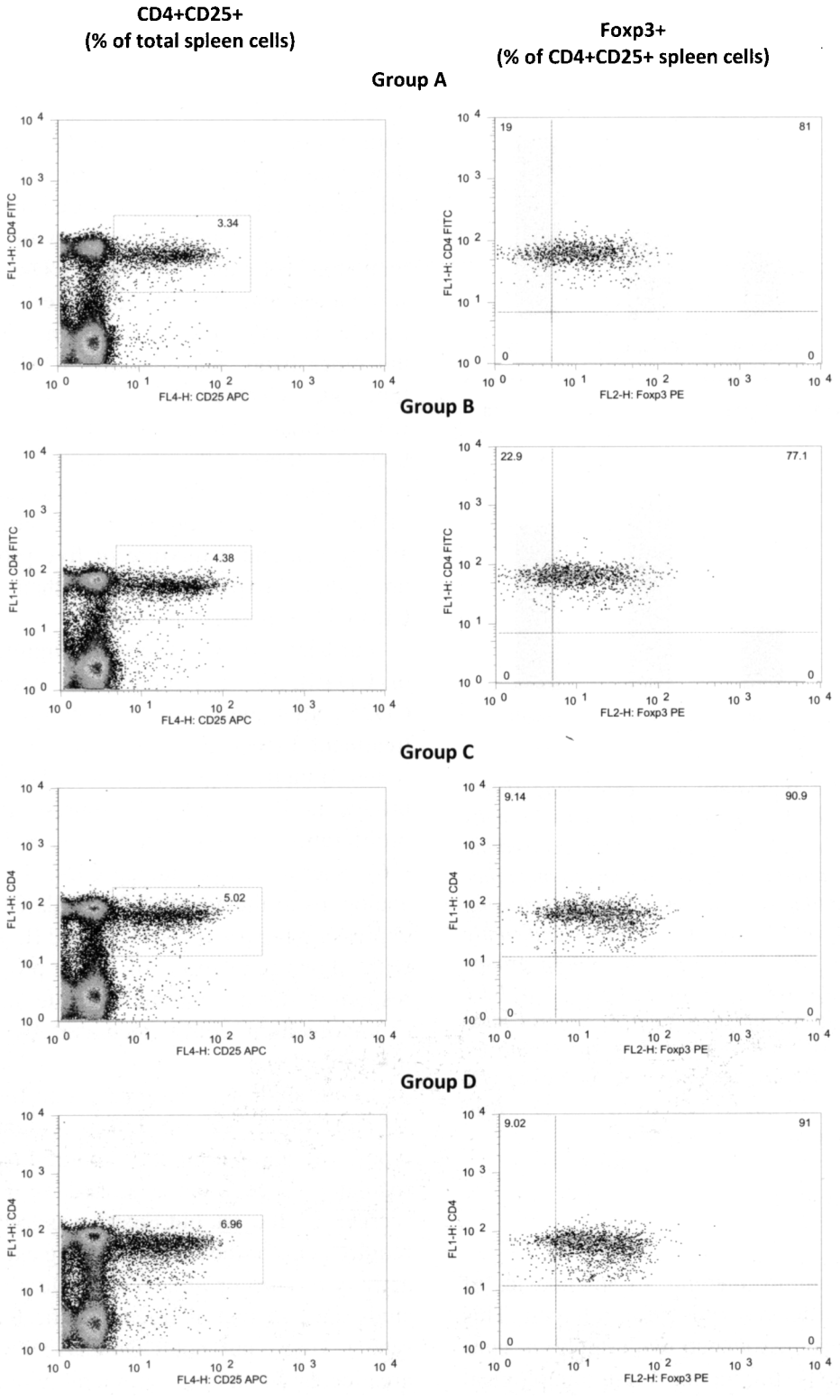


**Fig. 5.** *Representative cytofluorimetry dot plots of the frequency of CD4+CD25+ cells in total spleen cells (left side, boxed area), and their subpopulation expressing Foxp3 (right side, upper right quadrants) in the various groups of treatment. Group A: control mice (n=6); Group B: RW-sensitized mice (n=6); Group C: RW-sensitized and RW-treated mice (n=6); Group D: RW-sensitized and RW+Lp6-treated mice (n=6).*

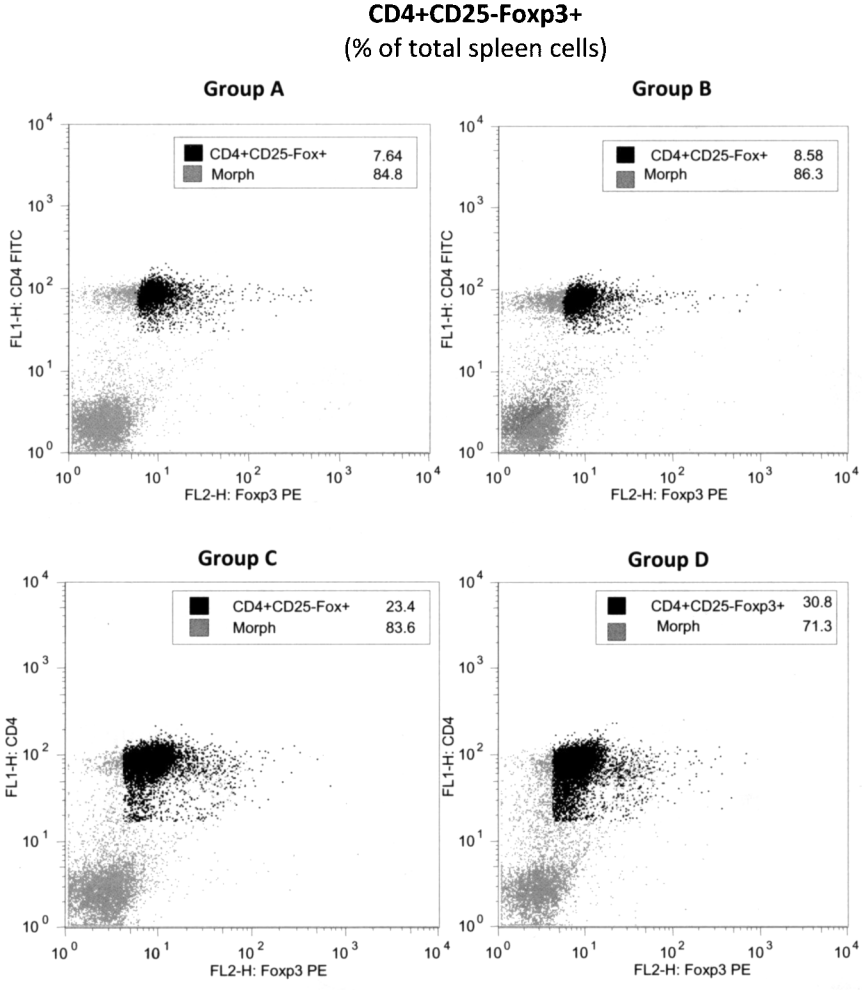


**Fig. 6.** *Representative cytofluorimetry dot plots showing the frequency of spleen CD4+Foxp3+ not expressing CD25 (dark gray dots) in the various groups of treatment. Group A: control mice (n=6); Group B: RW-sensitized mice (n=6); Group C: RW-sensitized and RW-treated mice (n=6); Group D: RW-sensitized and RW+Lp6-treated mice (n=6).*

analysis are found in the literature, relative to studies on inflammation in parasitic infection (21) and autoimmune conditions (22), reporting either lack of IL-10 in the first case, or the presence, in the latter.

In addition to Tregs, the association of Lp6 and RW-allergoid treatment induced the highest degree of suppression of allergen-driven IL-4 *in vitro* (Table II), the greatest reduction of IL-4/IFN-γ and IL-4/IL-10 ratios (Fig. 2) in respect to the other groups of mice. These effects are due to the adjuvant activity of Lp6 that, as other probiotics, is able to promote the induction of allergen-specific Treg cells (1), to polarize the immune response towards the Th1 pattern and to suppress the Th2 response *in vitro* (23-25) and in mouse models (8, 26-27).

In our experiments, Lp6 alone, supplemented to sensitized mice, induced an increase in the frequency of peripheral Tregs, expressing Foxp3 or IL-10 and a strong decrease of total IgE and a parallel reduction of allergen-specific IL-4, along with the induction of a systemic Th1 response, characterized by an increase of polyclonal IFN-γ (Table I). In combination with RW-allergoid treatment, Lp6 favored a significant greater reduction of allergen

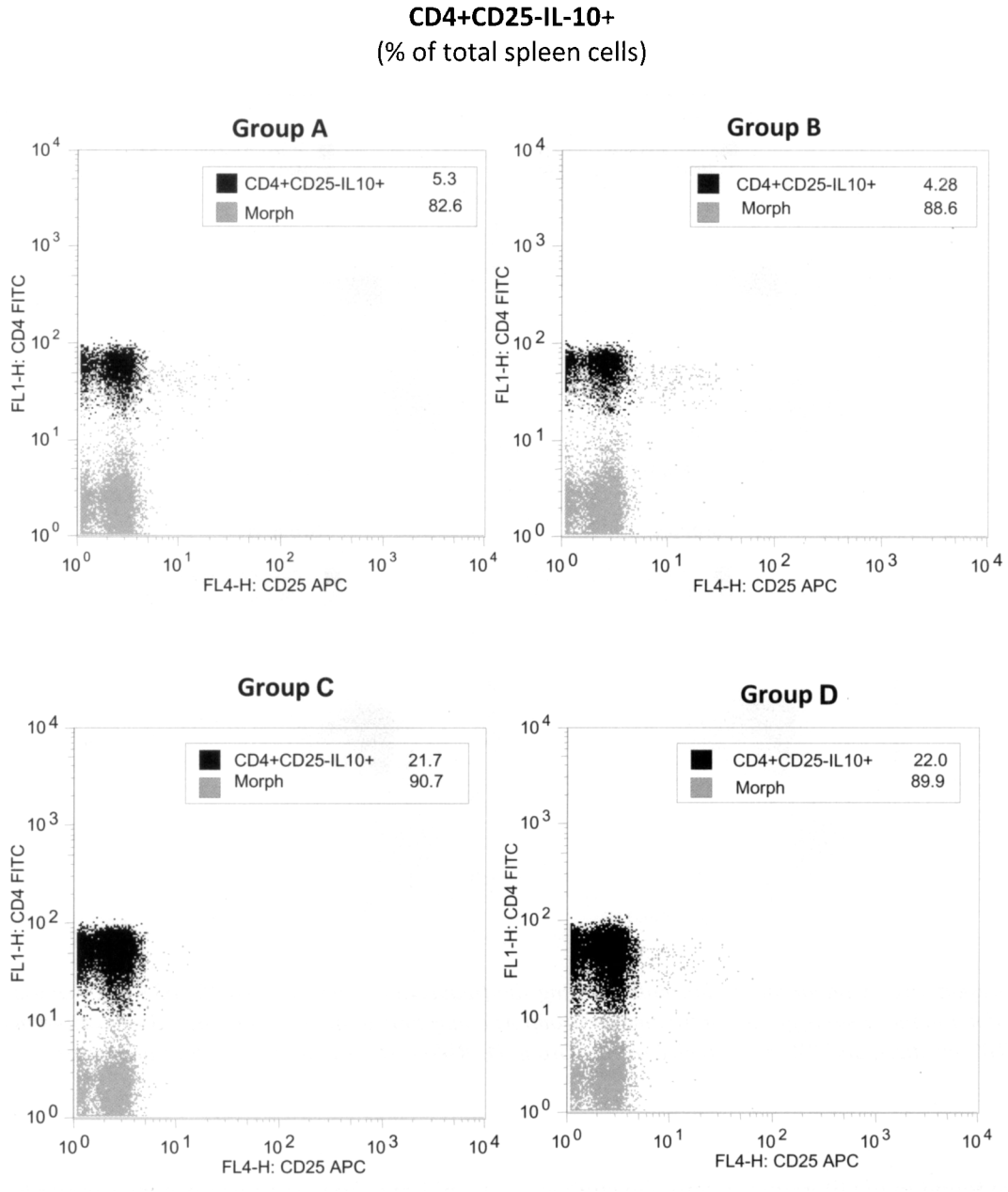


**Fig. 7.** *Representative cytofluorimetry dot plots of the frequency of CD4+IL-10+ spleen cells not expressing CD25 (dark gray dots) in the various groups of treatment. Group A: control mice (n=6); Group B: RW-sensitized mice (n=6); Group C: RW-sensitized and RW-treated mice (n=6); Group D: RW-sensitized and RW+Lp6-treated mice (n=6).*

driven IL-4 release by splenocytes and an increase of allergen-induced IFN-γ (and no change in polyclonal IFN-γ) significantly higher than that induced by the RW-allergoid.

In summary, this study demonstrates that Lp6 strengthens the immune modulation associated to allergoid-SIT in RW-sensitized mice, essentially characterized by a differential induction of Tregs associated to a reduction of IL-4; data converge to define a role of SIT adjuvant for Lp6.

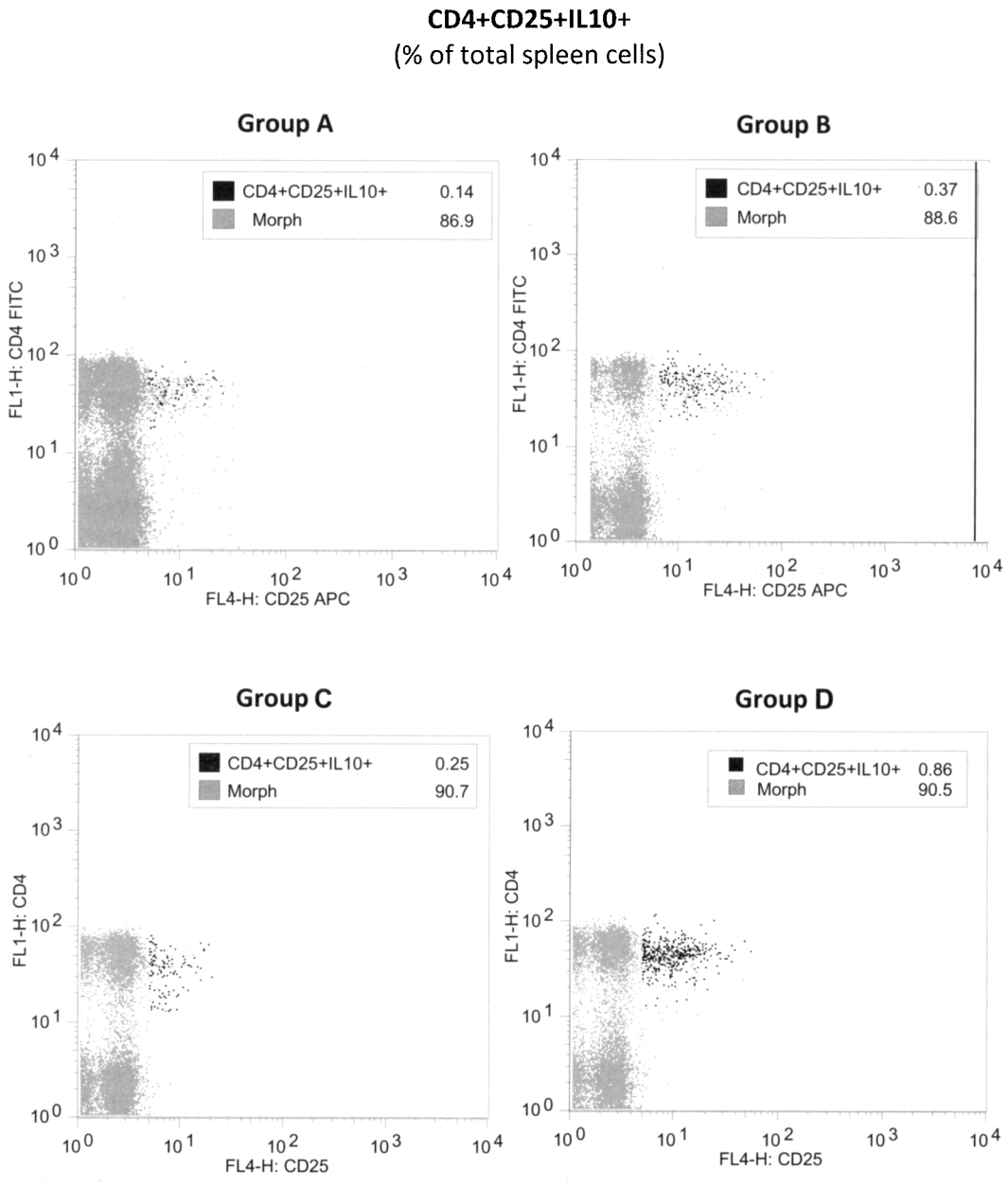


**Fig. 8.** *Representative cytofluorimetry dot plots of the frequency of CD4+CD25+IL-10+ spleen cells (dark gray dots) in the various groups of treatment. Group A: control mice (n=6); Group B: RW-sensitized mice (n=6); Group C: RW-sensitized and RW-treated mice (n=6); Group D: RW-sensitized and RW+Lp6-treated mice (n=6).*

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Conflict of interests: Dr Gianni Mistrello is an employee of Lofarma S.p.A., factory that supplied allergens for the study.

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