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Low-Dose IL-2 Induces Regulatory T Cell–Mediated Control of Experimental Food Allergy

Benjamin Bonnet,*,†,‡ James Vigneron,*,† Béatrice Levacher,*,† Thomas Vazquez,*,† Fabien Pitoiset,*,† Faustine Brimaud,*,† Guillaume Churlaud,*,†,‡ David Klatzmann,*,†,‡ and Bertrand Bellier*,†,‡

Regulatory T cells (Tregs) are pivotal for maintenance of immune self-tolerance and also regulate immune responses to exogenous Ags, including allergens. Both decreased Treg number and function have been reported in allergic patients, offering new therapeutic perspectives. We previously demonstrated that Tregs can be selectively expanded and activated by low doses of IL-2 (ld-IL-2) inducing immunoregulation without immunosuppression and established its protective effect in autoimmune diseases. In this study, we evaluated the ability of ld-IL-2 to control allergy in an experimental model of food allergy. Ld-IL-2 induced Treg expansion and activation that elicited protection against clinical manifestations of food allergy in two mouse models with OVA and peanut. This clinical effect was lost in Treg-depleted mice, demonstrating the major contribution of Tregs in ld-IL-2 efficacy. Mechanistic studies further indicated that protection from allergy could be explained by a Treg-dependent local modification of the Th1/Th2 balance and an inhibition of mast cell recruitment and activation. Preventive and therapeutic effects of ld-IL-2 were observed over a 7-mo-period, highlighting its long-term efficacy. This study demonstrated that ld-IL-2 is efficient to prevent and to treat allergic immune responses, and thus represents a promising therapeutic strategy for managing allergic diseases. ***The Journal of Immunology*****, 2016, 197: 188–198.**

Thymic-derived $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs) account for ~5–10% of circulating $CD4^+$ T cells, suppress autoreactive lymphocytes, and control innate and adaptive immune responses in mice and humans (1, 2). Treg impairment is associated with loss of tolerance, autoimmunity, and also allergy (3). Indeed, allergy is one of the clinical manifestations of patients with the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, a Treg deficit caused by mutations in *FOXP3* (4). Dysfunctional Tregs have been also identified in allergic individuals (5–7). Both decreased Treg numbers and function have been reported, particularly in children at high risk for allergic diseases based on maternal atopy compared with children at low risk (8). Studies investigating the pathophysiology of food allergy point to a functional role of Tregs in the development of normal tolerance to food allergens and the spontaneous resolution of milk allergy (9, 10).

In support for a role of Tregs in allergy, the induction of Tregs has been also demonstrated in successful allergen-specific immunotherapy (AIT), where they suppressed almost all aspects of Th2-mediated hypersensitivity (11, 12). In addition, transfer of Tregs inhibited and reversed airway hyperresponsiveness, gut inflammation, and related Th2 responses in murine models (13–15). Collectively, these data highlight that allergic diseases result from an inappropriate balance between allergen-activated effector Th2 cells and Tregs, and that treatments to enhance Treg responses might be useful in prevention and treatment of allergic diseases.

IL-2 is critical for Treg development, expansion, activity, and survival (16). IL-2 was originally discovered as a growth factor for activated T cells in vitro and has been extensively used to boost immune responses in patients with tumors (17) or chronic infection such as HIV (18). More recently, it was shown that ld-IL-2 increase specifically Treg numbers and function, and may be of therapeutic benefit in the treatment of autoimmune and inflammatory diseases. Pioneer clinical trials revealed the potential benefit of ld-IL-2 administration in alleviating chronic graft versus host disease (19, 20) and chronic hepatitis C–mediated vasculitis (21). This was then confirmed in alopecia areata (22) and other autoimmune diseases including systemic lupus erythematosus (23), confirming the potential of this approach. IL-2 is not only required for the survival of $Foxp3^+$ Tregs in the periphery, but also appears to be critical for maintenance of their suppressive functions (24). Thus, we sought to evaluate ld-IL-2 treatment in allergy control. Our results show that ld-IL-2 induces specific Treg expansion and activation, including in gut and lymphoid-associated tissues, which elicit protection against clinical manifestations of food allergy in mice. We demonstrated both preventive and therapeutic long-term effects of ld-IL-2. The mechanisms underlying this efficacy depend on the expansion and activation of Tregs that modify the Th1/Th2 balance and inhibit mast cell activation.

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Abbreviations used in this article: AIT, allergen-specific immunotherapy; Alum, aluminum hydroxide; ILC2, type 2 innate lymphoid cell; ld-IL-2, low dose of IL-2; MLN, mesenteric lymph node; mMCPT-1, murine mast cell protease type 1; NS, nonsensitized mice; p.o., oral; PP, Peyer's patch; pre-C, between mice sensitization and challenge; pre-S, before mice sensitization; SFU, spot-forming unit; SI, small intestine; Teff, effector T cell; Treg, regulatory T cell.

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Materials and Methods

Mice

Seven-week-old BALB/C (AnNR/J) female mice were purchased from Elevage Janvier (Le Genest-Saint-Isle, France). Mice were maintained in our animal facility under specific pathogen-free conditions in agreement with current European Union legislation on animal care, housing, and scientific experimentation. All procedures were approved by the local ethics animal committee. Mice were treated daily by i.p. or oral (p.o.) administration with the human rIL-2 (Proleukin; Novartis) during 5 consecutive days at the dose of 50,000 IU/injection in 200 or 100 μl, respectively.

Development of intestinal allergic inflammation in mice

OVA model. Based on published procedures (15) with specific modifications, BALB/c mice were sensitized twice at 1-wk interval by i.p. injection of 10 μg OVA (Ova A5503; Sigma-Aldrich) mixed with 500 μg aluminum hydroxide (Alum) gel (AlH_3O_3; Sigma) or PBS mixed with Alum as control (nonsensitized mice [NS]). Allergy was then induced 10 d later by OVA p.o. administrations (20 mg/mouse), five times within a 10-d period. Severity of allergic response was assessed 30–45 min after the last induction by measuring changes in body temperature using a rectal thermometer (Bioseb, Vitrolles, France) and by evaluating clinical score (component of the severity of diarrhea graded from 0 to 3 points and appearance of hirsute pelage marked out 0–2 points).

Peanut model. Crude peanut extract was prepared from defatted raw flour as previously described (25). Mice were sensitized i.p. three times at 1-wk interval with 500 μg peanut extract in 500 μg Alum. Control groups received PBS plus 500 μg Alum. Mice were then orally challenged with peanut extract (15 mg) every 2 d for a total of seven challenges. Severity of allergic response was assessed 30–45 min after the last challenge by measuring body temperature drops and clinical symptoms including: 1) repetitive mouth/ear scratching, 2) piloerection, 3) puffiness around eyes or mouth/eye edema, 4) decreased activity/lethargy, 5) diarrhea, and 6) death. Each clinical sign was noted with a score of 1; the maximum score per mouse is 6.

In vivo Treg depletion

Mice received two i.p. injections of 250 μg PC61 Ab (anti-CD25; Bio X Cell) at 3-d interval before OVA sensitization or challenge. As control, mice were treated with PBS or isotype (Antibodies Online). Blood samples were collected at day 10 post-PC61 treatment to control Treg depletion by flow cytometry.

Isolation of immune cells

Spleens were isolated and dissociated in PBS 5% FCS. RBCs were lysed with 1 ml ammonium-chloride-potassium buffer and then washed with 9 ml PBS 5% FCS. Mesenteric lymph nodes (MLNs) and Peyer's patches (PP) were isolated and then digested with 0.46 mg/ml Liberase TM and 0.1 mg/ml DNase (both from Roche Applied Science) in complete RPMI 1640 20% FCS medium for 10 min and filtered. Lamina propria lymphocytes from small intestine (SI) were isolated as previously described with minor modifications (26). In brief, SI was harvested and PP were removed. The tissue was then cut in small pieces and incubated with 10 μg/ml Liberase TM, 0.1 mg/ml DNase, and 0.5 mg/ml hyaluronidase (all from Roche Applied Science) for 25 min at 37°C in a shaker (150 rpm). The tissues were smashed, passed through a 70-μm cell strainer, and washed in complete RPMI 1640 3% FCS. Cells were centrifuged at 1300 rpm for 7 min at 4°C, washed again, passed through a 40-μm cell strainer, and finally suspended in complete RPMI 1640 20% FCS medium.

Flow cytometry

Isolated cells were stained with the following Abs at predetermined optimal dilutions for 20 min at 4°C: CD45-PETR, B220-FITC, IgM-allophycocyanin, IgD-PeCy7, SiglecF-PE, streptavidin-e780 (eBioscience), and IgE-biotin (Southern Biotech) to analyze B cells/eosinophils; Live Dead-e780, CD45-PETR, CD49b-FITC, FCεR1-PeCy7, c-Kit–allophycocyanin, B220-e450, SiglecF-PE, CD3-HorizonV500, NkP46-AlexFluor700 (eBioscience) to analyze mast cells and NK cells; and CD45-PETR, CD3-PE, CD8-Alexa700, CD4-HorizonV500, CD25-PeCy7, GITR-PE (eBioscience), and ICOS-allophycocyanin (Biolegend) to analyze T cells. Intracellular detection of Foxp3-e450 (eBioscience), CTLA4-biotin (eBioscience), and Ki67-FITC (eBioscience) were performed on fixed and permeabilized cells using a CytoFix/CytoPerm kit buffer (eBioscience). Cells were acquired on an LSR II (Becton Dickinson) and analyzed with FlowJo software (Tree Star). Dead cells were excluded by forward/side scatter gating. Tregs were defined as $Foxp3^+CD25^+CD4^+$ cells, activated Tregs as $ICOS^+Foxp3^+CD25^+CD4^+$ cells, and activated effector T cells (Teff) as $CD25^+Foxp3^-CD4^+$ cells. Memory and naive B cells were defined as IgM^-IgD^- and IgM^+IgD^+ cells among $B220^+$ cells, respectively. Eosinophils and mast cells were characterized as $SSC^{hi}CD45^+SiglecF^+$ and $FceR1^+c\text{-}Kit^+CD49b^-$, respectively.

In vitro basophil activation assay

Heparinized blood sample was collected 2 d after the last OVA p.o. administrations and erythrocytes were lysed. Cells were stimulated with RPMI 1640 (negative control), 500 ng/ml anti-IgE (positive control; Southern Biotech), or 1 μg/ml OVA for 2 h at 37°C. Cells were then stained with CD45-PETR, CD49b-FITC, FcεR1-PeCy7, and IgE-biotin to identify the basophil population and CD63-allophycocyanin (eBioscience) to measure degranulation.

ELISPOT assays

OVA-specific production of IFN-γ, IL-4, and IL-5 were determined by murine ELISPOT assay (Mabtech, Nacka Strand, Sweden). In brief, 5×10^5 cells/well (or 2×10^5 for IFN-γ) were restimulated for 48 h in duplicates at 37°C in 5% CO_2 with 100 μg OVA protein (0.5 mg/ml final). Medium alone and Con A (Sigma) at 2 μg/ml final were used as negative and positive controls, respectively. Spots were counted with an AID ELISPOT reader (ELR03; AID Autoimmun Diagnostika, Strassberg, Germany). Results, expressed as spot-forming units (SFU) per 10^6 cells, represent the mean of duplicates.

ELISA assays

To measure murine mast cell protease type 1 (mMCPT-1) levels, we harvested blood samples 30 min after the last OVA challenge and isolated sera by centrifugation. Samples were frozen and kept at −20°C until use. Level of mMCPT-1 in serum was measured according to the manufacturer's recommendations using specific mouse ELISA kits (eBioscience).

IFN-γ, IL-4, and IL-5 secretion levels were measured by murine ELISA assays (eBioscience) in supernatants of cells (5×10^5 cells/well) cultured during 72 h in 96-well plates (Nunc, Denmark) in complete RPMI 1640 with 100 μg OVA (0.5 mg/ml final).

OVA-specific IgE titers were quantified in serum of sensitized mice by ELISA. OVA (500 ng/well) was coated in flat-bottom, 96-well plates (Nunc, Denmark) at 4°C overnight. After washing and blocking nonspecific binding sites with PBS + 1% BSA for 1 h at room temperature, 100 μl diluted serum samples were added to each well and incubated for 2 h at room temperature. OVA-specific IgE were detected with biotinylated anti-IgE (Southern Biotech) and revealed with peroxidase-conjugated streptavidin (Sigma-Aldrich) and TMB at room temperature for 5 min. The reaction was stopped by adding 100 μl HCl (1M), and the OD was read at 450 nm using an automatic ELISA plate reader (DTX 880, Multimode detector; Beckman Coulter).

Intestine quantitative RT-PCR

Segments of duodenum were flushed with PBS 5% FCS and immersed in 1 ml RNA-later (Qiagen, Hilden, Germany) and stored at −80°C. For RNA extraction, total tissues were transferred in 1 ml TRIzol Reagent (Life Technologies, France), and disruption and homogenization were done with TissueLyser II (Qiagen). Total RNA was isolated by using RNeasy Mini Kits (Qiagen). cDNA was generated using SuperScript III (Life Technologies) according to the manufacturer's instructions. Quantitative PCR was performed using the 7500 Fast Real-Time PCR System (Life Technologies) with Fast Master mix (Life Technologies). The probe IDs are: m1,TNF-α: Mm00443258_m1, IFN-γ: Mm01168134_m1, IL-4: Mm00445259_m1, IL-5: Mm00439646_m1, IL-6: Mm00446190_m1, IL-10: Mm00439614_m1, IL-12p40: Mm01288992_m1, IL-13: Mm00434204_m1, 18S: Mm03928990_g1, and GAPDH: Mm99999915_g1. PCRs were performed in triplicate, and the mRNA levels were normalized to that of GAPDH and 18S mRNA levels. Gene expressions in control and ld-IL-2–treated groups were expressed as relative transcript abundance, relative to the GAPDH reference gene.

Histology

One centimeter length of duodenum was removed and immersed in 4% formaldehyde buffer. Tissues were further dehydrated in alcohol solutions using an automatic tissue processor (STP 120; Microm Microtech, Francheville, France) and included in paraffin. Transverse sections were cut at 4 mm by HM340E microtome (Thermo Scientific). Sections were stained with toluidine blue, and mast cells were visualized with a Leica DMRBE microscope. A total of four sections per group were randomly analyzed (original magnification ×400), and results were expressed as number of mast cells per millimeter.

Statistical analyses

Statistical significances were evaluated between the treated groups and control subjects using GraphPad Prism (GraphPad Software, San Diego,

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CA) using the Mann–Whitney *U* test (comparison of rank, unpaired test, nonparametric test, two-tailed *p* value) with $*p < 0.05$ taken as statistical significance ($**p < 0.01$, $***p < 0.001$). Statistical significances for long-term ld-IL-2 therapeutic effect were evaluated using one-way ANOVA test with $*p < 0.05$ taken as statistical significance ($***p < 0.001$).

Results

Ld-IL-2 treatment induces expansion and activation of Tregs in spleen and GALTs

We evaluated the effect of ld-IL-2 on immune cell populations at various locations, focusing mainly on the GALTs. BALB/c mice were injected daily for 5 d with 50,000 IU IL-2 per injection or PBS as a negative control. $CD4^+$, $CD8^+$ T cells, B cells, Tregs, and Teff were monitored in spleen, MLN, PP, and SI mucosa. We observed that ld-IL-2 had no significant impact on $CD4^+$, $CD8^+$ T cell, and B cell populations in spleen, PP, and SI in terms of percentages and absolute numbers (Fig. 1A). Only a significant expansion of total cells in MLN was observed in ld-IL-2–treated mice, which did not affect the relative values of B, $CD4^+$, and $CD8^+$ T cells (Fig. 1A). As expected, ld-IL-2 induced expansion of total $CD4^+FOXP3^+$ cells (Fig. 1B, *upper panels*), Tregs ($CD4^+CD25^+FOXP3^+$; data not shown), and $CD8^+$ Tregs ($CD8^+FOXP3^+$; Supplemental Fig. 1) in the different organs. Interestingly, the highly suppressive $ICOS^+$ Tregs (27) were robustly expanded in percentage (Fig. 1B, 1C) and absolute number (data not shown) in local and peripheral lymphoid tissues. Ld-IL-2 induced both Treg proliferation and activation as shown by a significant increase of the proportion of $Ki67^+$, $ICOS^{hi}$, and $CTLA\text{-}4^+$ Tregs in spleen and MLN (Fig. 1E). In contrast, ld-IL-2 had no significant impact on $CD4^+CD25^+FOXP3^-$ Teff, except a moderate but significant increase in spleen (Fig. 1C). Nevertheless, the Treg/Teff ratio was significantly increased in all organs of treated mice as compared with control subjects (Fig. 1D). Importantly, other cell populations such as mast cells, eosinophils, type 2 innate lymphoid cells (ILC2), naive B cells, or IgE^+ B cells are not significantly expended under ld-IL-2 except for moderate eosinophilia in spleen and NK cells (Supplemental Fig. 1). A small increase of IgE^+ B cells in spleen and mast cells in SI were also observed in IL-2–treated mice but at lower levels than allergic mice. Taken together, these results show that ld-IL-2 induces expansion and activation of Tregs in naive mice, notably in gut-associated tissues.

Effective induction of food allergy in OVA-sensitized mice

A murine food allergy model with high degree of anaphylaxis was established by repeated p.o. administration of OVA protein in OVA-sensitized mice (Fig. 2). Severe allergic reactions were observed in sensitized mice and scored based on diarrhea and fur characteristics (Fig. 2A). A significant decrease in body temperature was also induced in OVA-sensitized mice and was positively correlated with the severity of allergic symptoms (Fig. 2A, 2B). Elevated mMCPT-1 levels were also detected in serum of sensitized mice after OVA challenges and can be related with an increase of mast cells in the gut (Fig. 2C). The robustness of this food allergy model was also evidenced by the severe allergic reactions observed in sensitized mice when they were challenged 30 or 60 d after the last sensitization (data not shown). Moreover, a strong systemic and local Th2 bias was observed in OVA-sensitized mice as shown by high levels of IL-4– and IL-5–secreting cells in spleen, MLN, and PP as compared with the NS (Fig. 2D and data not shown). We also detected OVA-specific IgE Abs in serum and feces in sensitized mice that can be related to a significant increase of IgE^+ memory B cells in allergic mice compared with NS (Fig. 2E). Taken together, these results show that our protocol is effective to establish food allergy with a high degree of anaphylaxis and strong Th2-biased immune responses.

Ld-IL-2 treatment prevents food allergy in OVA and peanut allergy models

We first investigated the preventive efficacy of ld-IL-2 in the OVA-specific allergy model. Mice were daily i.p. injected with IL-2 or PBS as control for 5 consecutive days and then sensitized and challenged with OVA according to our protocol. We observed that i.p. ld-IL-2 prevents food allergy because 90% of IL-2–treated mice were fully protected (score ≤ 2), whereas control mice had severe allergic reactions with a median score of 5 (Fig. 3A). The protective effect of ld-IL-2 was confirmed by the prevention of body temperature decline in IL-2–treated mice in contrast with PBS-treated controls (Fig. 3A) and a significant decrease of serum levels of mMCPT-1 (Fig. 3B) that were both positively correlated ($p < 0.001$, $r = 0.746$). Notably, the protective effect was robust because IL-2–treated mice were still protected against severe hypothermia (temperature declines $>2^\circ C$) after repeated OVA challenges (Fig. 3C).

Protective effect of ld-IL-2 was also demonstrated in a second experimental model of food allergy using peanut protein extract as allergen and using specific scoring system based on anaphylactic symptoms to measure the severity of allergic reactions (Fig. 3D and *Materials and Methods*). We observed that IL-2–treated mice were protected against peanut allergy because very few mice presented anaphylactic symptoms, whereas pruritus, piloerection, swollen eyes, lethargy, and diarrhea were observed in the PBS group after peanut extract challenge. Absence of temperature decline in IL-2–treated mice confirmed the preventive effect of IL-2 (Fig. 3D).

Ld-IL-2 control of food allergy is Treg mediated

To evaluate the role of Tregs in ld-IL-2 treatment efficacy, we performed similar experiments in which Tregs were depleted. Mice were first treated by i.p. ld-IL-2 or PBS during 5 d and then injected or not with PC61 mAb (anti-CD25 IgG1) to eliminate Tregs. Mice were then sensitized and challenged with OVA to induce food allergy. PC61-mediated specific Treg depletion was documented by the major loss of $CD4^+FOXP3^+CD25^+$ cells in blood of treated mice at day 10 and confirmed by the significant decrease of total $Foxp3^+$ cells in comparison with PBS or isotype controls (Supplemental Fig. 2). Notably, no variations in blood eosinophil and NK cell counts or ILC2 cells in PP were observed (Supplemental Fig. 2).

After OVA challenges, we observed that sensitized control mice in which Tregs have been depleted after PC61 treatment had more severe allergic symptoms than controls, highlighting that Tregs downmodulate allergic immune responses in PBS-treated control mice (Fig. 3E). More importantly, we demonstrated that Treg depletion abrogates the efficacy of ld-IL-2 as shown by severe allergic symptoms, an important decrease in body temperature (Fig. 3E) and significant increase of mMCPT1 levels (Fig. 3G) in Treg-depleted IL-2–treated mice. Interestingly, the Treg counts observed before mice sensitization (pre-S) can be correlated with the severity of the allergic reactions (Fig. 3F). However, no significant variation of OVA-specific Abs was observed in serum of treated mice at this early time point, including when OVA-specific IgE, IgG2a, IgG1, and IgA isotypes were measured (Fig. 3H and data not shown).

To formally demonstrate that the protective effect induced by ld-IL-2 is Treg mediated, we realized experiments in which Tregs were depleted in protected mice, 1 mo after ld-IL-2 treatment. After additional OVA challenges, we observed that late PC61 treatment abolished protection because treated mice presented severe allergic reactions with clinical scores comparable with PBS control groups (Fig. 3I).

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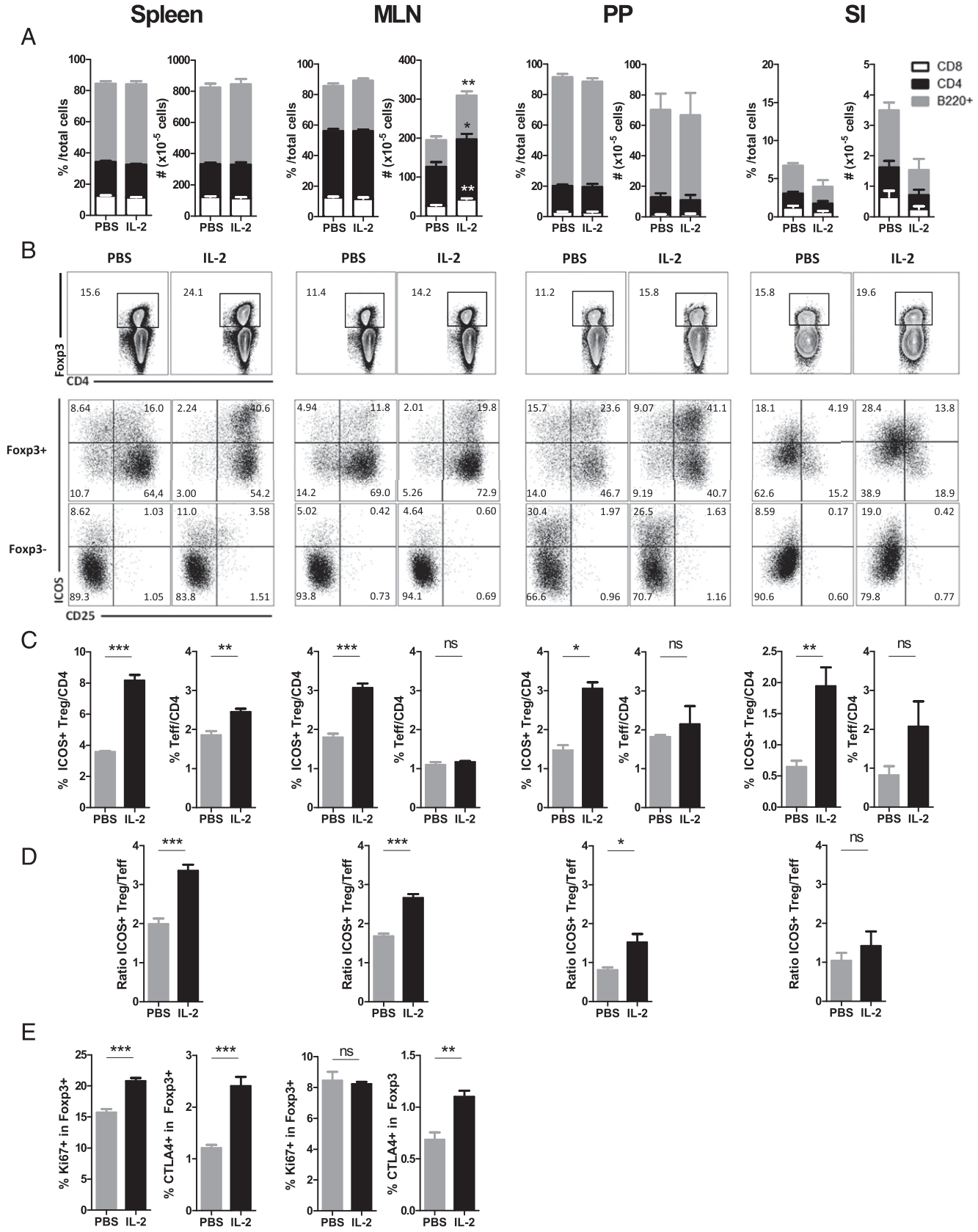

FIGURE 1. Immune characterization of ld-IL-2 treatment in mice. Mice were daily i.p injected with ld-IL-2 (IL-2) or PBS as control during 5 d, and cell populations were analyzed by flow cytometry in spleen, MLNs, PP, and SI 2 h after the last injection. (**A**) Cumulative percentages and absolute numbers of $CD8^+$, $CD4^+$ T cells and B cells in the different organs. (**B**–**E**) Effect of ld-IL-2 on Treg and Teff compartments. (B) Representative FACS contour plots of $Foxp3^+$ among $CD4^+$ T cells (*upper panels*) and ICOS/CD25 expression analysis among $Foxp3^+$ (*middle panels*) and $Foxp3^-$ (*lower panels*) $CD4^+$ T cells in IL-2– and PBS-treated mice. (C) Proportions (%) of activated Tregs ($ICOS^+CD25^+$ $FOXP3^+$) and effector T cells ($CD25^+FOXP3^-$, Teff) among $CD4^+$ T cells in the different organs. (D) Ratio of $ICOS^+$ Tregs over Teff cells after treatment with ld-IL-2 or PBS as control. (E) Proportion (%) of $Ki67^+$ and CTLA-4^+ cells among Tregs in spleen and MLN. Data represent one of three independent experiments. Each value represents the mean ± SEM of 6–10 mice ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus control, Mann–Whitney *U* test). ns, nonsignificant.

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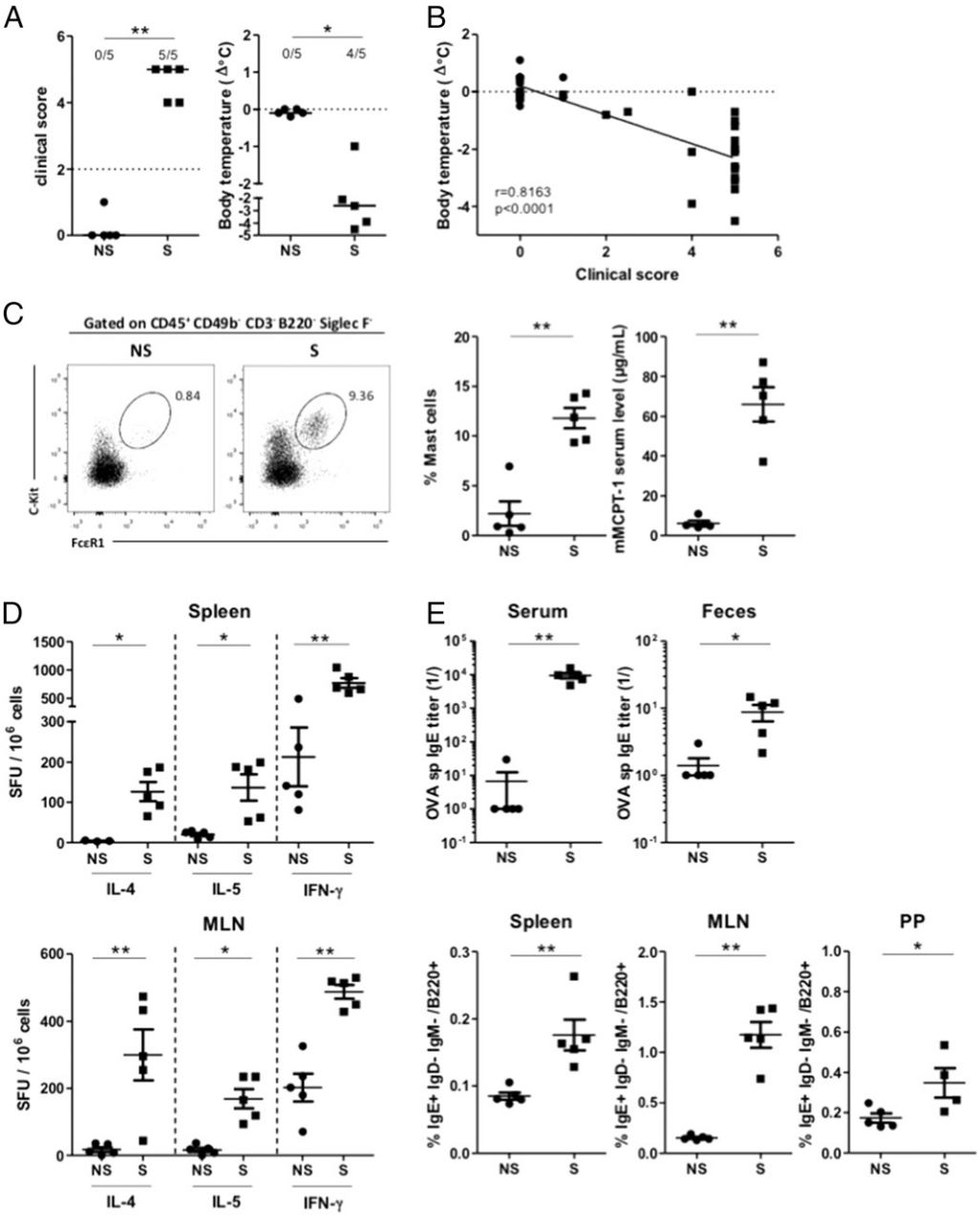

FIGURE 2. Ova-specific food allergy model. Mice (n = 5 per group) were sensitized by two i.p. administrations of OVA/alum or PBS/alum as control (NS group) and challenged by five p.o. administrations of Ova every 2 d to induce food allergy. (**A**) Allergic reactions were monitored after the fifth p.o. challenge. Clinical scores (*left panel*) and body temperature variations (*right panel*) measured 30 min after the last challenge are shown for each individual mouse. Clinical protection threshold has been defined for clinical score $\leq$2 and hypothermia when the body temperature declines $>$2°C. The numbers of unprotected and hypothermic mice are indicated for each group. (**B**) Correlation between clinical score and body temperature variations in sensitized mice (square) and NS (circle). Pearson correlation coefficient (r) is done. Data represent a pool of four different experiments. (**C**) Representative FACS dot plots (*left panels*) and quantification of frequencies (*middle panel*) of C-kit$^+$FcεR1$^+$ mast cells among CD45$^+$CD49b$^-$CD3$^-$B220$^-$SiglecF$^-$ in SI of naive and sensitized mice. Serum mMCPT-1 levels (*right panel*) were quantified in naive and sensitized mice after OVA challenges. (**D**) OVA-specific T cell immune responses were measured in the spleen (*upper panels*) and MLNs (*lower panels*) of sensitized mice and NS by IL-4, IL-5, and IFN-γ ELISPOT assays. Individual results are expressed in SFUs per 10^6 total cells, and means ($\pm$ SEM) of each group are represented. (**E**) OVA-specific IgE-mediated immune responses. Ova-specific IgE Ab titers (*upper panels*) were measured in serum (*left panel*) and feces (*right panel*) by ELISA assay, and IgE$^+$ memory B cells (*lower panels*) were quantified in spleen (*left panel*), MLN (*middle panel*), and PP (*right panel*) by flow cytometry. Each dot represents individual value, and mean $\pm$ SEM of the five mice are shown (*$p < 0.05$, **$p < 0.01$, Mann–Whitney U test; sensitized [S] versus NS groups). ns, nonsignificant.

Ld-IL-2 treatment modifies the local Th1/Th2 balance and lowers the mast cell and basophil degranulation

To better characterize the mechanisms associated to the clinical protection induced by ld-IL-2, we analyzed the immune profile of treated and untreated mice 2 d after the last OVA challenge. While ld-IL-2–treated mice were protected from allergic responses, the percentages of total CD4$^+$FOXP3$^+$ T cells or activated CD4$^+$CD25$^+$ FOXP3$^+$ICOS$^+$ Tregs (Fig. 4A) were not increased in the different organs as compared with control mice. We also did not observe variation of IL-4– and IL-5–secreting cell numbers measured by ELISPOT in spleen, PP (data not shown), and MLN (Fig. 4B, *upper panels*). However, when IL-4 and IL-5 productions were measured by

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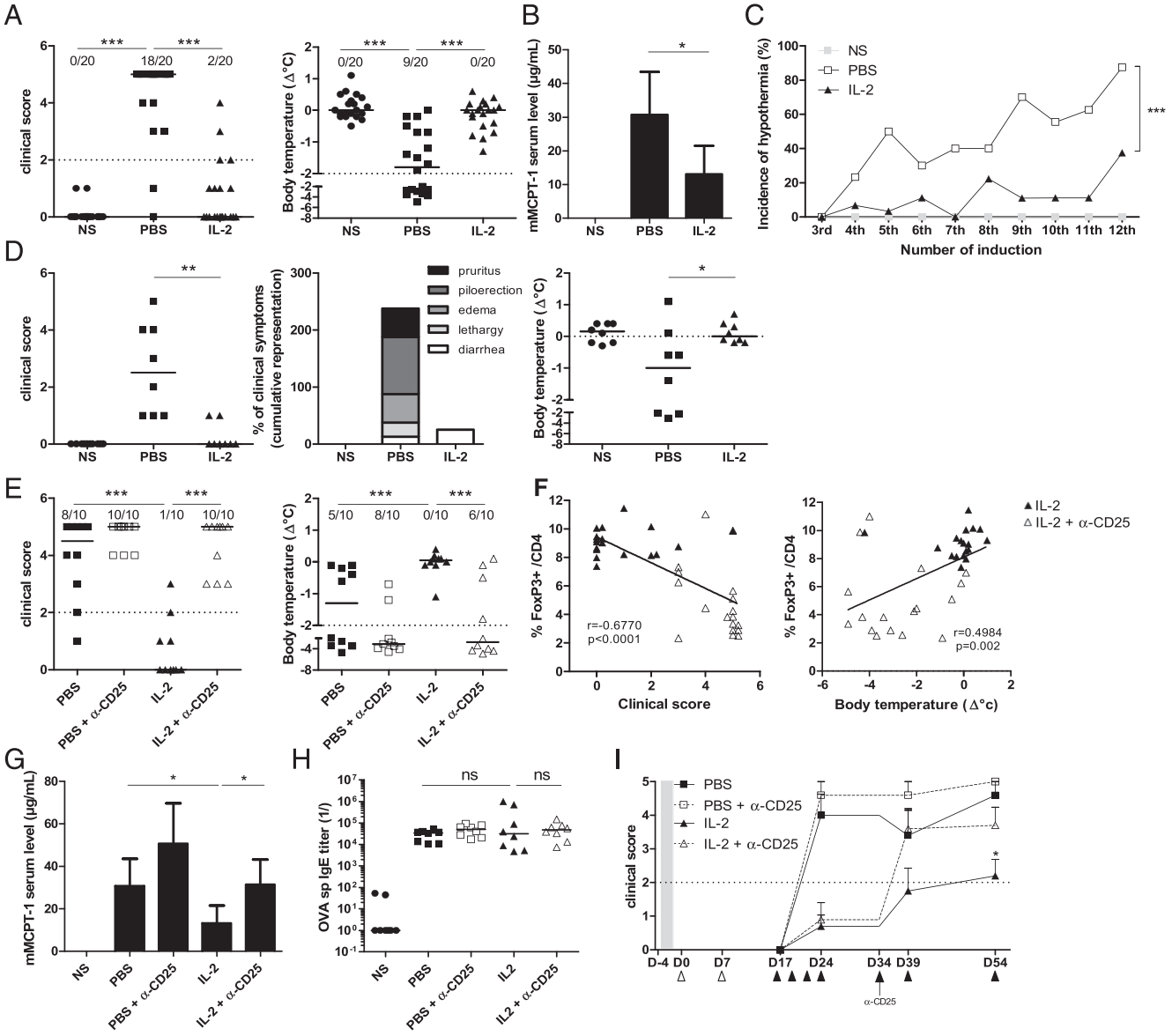

FIGURE 3. Ld-IL-2 provides Treg-mediated protection against clinical manifestations of food allergy. (**A**–**C**) OVA-specific allergy model. Mice were treated for 5 d with ld-IL-2 (IL-2) or PBS, i.p. sensitized or not (NS), and then challenged by repeated p.o. administration of OVA. (A) Clinical score (*left panel*) and body temperature (*right panel*) measured 30 min after the fifth p.o. challenge are shown for each individual mouse. Each dot represents individual value for each mouse, and group medians are shown. Clinical protection threshold has been defined for clinical score ≤2 and hypothermia when the body temperature declines >2°C. The numbers of unprotected and hypothermic mice are indicated for each group. Data represent a pool of four independent experiments of $n = 5$ mice per group. (B) Serum mMCPT-1 levels were measured after the last OVA challenge. Bars represent group means (± SEM, $n = 10$ per group). (C) Incidence of hypothermia was measured after multiple OVA challenges, repeated every 2 d (***$p < 0.001$, ANOVA; IL-2 versus PBS, $n = 10$ per group). (**D**) Peanut-specific allergy model. Mice were treated for 5 d with ld-IL-2 (IL-2) or PBS, sensitized with peanut protein extract, and then orally repeatedly challenged with peanut extract. As control, untreated NS are included and challenged with peanut extract. Clinical scores (*left panel*), cumulative incidence of symptoms (*middle panel*) and body temperature variation (*right panel*) measured 30 min after the seventh challenge, are shown. Each dot represents individual mouse data and group medians are shown ($n = 8$ per group). (**E**–**I**) Role of Tregs in IL-2–induced prevention of OVA-specific allergy. (E) Tregs were depleted after ld-IL-2 or PBS treatment by i.p. administration of anti-CD25 PC61 mAb (α-CD25). OVA sensitization and p.o. challenges were then performed to induce food allergy. Clinical scores and body temperature variations measured 30 min after the fifth challenge are shown for each individual mouse. The numbers of unprotected and hypothermic mice are indicated for each group. Data represent one of two independent experiments of $n = 10$ mice per group. (F) Correlation between % Tregs in blood at day 5 and clinical scores (*left panel*) or body temperature variations (*right panel*) measured after OVA challenges in IL-2– (black symbols) and IL-2 + anti-CD25 (white symbols)–treated mice. Pearson correlation coefficient (r) and p values are noted. Data represent a pool of two independent experiments of $n = 8$–10 mice per group. (G) Levels of mMCPT-1 and (H) Ova-specific IgE Abs were measured in serum. Bars represent group means (± SEM, $n = 8$ per group). (I) Impact of Treg depletion during the OVA-challenge phase. The 5-d ld-IL-2 cure (gray bar) was initiated before OVA sensitization (white arrows), and mice were repeatedly challenged (black arrows) to induce allergic symptoms (measured at day 24). Ten days later, mice were injected or not with anti-CD25 PC61 mAb (α-CD25) to deplete Tregs and then challenged with OVA at days 39 and 54. Clinical scores (mean ± SEM) are shown for each group ($n = 10$ per group). (A–H) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Mann–Whitney U test, PBS- versus IL-2–treated group. (I) *$p < 0.05$, Mann–Whitney U test, IL-2 + anti-CD25 versus IL-2–treated group. ns, nonsignificant.

ELISA in supernatants after Ag-specific restimulation, we reported a decrease of Th2 cytokine secretions in MLN of IL-2–treated mice (Fig. 4B, *lower panels*). In contrast, we observed an increase of OVA-specific IFN-γ–secreting cell numbers and IFN-γ production in MLN and PP of IL-2–treated mice compared with control mice (Fig. 4B and data not shown). As a result, ld-IL-2 treatment

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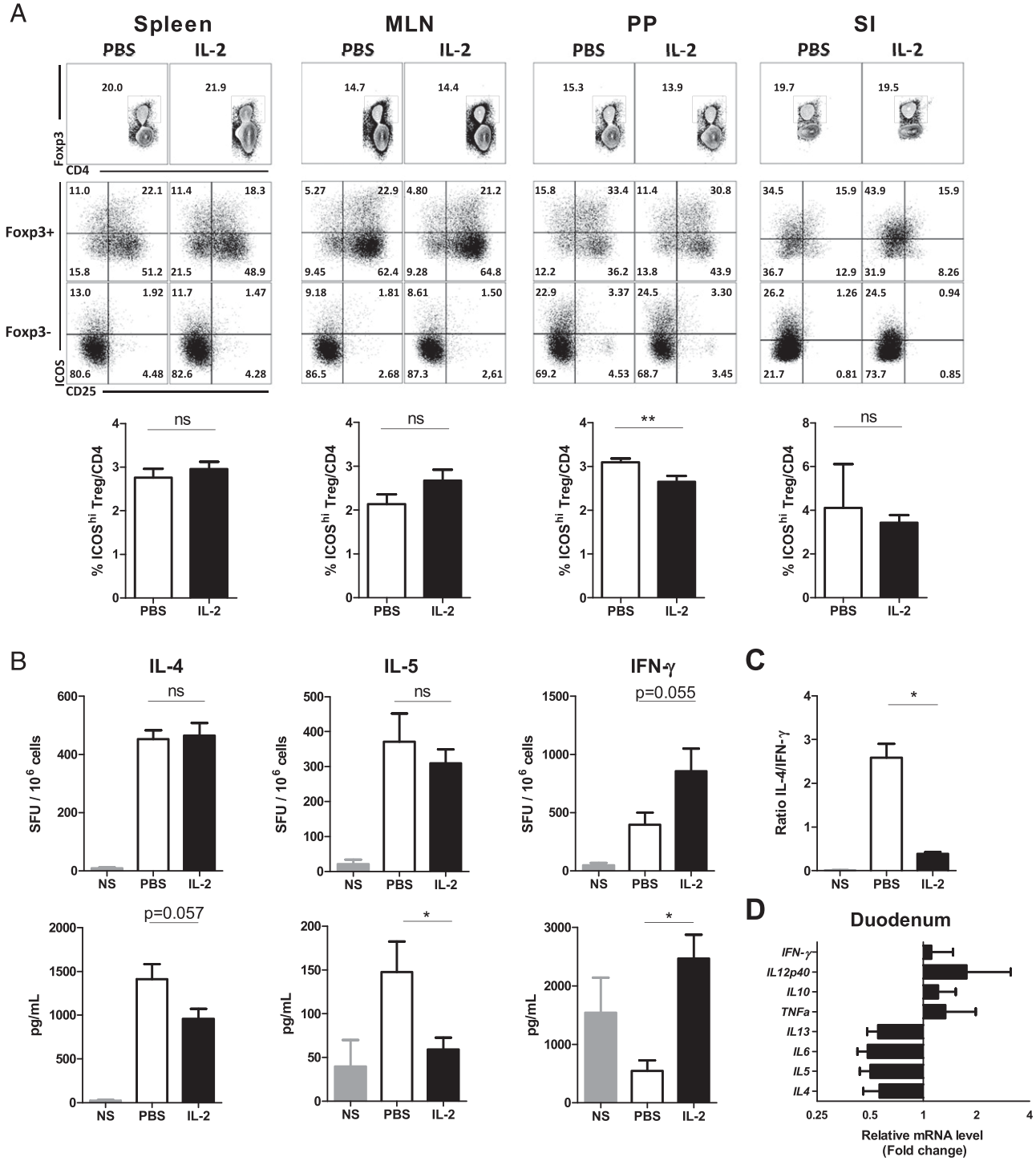

FIGURE 4. Ld-IL-2 treatment locally modulates the Th1/Th2 balance. Tregs enrichment and immune responses were analyzed in spleen, MLNs, PP, and SI harvested from IL-2– or PBS-treated sensitized mice after the fifth OVA challenge. (**A**) Representative FACS contour plots of $Foxp3^+$ cells among $CD4^+$ T cells (*upper panels*); dot plots of ICOS/CD25 expression among $FOXP3^+CD4^+$ T cells or $FOXP3^-CD4^+$ T cells (*middle panels*) and quantification of frequencies of activated Tregs ($ICOS^+FOXP3^+CD25^+$) (*lower panels*) in PBS- and IL-2–treated mice are shown. Bars represent the mean ± SEM of 10 mice. (**B**) OVA-specific T cell response was measured in MLN by IL-4, IL-5, and IFN-γ ELISPOT (*upper panels*) and ELISA (*lower panels*) assays after OVA restimulation. As control, NS challenged mice were included. Results represent the average of duplicates of individual mouse as SFUs per 10^6 total cells, and group means (± SEM; $n = 5$ per group) are indicated. (**C**) Ratio of IL-4:IFN-γ–secreting cells in MLN. (**D**) Cytokine immune responses analyzed by qRT-PCR in duodenum of PBS and ld-IL-2–treated mice. Graph showing the mean values of fold change for IFN-γ, IL-12p40, IL-10, TNF-α, IL-13, IL-6, IL-5, and IL-4 mRNA levels in ld-IL-2–treated mice relative to PBS-treated mice (control). Gene expressions in control and ld-IL-2–treated groups were expressed as relative transcript abundance, relative to the GAPDH reference gene. The fold changes of gene expression in ld-IL-2 mice relative to PBS controls are shown. Data represent a pool of two independent experiments of $n = 5$ mice per group. $^*p < 0.05$, $^{**}p < 0.01$, Mann–Whitney U test ($n = 5$–10 per group). ns, nonsignificant; S, sensitized mice.

led to a modulation of Th2/Th1 balance in draining lymphoid organs highlighted with the decline of the IL-4/IFN-γ ratio in PP (data not shown) and MLN (Fig. 4C). We also observed specific local modifications of Th2 cytokine expression levels assessed in SI by quantitative RT-PCR (qRT-PCR). IL-4, IL-5, IL-6, and IL-13 gene expression were decreased in the duodenum of the IL-2–treated

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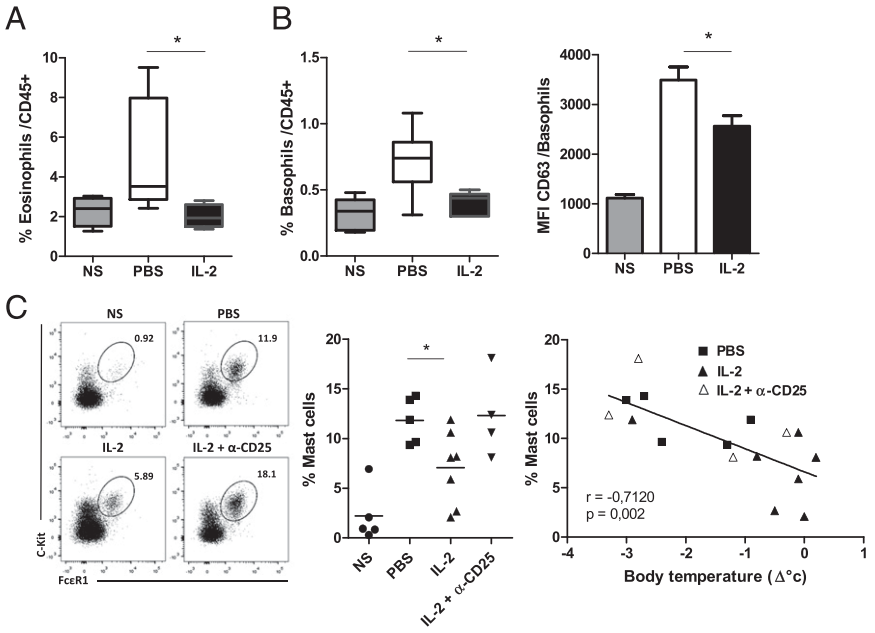

FIGURE 5. Ld-IL-2 prevents recruitment and activation of allergic effector cells. (**A** and **B**) Blood eosinophils and basophils were analyzed by flow cytometry 3 d after the last OVA challenge in ld-IL-2– (IL-2) or PBS-treated mice. (A) Percentage of eosinophils among total $CD45^+$ cells. (B) Percentage of basophils among total $CD45^+$ cells and level of CD63 expression (MFI) were measured after 4 h of incubation with OVA. (**C**) Mast cells were analyzed 10 d after the last OVA challenge in SI of IL-2–treated mice and Treg-depleted (α-CD25) or not mice before allergy induction. Representative FACS dot plots (*left panels*) and percentages (*middle panel*) of c-$Kit^+FcεR1^+$ cells among $CD45^+CD49b^-CD3^-B220^-SiglecF^-$ cells in NS, sensitized PBS-treated (PBS), IL-2–treated (IL-2), and Treg-depleted IL-2–treated mice (*left panels*) are shown. Correlation between percentage of mast cells and body temperature variations in sensitized mice including IL-2 (black triangle), IL-2+anti-CD25 (white triangle), and PBS (black square)–treated mice (*right panel*) is done. Pearson correlation coefficient (r) and p values are noted. Data represent one of two independent experiments (n = 5 mice per group, $*p < 0.05$, Mann–Whitney U test).

group (Fig. 4D), whereas IFN-γ, IL-12, TNF-α, and IL-10 gene expression levels were increased (Fig. 4D).

We also investigated the effect of ld-IL-2 treatment on the different effector cells of allergic disease. We observed that ld-IL-2 prevented the eosinophil and basophil recruitment as shown by the lower cell counts in blood of treated mice compared with the PBS group (Fig. 5A, 5B). Interestingly, we showed in ex vivo assay that ld-IL-2 reduced basophil degranulation as shown by the low expression of CD63 in IL-2–treated mice compared with control mice. More importantly, we also observed in SI of treated mice lower frequency of mast cells that can be correlated with limitation of temperature decline (Fig. 5C). The control of mast cell infiltrate induced by IL-2 is Treg mediated because the percentages of mast cells were high, reaching similar levels than allergic animals in PC61-treated mice (Fig. 5C). These results show that ld-IL-2 prevents food allergy by a Th1 shift of the Th1/Th2 balance and reduced activation of effector cells.

Ld-IL-2 induces long-term protection against food allergy reaction in preventive and therapeutic settings

To further evaluate ld-IL-2 efficacy, we investigated the long-term effect of IL-2 treatment for food allergy protection and evaluated its preventive and therapeutic effects. In addition to the 5-d ld-IL-2 course performed pre-S, a 5-d ld-IL-2 course between mice sensitization and challenge (pre-C) and a therapeutic 5-d ld-IL-2 course after p.o. challenge were also tested (Fig. 6).

When long-term efficacy of the pre-S treatment was evaluated, we confirmed that IL-2–treated mice are immediately protected from allergic symptoms ($p < 0.05$, Mann–Whitney compared with PBS group at day 23; Fig. 6A) and remains protected against four additional cycles of OVA challenges (between days 36 and 100; Fig. 6A). After another 5-d ld-IL-2 course initiated 3 mo after the first one, long-term treatment efficacy was observed because mice resisted to 10 additional OVA challenge cycles. Altogether, we observed that ld-IL-2 treatment could control allergic reactions over a 10-mo period, with no progressive loss of efficacy over time (Fig. 6A) that was confirmed by absence of body temperature variation (Supplemental Fig. 3).

A between mice sensitization and challenge treatment showed that protection was not immediately effective (score >2 at day 23; Fig. 6B) but was elicited as from the second cycle of OVA challenges (day 36). As previously and because of a partial loss of the protective effect at days 79 and 100, a new 5-d ld-IL-2 course was performed, and the long-term efficacy of the treatment was then tested. We observed that IL-2 could control allergic reactions over a 7-mo period with high efficacy because >80% of mice were fully protected (score ≤2; Fig. 6B) and no significant temperature decline was observed (Supplemental Fig. 3).

In the same experiment, we observed that allergic mice treated with IL-2 after p.o. challenge were also not immediately protected from allergic reactions induced by OVA challenges (day 36 and day 56; Fig. 6C). However, mice became resistant to next OVA challenges (day 79 and day 100). Additional cure of ld-IL-2 was performed like other groups 3 mo after the sensitization, and long-term efficacy was investigated over an extra period of 7 mo. We observed that IL-2 could control allergic reactions with similar efficacy and long-term maintenance than the pre-S treatment (Fig. 6A, 6C). Importantly, therapeutic efficacy of IL-2 was confirmed by absence of body temperature variation and low level of MPCT1 in IL-2–treated mice compared with control mice (Supplemental Fig. 3). However, no significant variations of OVA-specific IgE titer were observed at early (day 73) or late (day 320) time points in IL-2–treated mice compared with control mice (Supplemental Fig. 3).

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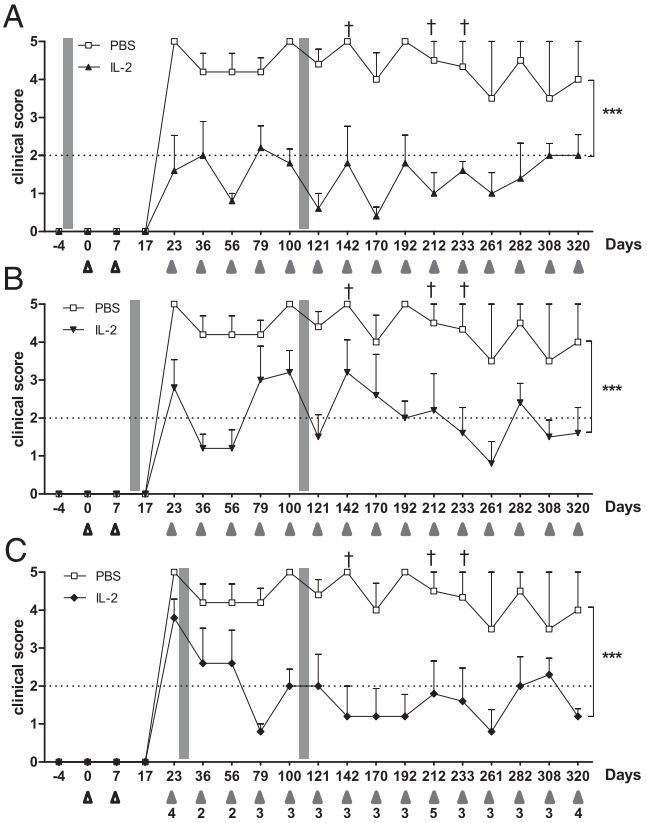

FIGURE 6. Ld-IL-2 induces a sustainable protection against food allergy reaction in preventive and therapeutic settings. Mice were sensitized at days 0 and 7 (white arrows) with OVA and then challenged with OVA by repeated p.o. administrations. Food allergy was scored (gray arrows) after two to five p.o. inductions to induce severe allergic reactions (score ≥4) in 75% of PBS-treated sensitized mice (group S). Number of challenges is indicated below the symbols. The 5-d ld-IL-2 (gray bars) cure was initiated (**A**) pre-S, (**B**) pre-C, or (**C**) after allergy induction. One additional cure of ld-IL-2 was performed in treated mice at day 110. Allergic status of IL-2– or PBS-treated mice was analyzed over a 10-mo period. Data represent one of two independent experiments. Dagger indicates a dead mouse. Each value represents the mean + SEM of five mice (***$p < 0.001$, one-way ANOVA).

Ld-IL-2 administrated by p.o. route is effective to induce protection against food allergy reaction

We wondered whether alternative and more convenient routes of administration could be used to perform ld-IL-2 treatment. We demonstrated that ld-IL-2 p.o. treatment induced protection against allergy as shown by the lower susceptibility of IL-2–treated mice to allergy induction compared with control mice (Fig. 7A). A robust protection was observed because mice were fully protected after three cycles of challenges over a 2-mo period (data not shown). We also demonstrated that the protective effect is Treg-mediated because protection was abolished when Tregs have been depleted after IL-2 p.o. treatment (Fig. 7A). Notably, IL-2 p.o. induced protection is associated with a decrease of mast cell frequency in the duodenum, whereas large infiltrates were shown in Treg-depleted mice after OVA challenges (Fig. 7B).

Altogether, these results show that both preventive and curative administration of IL-2 provide long-term clinical protection against food allergy, including when IL-2 is administrated by more convenient route.

Discussion

Previous investigations have established that Tregs play a central role both in the development of natural p.o. tolerance (28, 29) and in the mechanism of AIT efficacy (12, 30, 31). However, specific Treg-based immunotherapies have not been evaluated in the context of food allergy. For this purpose, we established an experimental model based on previous work (15), comprising sensitization with OVA/alum i.p. injections followed by administration of high amounts of OVA by repeated intragastric delivery. This induces gastrointestinal hypersensitivity characterized notably by temperature decline, acute diarrhea, local recruitment, and degranulation of effector cells, associated with a Th2 immune response profile as shown by high levels of IL-4– and IL-5–secreting cells in peripheral and gut-associated tissues. This Th2 profile was confirmed by high levels of OVA-specific IgE and IgG1 Abs after allergen challenges (Fig. 2 and data not shown). As discussed recently by Liu et al. (32), the genetic background, the nature of allergen, and the route of allergen exposure greatly influence the phenotype of mouse models. We have tested different inbred mouse strains and selected the BALB/c AnN mice in which we succeed to induce severe allergic reactions. Indeed, in contrast with a previous report (33), we observed that a significant proportion of sensitized BALB/c AnN mice were diarrheic and were hypothermic after a limited number of OVA challenges (Figs. 2, 3) whatever the animal provider (Janvier Labs or Charles River; data not shown).

Using this mouse model, we showed that ld-IL-2 treatment efficiently prevents and controls food allergy reactions that can be explained by the capacity of Tregs to rebalance the Th2-dominant local immune responses. Indeed, we observed in protected mice a significant decrease of Th2-associated gene expression and cytokine production in gut and lymphoid-associated tissues, whereas the Ag-specific Th1 immune responses in MLN and PP were amplified (Fig. 4 and data not shown). Because IL-4 has been shown to exacerbate anaphylaxis (34), the reduction of IL-4 levels induced by ld-IL-2 during the effector phase of immediate hypersensitivity may rapidly alleviate allergic symptoms. Interestingly, the Th1/Th2 immune deviation cannot be related with the potential immunogenicity of human IL-2 used in these experiments because control of Th2 allergic responses was also observed in mice treated with murine IL-2 (Supplemental Fig. 4 and data not shown). In contrast, we showed that mice treated with heat-denatured human IL-2 were not protected against Th2-mediated allergic reaction. A switch to Th1 immune responses was already reported in AIT (35), including in food allergy treatment (36). Even though Th1 redirection induced by AIT with allergen extracts or peptides continues to be reported in more recent publications (37, 38), other groups, however, did not observe this effect (39). More recently, the induction of allergen-specific Tregs producing IL-10 has been considered as a main event causing peripheral T cell tolerance (35). Importantly, the mechanisms of immunodeviation and immunoregulation should not be considered as alternative and mutually exclusive events. Indeed, it was reported that Th2 to Th1 recommitment of T cells correlates with the induction of $CD4^+CD25^{high}$ and IL-10–producing Tregs in wasp venom immunotherapy (40). Accumulation of $IL\text{-}10^+$ Tregs and Th1 cells in allergen-challenged skin sites was also shown after tolerogenic vaccination with Fel d 1 peptides (41). In our results, we observed an increase of both IL-10 and IFN-γ responses in PP and duodenum (data not shown). Regulatory IFN-γ/IL-10 double-positive $Foxp3^-$ $CD4^+$ T cells that have been described in mice undergoing chronic Ag stimulation (42) might be induced after ld-IL-2 treatment, thus explaining the unexpected absence of $Foxp3^+$ Tregs expansion after p.o. challenges in the protected mice (Fig. 4A). In this line, Wilson et al. (43) report that the preventive protective effect of IL-2:anti–IL-2–based therapy in the mouse asthma model was correlated with an increase of $IL\text{-}10^+CD4^+$ cells. There are other lines of evidence suggesting that IL-10 and Tregs play interrelated roles in the regulation of effector phase of the allergic re-

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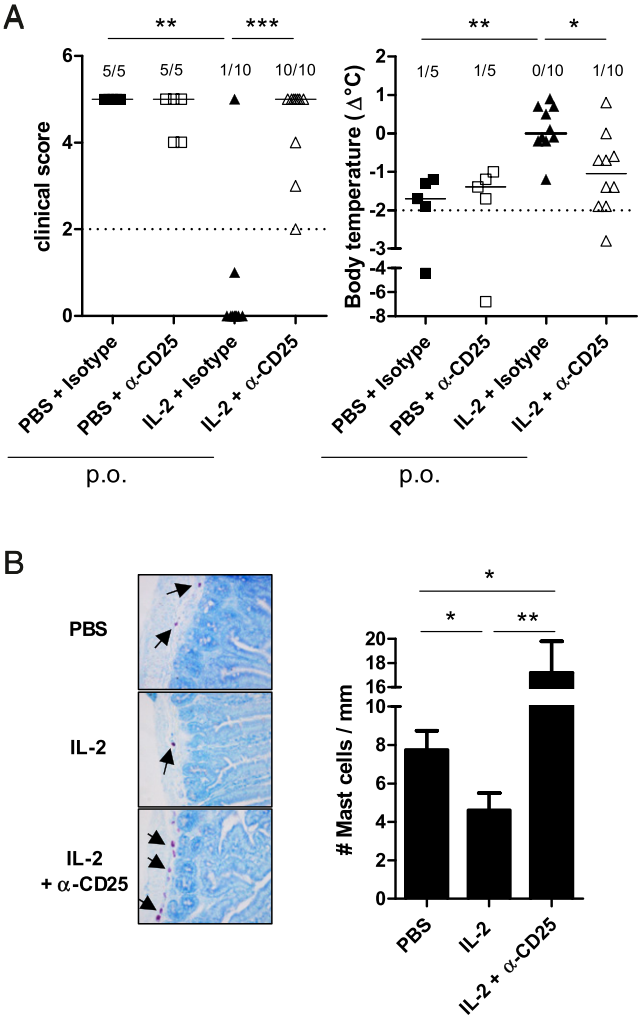

FIGURE 7. Ld-IL-2 by p.o. route provides protection against clinical manifestations of food allergy and prevents mast cells recruitment in Treg-dependent manner. Mice were orally treated (p.o.) with ld-IL-2 (IL-2) or PBS during 5 d, injected or not at day 5 with anti-CD25 PC61 mAb (anti-CD25) to depleted Tregs, and then sensitized and challenged with OVA. (**A**) Clinical score (*left panel*) and body temperature (*right panel*) measured 30 min after the sixth p.o. challenge are shown for each individual mouse. Each dot represents individual value for each mouse, and group medians are shown. Clinical protection threshold has been defined for clinical score ≤ 2 and hypothermia when the body temperature declines $>2^{\circ}C$. The numbers of unprotected and hypothermic mice are indicated for each group ($n = 5$–10 mice per group). (**B**) Mast cell infiltrates were analyzed by immunohistochemistry in the duodenum of IL-2–, IL-2+α-CD25–, and PBS-treated mice. Representative photomicrographs (original magnification ×400) are shown. Arrows indicate mast cells. A total of four sections were randomly analyzed per group, total mast cells were counted, and results were expressed as mast cells per millimeter. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Mann–Whitney U test.

sponses. IL-10 produced by increased numbers of induced Tregs during AIT downregulates eosinophil function and activity, and suppresses IL-5 production by Th2 cells (44, 45). As a result, local IL-10 production might have a major contribution in ld-IL-2 treatment efficacy, as suggested by the established correlation between IL-10 secretion in PP and the control of degranulation revealed by low mMCPT-1 concentration in serum of ld-IL-2–treated mice (data not shown).

Nevertheless, we can state that ld-IL-2 acts primarily through Treg expansion and activation. The fundamental role of Tregs in protective phenotype was demonstrated by the abolition of protection after anti-CD25 PC61 mAb treatment, which induced Treg depletion but preserved ILC2 and eosinophil populations that are known to promote allergic reactions. Interestingly, we observed that ld-IL-2 induced initial Treg expansion in systemic (spleen) and local compartments (MLN, PP) including SI where Tregs express a lower level of CD25, whereas their proliferation occurred mainly at a systemic level as shown by the increased percentages of KI67^{+}, ICOS^{hi}, and CTLA4^{hi} Tregs in the spleen (Fig. 1). In contrast, a few weeks after the end of ld-IL-2 treatment, we did not observe any sustained CD4^{+}Foxp3^{+} or CD4^{+}CD25^{+}Foxp3^{+}ICOS^{+} Treg expansion in the different organs of protected mice after OVA challenges (Fig. 4A). These observations suggest that: 1) alternative local immune-suppressive mechanisms might be induced by IL-2–activated Tregs, including suppressive Foxp3^{-} T cells; or 2) Ag-specific Tregs might be selected and persisted at low frequencies. Interestingly, a relationship between the ICOS^{hi} Tregs and the suppressive IL-10^{+}Foxp3^{-} Tr1 cells has been already described (46). However, the loss of protection observed when PC61 mAb was administered between two OVA challenges (Fig. 3I) highlights the role of Tregs in the long-term protected phenotype. Further investigations are necessary to address this specific issue. We especially plan to investigate whether association of ld-IL-2 with allergen could improve therapeutic efficacy by generating allergen-specific Tregs. Alternatively, specific IL-2 treatment regimens to maintain a high level of Tregs over a long-term period could be proposed. Importantly, we previously demonstrated that sustained stimulation and expansion of Tregs does not induce general immunosuppression because we showed that long-term IL-2 treatments control immune disorders in mice without impairing immune responses to infection, vaccination, and cancer (47). Alternative and more convenient routes of ld-IL-2 administration can be proposed. In this article, we have shown for the first time, to our knowledge, that p.o. administration of IL-2 can induce Treg activation and immune protection against food allergy that can be related to a local modification of Th1/Th2 balance (i.e., decrease of IL-4 and increase of IFN-γ responses in PP of IL-2–treated mice; data not shown) and a control of mast cell infiltration in gut (Fig. 7). Accordingly with previous work (48), our results emphasize the suppressive function of Tregs on mast cell compartment because Treg depletion induces an intestinal mastocytosis that can be correlated with the severity of allergic reactions (Figs. 5C, 7B). Whatever the route of IL-2 administration, we have shown that ld-IL-2 induced a Treg-mediated control of allergic diseases that may be explained by Th2 and mast cell suppression (34, 48).

Altogether, the present data show for the first time, to our knowledge, the therapeutic potential of ld-IL-2 for the treatment of food allergy and should prompt further investigations of this potential to re-establish long-term immune tolerance to allergens.

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Disclosures

The authors have no financial conflicts of interest.

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