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Allergen alters IL-2/αIL-2-based Treg expansion but not tolerance induction in an allergen-specific mouse model

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Abstract

Background: Regulatory T lymphocytes (Treg) play an important role in preventing allergic diseases. We characterized Treg expansion kinetics, marker profiles, and recirculation behavior in allergen-challenged mice, which had been pretreated with IL-2/αIL-2 complexes in the presence or absence of allergen. Moreover, the ability of induced Treg to control airway hyperreactivity and effector functions of lung T cells was determined.

Methods: Humanized TCR/HLA-transgenic allergy mice were treated in vivo with recombinant IL-2 complexed to the anti-IL-2 mAb JES6-1 in the presence or absence of mugwort pollen extract (MPE) on days 0-2. Afterward, they were intranasally challenged with MPE (days 13-15) followed by determination of airway hyperreactivity and lung T cell effector functions. Multiparametric flow cytometry on peripheral blood T cells was performed on a daily basis.

Results: IL-2/αIL-2 complexes highly efficiently expanded peripheral Treg cells, while concomitant allergen exposure altered the phenotype of expanded Treg cells. Notably, application of allergen together with IL-2/αIL-2 complexes induced expression of Treg marker molecules CTLA4, NRP1, Helios, and GITR on conventional T cells. Apart from CD25, GARP was identified as the most reliable surface-expressed lineage discrimination marker of Treg expanded in the presence of IL-2/αIL-2 complexes and allergen. Finally, IL-2/αIL-2 complex-expanded Treg cells could be recalled upon allergen challenge, which was associated with suppression of lung-specific Th2 responses long after initial treatment.

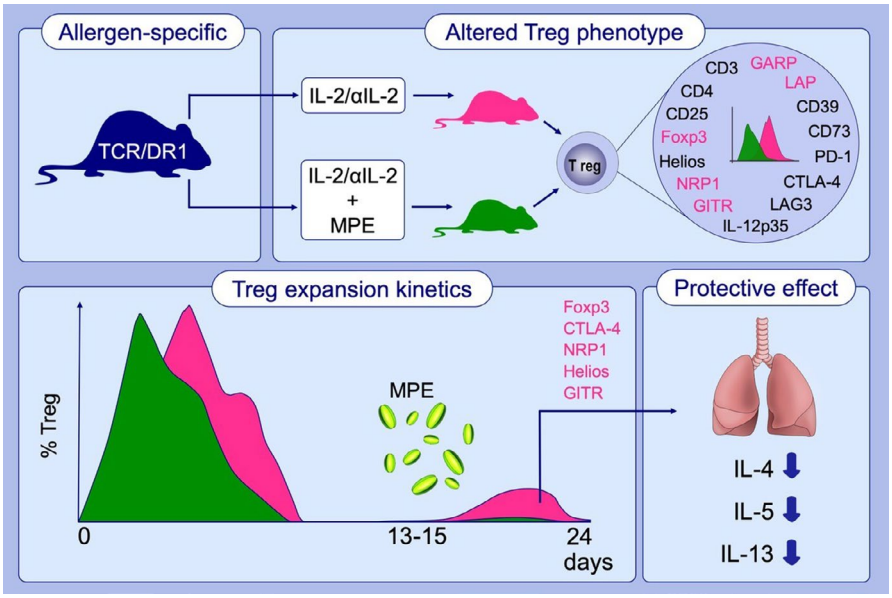
Conclusion: The characterization of reliable surface and transcription markers of IL-2/αIL-2 complex-expanded Treg along with their expansion kinetics and function will help to identify protocols for their long-term expansion in vivo.

Abbreviations: AHR, airway hyperreactivity; APC, antigen-presenting cell; CD, cluster of differentiation; CTL, cytotoxic T lymphocytes; DC, dendritic cells; FOXP3, forkhead box protein 3; HLA, human leukocyte antigen; IFN, interferon; Ig, immunoglobulin; IL, interleukin; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; MPE, mugwort pollen extract; NK, natural killer cell; PB, peripheral blood; PHA, phytohemagglutinin; RAG, recombination activation gene; TCR, T-cell receptor; tg, transgenic; Th, T helper cell; TNF, tumor necrosis factor; WBP, whole-body plethysmography; Penh, enhanced pause; Teff, effector T cell; Treg, regulatory T cell.

Köhler, Smole and Kratzer contributed equally to the work

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KEYWORDS
allergy treatment, IL-2/αIL-2 complexes, immunotherapy and tolerance induction, T cells, Treg cells



GRAPHICAL ABSTRACT
Application of IL-2/αIL-2 complexes changes the phenotype and function of peripheral blood T cells by inducing a wave of recirculating Treg cells. Presence of MPE changes the expansion kinetics and the phenotype of the expanded Treg population and reveals lack of specificity of several Treg marker molecules. IL-2/αIL-2 complex-induced Tregs can be recalled when mice are allergen challenged on days 13-15 and protect from allergic lung inflammation long after initial treatment.

1 | INTRODUCTION

Allergic diseases represent exaggerated type 2 immune responses to otherwise innocuous, environmental antigens in genetically susceptible individuals.[1-6] The development of allergies might be caused, in part, by an imbalance between allergen-specific T helper 2 (Th2) and T regulatory (Treg) cell responses.[3,7] Previous evidence suggests that allergic individuals may suffer from a systemic paucity of Treg cells,[8,9] which can be (partially) corrected by allergen-specific immunotherapy that aims at alleviating allergic diseases through increasing the numbers of Tregs.[10] In fact, Tregs have been shown to contribute to protective immune responses against allergens during normal immunity but also during allergen-specific immunotherapy (AIT).[11]

Under physiological conditions, homeostatic Treg numbers are maintained by low level production of IL-2 from conventional $CD4^+$ T cells (Tconv).[12] However, this balance may become disturbed upon (repeated) exposure to foreign antigens (*eg*, during infections, exposure to allergens, etc. [11,13]), resulting in exaggerated $CD4^+$ Tconv cell expansion and excess IL-2 production, which, in turn, leads to compensatory Treg cell expansion.[12] Whether and how antigen directly contributes to Treg development and expansion has been controversially discussed in the past. While low antigen levels or impairment of TCR signaling are thought to favor peripheral Treg differentiation,[14-18] high antigen levels have been reported to be required for thymic Treg development[19,20]; however, also the exactly opposite was claimed recently.[21] The matter becomes even more complex when TCR-independent Treg expansion forces come into play, for example, those provided by strong IL-2R and/or STAT5 agonists.[22] In these situations, antigen seems to be entirely dispensable for sustained Treg cell expansion.[22]

Interleukin(IL)-2 complexed to distinct anti-IL-2 antibodies represents such a strong IL-2R agonist.[23,24] Distinct anti-IL-2 mAbs, which bind to functionally important epitopes of IL-2, not only increase the half-life of IL-2 but also direct the selective expansion of certain T cell subsets, most prominently Tregs.[23,25] This would suggest IL-2/αIL-2 complexes as a treatment option for allergic diseases. Indeed, a recent prospective study on childhood asthma showed a correlation between low IL-2 responses early in life and elevated total IgE levels and allergic rhinitis later on.[26] This highlights the importance of sufficient IL-2 levels to support peripheral tolerance induction.[12] However, individuals suffering, for example, from perennial allergies would have to undergo IL-2/αIL-2 complex-based Treg expansion in the constant presence of allergen exposure. Whether and how IL-2/αIL-2 complex-based expansion of allergen-specific Treg would be influenced by the presence of allergens remained enigmatic so far.

Therefore, we here evaluated in a humanized preclinical model of allergy the co-administration of IL-2/αIL-2 complexes and allergen and

how this would impact on the course of the allergic disease. We took advantage of a recently established humanized allergy model, which is specific for mugwort allergy and based on the co-expression of a human TCR and HLA-DR1.[4] Mice were exposed to IL-2/αIL-2 complexes [23] in the presence or absence of mugwort pollen extract (MPE, days 0-2), while control mice were exposed to MPE or PBS alone. Subsequently, we investigated whether the different treatments would lead to differences in the numbers and phenotypes of Tregs,[27] and whether such pretreatment would impact on AHR and T-cell effector functions in lungs upon allergen challenge. Furthermore, we wanted to elucidate the specificity of the described Treg markers CD25, Foxp3, CD73, GARP, GITR, NRP1, CTLA-4, Helios, LAG3, CD39, PD-1, IL-12p35 and LAP in such expanded Treg populations and whether they are useful to define those cells.[27]

2 | MATERIALS AND METHODS

2.1 | Preparation of MPE

Artemisia vulgaris pollen (Greer, Lenoir, NC, US) was used for preparation of mugwort pollen extract (MPE) as described previously.[4] Briefly, 10 g of mugwort pollen were incubated in 100 mL of PBS under constant stirring at 4°C for 24 hours. After centrifugation at 52 000 *g* at 4°C for 60 minutes, the supernatants were filtered and dialyzed (Spectra/Por Dialysis Membrane, MWCO: 6-8000, Spectrum Laboratories) against PBS for 48 hours. Total protein concentration was determined by standard procedures (BCA-bicinchoninic acid protein Kit, Pierce). The lipopolysaccharide (LPS) content of the mugwort pollen extract was ≤0.024 U/mg. Extracts were lyophilized and aliquots stored at −80°C.

2.2 | Preparation of IL-2/αIL-2-complexes

IL-2 complexes were generated by incubating 1 µg murine recombinant IL-2 (Peprotech) with 5 µg αIL-2 antibody JES6-1A12 (Life Tech Austria) at 37°C for 30 minutes.

2.3 | Preparation of MPE immunization solution

Immunization solution was prepared by incubation of 50 µg MPE in the presence of 75 µL aluminum hydroxide (alum; 5.9-7.1 mg/mL SERVA Electrophoresis GmbH) and 60 µL PBS w/o Ca^{2+} and Mg^{2+} at RT on a shaker for 30 minutes and subsequently used for in vivo application.

2.4 | Mice and animal experimental procedures

TCR/DR1 transgenic mice[4] (>12-times backcrossed to C57BL/6J) were housed in a conventional animal facility at the Institute of Immunology (Medical University Vienna). Age-matched female mice (6-8 weeks of age) were used for experiments. Mice received food and water ad libitum. Sentinel mice were screened for and found free of mouse pathogenic viruses, bacteria, and parasites according to FELASA 2014 recommendations.[28] Experimental procedures were reviewed and approved by the Institutional Review Board of the Medical University Vienna and approved by the Federal Ministry of Science, Austria (BMWF-66.009 0118-II 3b 2012).

2.5 | In vivo expansion of Tregs by IL-2/αIL-2 complexes

TCR/DR1 transgenic mice were treated i.p. with IL-2/αIL-2 complexes (1 µg mIL-2 complexed to 5 µg αIL-2) on three consecutive days alone or in combination with MPE (50 µg/d). Treatment of mice with PBS or antigen alone was used for control groups.

2.6 | Statistics

Data were analyzed using the GraphPad Prism 6 (La Jolla, CA, US) software. Unless indicated, all values indicate mean values ± SEM. Multiple *t* tests using the Holm-Sidak method were used in Figure 2C and D, Figure 4C, and Figure S4A-C. Unpaired *t* test was used for comparisons between two groups in Figure 3B. Two-way ANOVA followed by Dunnet's correction was used for repeated measurements (Figures 5 and 6). *P* values < .05 were considered significant.

Further experimental details are provided in the Materials and Methods section in this article's Supporting information.

3 | RESULTS

3.1 | Phenotypic characterization of Treg generated in vivo upon exposure to IL-2/αIL-2 complexes in the presence or absence of antigen

We here tested whether and how IL-2/αIL-2 complex-based expansion of allergen-specific Tregs would be influenced by the systemic co-administration of allergen according to the protocol shown in Figure S1. We found that T cells expressing the high-affinity IL-2 receptor α-chain (CD25), representing the first described Treg marker on $CD3^+CD4^+$ Th cells,[29] increased from day 2 onwards. The numbers of $CD3^+CD4^+CD25^+$ Treg peaked at 32.3 ± 3.9% on day 5 (Table S1) (6.05 ± 0.7-fold expansion, *P* < .001 compared with baseline) (Figure 1A) and returned to baseline levels on day 10. Injection of IL-2/αIL-2-complexes in combination with allergen (MPE) led to an earlier peak already on day 4 with 34.0 ± 4.9% $CD3^+CD4^+CD25^+$ Treg cells, and a 7.0 ± 1.1-fold expansion (*P* < .01 compared with baseline). Peripheral blood (PB) $CD3^+CD4^+CD25^+$ T cell numbers returned to baseline levels on day 9.

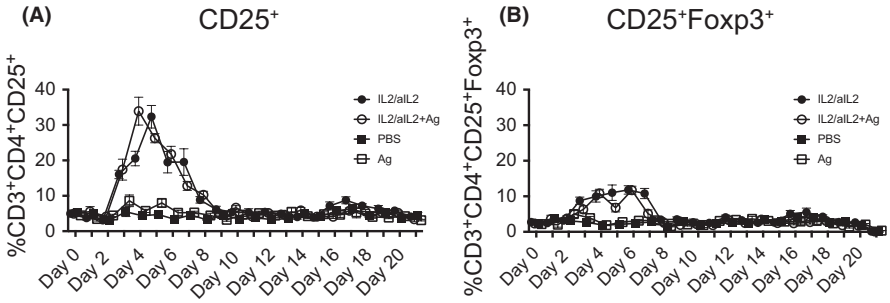


FIGURE 1 Expansion kinetics of Tregs. Shown are (A) $CD3^+CD4^+CD25^+$ and (B) $CD3^+CD4^+CD25^+Foxp3^+$ PB T cells in mice treated with IL-2/αIL-2 complexes in the presence or absence of specific allergen. Shown are the percentages (mean ± SEM) of $CD3^+CD4^+CD25^+$ and $CD3^+CD4^+CD25^+Foxp3^+$ PB T cells from days 0-21 derived from mice i.p. injected with IL-2/JES6-1 complexes in the absence (IL-2/αIL-2) or presence of alum-adsorbed MPE (IL-2/αIL-2 + MPE). Control groups received PBS (PBS) or allergen (MPE) alone. Data show the summary of two independently performed experiments with n = 6 for IL-2/αIL-2 and IL-2/αIL-2 + MPE, n = 5 for MPE and n = 4 for PBS mice per group

Foxp3, a Treg lineage-specific transcription factor in mice,[30] was also significantly upregulated in PB $CD3^+CD4^+CD25^+$ T cells after IL-2/αIL-2 treatment, peaking on day 5 (6.5 ± 1.2-fold expansion, $P < .05$ compared with baseline) and returning to baseline levels on day 10 (Figure 1B). In contrast, administration of IL-2/αIL-2 complexes in combination with antigen (MPE) resulted in a multi-phasic upregulation of Foxp3 in PB $CD3^+CD4^+CD25^+$ Treg cells, with maximal peaks on day 4 and day 6 after initial treatment (10.8 ± 3.3-fold and 9.8 ± 2.6-fold expansion; $P < .05$ and $P < .05$ compared to baseline, respectively). Foxp3 expression returned to baseline levels on day 8. Control groups, treated with PBS or allergen alone, did not show changes in the percentages of $CD3^+CD4^+CD25^+$ or $CD3^+CD4^+CD25^+Foxp3^+$ cells in PB (Figure 1).

The Treg surface markers CTLA-4, GITR, NRP1, and LAP followed expression kinetics as CD25 upon TCR co-engagement, ie, in the presence of allergen, with earlier but similar peak expression levels when compared to mice treated with IL-2/αIL-2 complexes alone (Figure S2). In contrast, peak expression levels for Helios, GARP, and CD73 were modestly lower in the presence of TCR co-ligation (Figure S3).

CTLA-4, GITR, and GARP were found to be co-regulated with CD25 (Figure 2A), with co-expression on >80% of $CD3^+CD4^+CD25^+$ T cells on day 4 (Figure 2B). In contrast, NRP1, Helios, Foxp3, and CD73 were co-expressed only on a subset of $CD3^+CD4^+CD25^+$ T cells (75.9 ± 4.1%; 75.4 ± 14.8%, 57.8 ± 5.9%, and 13.7 ± 5.0%, respectively (Figure 2A and B). This changed until day 6 when >90% of $CD3^+CD4^+CD25^+$ T cells co-expressed Helios (90.2 ± 1.9), while NRP1 and Foxp3 were still expressed on <80% of $CD3^+CD4^+CD25^+$ T cells (Figure 2F and G). The subset of $CD3^+CD4^+CD25^+$ T cells co-expressing CD73 remained small (6.05 ± 1.4%) (Figure 2B and G). However, administration of IL-2/αIL-2 complexes in the presence of TCR ligation (MPE) significantly increased the fraction of $CD3^+CD4^+CD25^+$ T cells, which remained negative for NRP1, GARP, and Foxp3, respectively (Figure 2A and B). In fact, NRP1, GARP, Foxp3, and LAP were found co-expressed only on 54.6 ± 2.3%, 44.1 ± 10.6%, 39.1 ± 13.8%, and 16.1 ± 5.1 of $CD3^+CD4^+CD25^+$ T cells, respectively, which was significantly less when compared to $CD3^+CD4^+CD25^+$ Th cells upon exclusive treatment of mice with IL-2/αIL-2 complexes on day 4 (Figure 2A and B). A similar MPE-induced reduction of marker expression on $CD3^+CD4^+CD25^+$ T cells was observed on day 6 for GARP, Foxp3, and LAP (Figure 2F and G). In contrast to day 4, NRP1 was expressed at similar levels on day 6 when compared to mice treated in the absence of MPE allergen. Control groups (PBS or MPE) did not show such changes (Figure S4). When looking at the subset of $CD25^+$Treg marker$^+$ T cells as a fraction of overall $CD3^+CD4^+$ T cells on day 4, the treatment-related differences were less apparent due to the faster expansion kinetics of Tregs in the presence of allergen (MPE), while they were evident on day 6 (Figure 2C and H). Notably, the observed effects were allergen (MPE)-specific and not induced by alumn used as adjuvant, since no such reductions of $CD25^+$Treg marker$^+$ cells were observed when IL-2/αIL-2 complexes were administered together with BSA complexed to alumn (not shown). Moreover, TCR signaling also seemed to sustainably inhibit maximal expression levels on $CD3^+CD4^+CD25^+$ T cells of Helios, and GARP on day 6 (Figure 3). When gating on $CD3^+CD4^+Foxp3^+$ T cells, all Treg markers, except NRP1 (day 4) and GARP (day 6), were similarly expressed in the presence or absence of allergen (Figure S5A and B). IL-2/αIL-2 complexes, irrespective of the presence or absence of allergen, induced only weak expression (<5% cells) of LAG-3, CD39, PD-1, and IL-12p35 on $CD3^+CD4^+CD25^+$ Th cells (not shown).

3.2 | Specificity of a panel of Treg marker molecules upon IL-2/αIL-2 complex-based expansion of Tregs in the presence or absence of allergen

Next, we evaluated the Treg-specificity of CTLA-4, NRP1, Helios, GITR, and GARP, by gating on PB $CD3^+CD4^+CD25^-$ or $Foxp3^-$ T cells. In the presence of TCR signaling, significantly larger fractions of $CD3^+CD4^+CD25^-$ Tconv cells co-expressed CTLA-4, NRP1, Helios, and GITR on day four as compared to IL-2/αIL-2-treated mice (Figure 2A and D, E, Table S2); however, expression levels were generally lower when compared to $CD3^+CD4^+CD25^+$

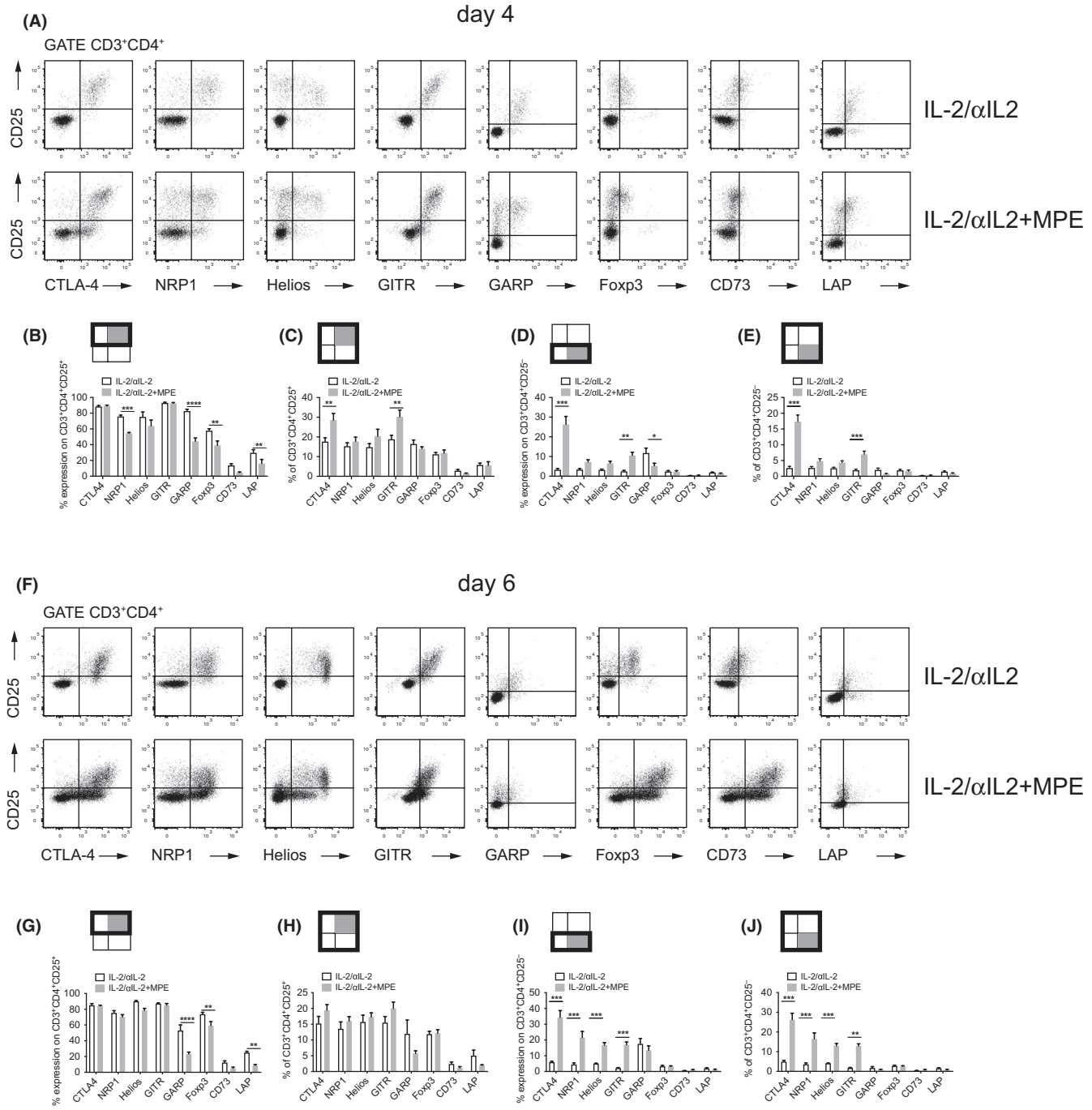


FIGURE 2 Effects of IL-2/αIL-2 complexes and allergen co-exposure on Treg marker expression. Representative flow plots show CD25 versus CTLA4, NRP1, Helios, GITR, GARP, Foxp3, CD73, and LAP expression, respectively, on $CD3^+CD4^+$ PB T cells on day 4 (A) or day 6 (F) derived from mice i.p. injected with IL-2/αIL-2 complexes in the absence (IL-2/αIL-2) or presence of alum-adsorbed MPE (IL-2/αIL-2 + MPE). Graphs show Treg marker positive cells (mean ± SEM) on day 4 (B-E) and 6 (G-J) related to either $CD3^+CD4^+CD25^+$ T cells (B, G) or $CD3^+CD4^+CD25^-$ T cells (D, I). Moreover, Treg marker positive and $CD25^+$ (C, H) or $CD25^-$ (E, J) T cells have been related to the $CD3^+CD4^+$ T cell population. The reference populations are indicated by bold frames, the tested populations by gray quadrants. Data are either representative (A and F) or show the summary (B-E and G-J) of two independently performed experiments with n = 6 mice per group for IL-2/αIL-2 and IL-2/αIL-2 + MPE. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$ comparing IL-2/αIL-2 and IL-2/αIL-2 + MPE treatment group (multiple *t* tests assuming all rows are sampled from populations with same scatter. Holm-Sidak method was used to correct for multiple comparisons)

T cells (Figure 4). Similar findings were made upon gating on $CD3^+CD4^+Foxp3^-$ Tconv cells in which, upon TCR ligation, a significantly larger fraction co-expressed CTLA-4, GITR, and NRP1, while Helios expression was unaffected on day 4 (Figures S5C and Figure S6A, Table S3). Differential expression became even more pronounced on day 6, irrespective of gating on either $CD3^+CD4^+CD25^-$ (Figure 2F and I, J, Table S2) or $CD3^+CD4^+Foxp3^-$ Tconv (Figure S5C, Table S3). Notably, expression levels of Treg

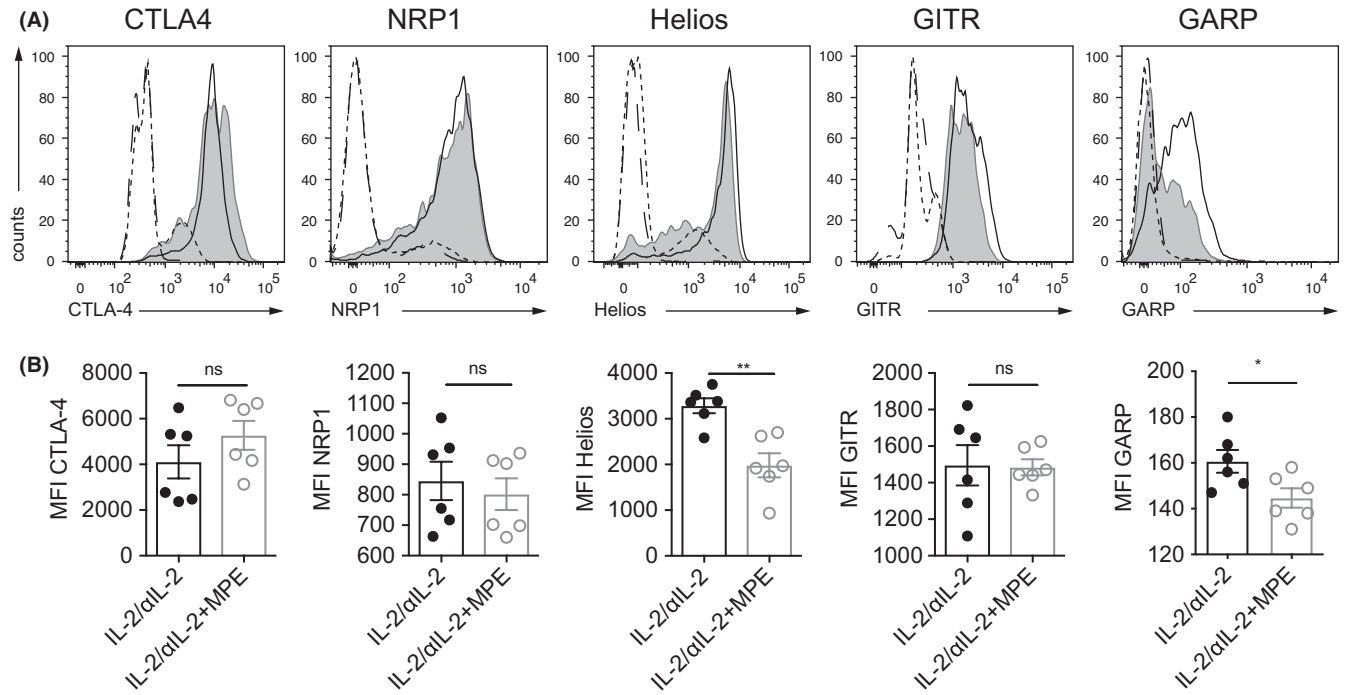


FIGURE 3 Decreased expression levels of Treg markers in the presence of allergen. A, Histogram overlays representing Treg marker expression on day 6 on $CD3^+CD4^+CD25^+$ or PB T cells (open and gray histogram) or $CD3^-CD4^-$ lymphocytes (dotted and dashed histograms) from mice treated with IL-2/αIL-2 in the absence (open and dotted histogram) or presence (gray and dashed histogram) of alum-adsorbed MPE. B, Summary plots of mean fluorescence intensity (MFI) data representative of two independently performed experiments with n = 3 mice per group for IL-2/αIL-2 (open bars) and IL-2/αIL-2 + MPE (gray bars). **P* < .05, ***P* < .01, ****P* < .001 comparing IL-2/αIL-2 and IL-2/αIL-2 + MPE treatment group (unpaired *t* test)

markers on $CD3^+CD4^+Foxp3^-$ were generally lower when compared to $CD3^+CD4^+Foxp3^+$ T cells (Figure S6).

These analyses suggested that neither CTLA-4, nor GITR, Helios or NRP1 can be regarded as *bona fide* Treg markers upon IL-2/αIL2 complex-based Treg expansion in the presence of TCR ligation. However, in the absence of TCR ligation, ie, upon exclusive IL-2/αIL-2 complex-based expansion of Tregs in vivo, these makers were almost exclusively expressed on all (or subsets) $CD25^+$ and/or $Foxp3^+$ $CD3^+CD4^+$ Th cells. GARP was confirmed herein as a specific Treg cell marker molecule, being co-expressed with CD25 on $CD3^+CD4^+$ T cells when compared to $CD3^+CD4^+CD25^-$ T cells, irrespective of whether Treg expansion was promoted by IL-2/αIL-2 complexes alone or in the presence of TCR ligation (88.2 ± 6.1% vs 94.7 ± 3.2% on day 4; and 82.4 ± 8.4% vs 86.6 ± 7.0% on day 6).

3.3 | Treg cells initially expanded by IL-2/αIL-2 complexes recirculate more efficiently upon allergen challenge in vivo at later time points

Next, we tested whether initial expansion of Tregs with IL-2/αIL-2 complexes has long-term effects. For that purpose, mice were i.n. challenged with MPE on days 13-15 and subsequently exposed to MPE aerosol followed by daily determination of PB T cell phenotypes (Figure S1). Using day 15 as baseline, IL-2/αIL-2 complex-treated mice showed a significant increase of recirculating $CD3^+CD4^+CD25^+$ T cells co-expressing the Treg marker molecules Foxp3, CTLA-4, NRP1, Helios and GITR, but not GARP, respectively, already on day 16 (Figure 5A-F), which peaked and became significant on day 17 (Figure 5G-L and Figure S7) and continued to be clearly elevated through day 19 (Figure 5). For instance, $CD3^+CD4^+Foxp3^+$ Treg showed a 2.2 ± 0.2-fold expansion (increasing from 2.5 ± 0.3% on day 15 to 5.4 ± 1.8% on day 17; *P* < .01) in PB with similar increases found for other Treg marker molecules (Table S4). Interestingly, mice treated with IL-2/αIL-2 complexes in the presence of TCR signaling (*ie*, upon co-administration of allergen), comparable to mice treated with PBS or allergen alone, did not show similar increases of PB Treg cell numbers after MPE challenge.

3.4 | Exclusive treatment of mice with IL-2/αIL-2 complexes alleviates AHR upon challenge with allergen 2 weeks later

Finally, we analyzed the impact of IL-2/αIL-2 complex treatment on lung inflammation and function upon intranasal-followed by aerosol-based allergen challenge (Figure S1). Notably, BAL fluid cellularity was lowest in the IL-2/αIL-2 pretreatment group, which was paralleled by low numbers of eosinophils, neutrophils and T

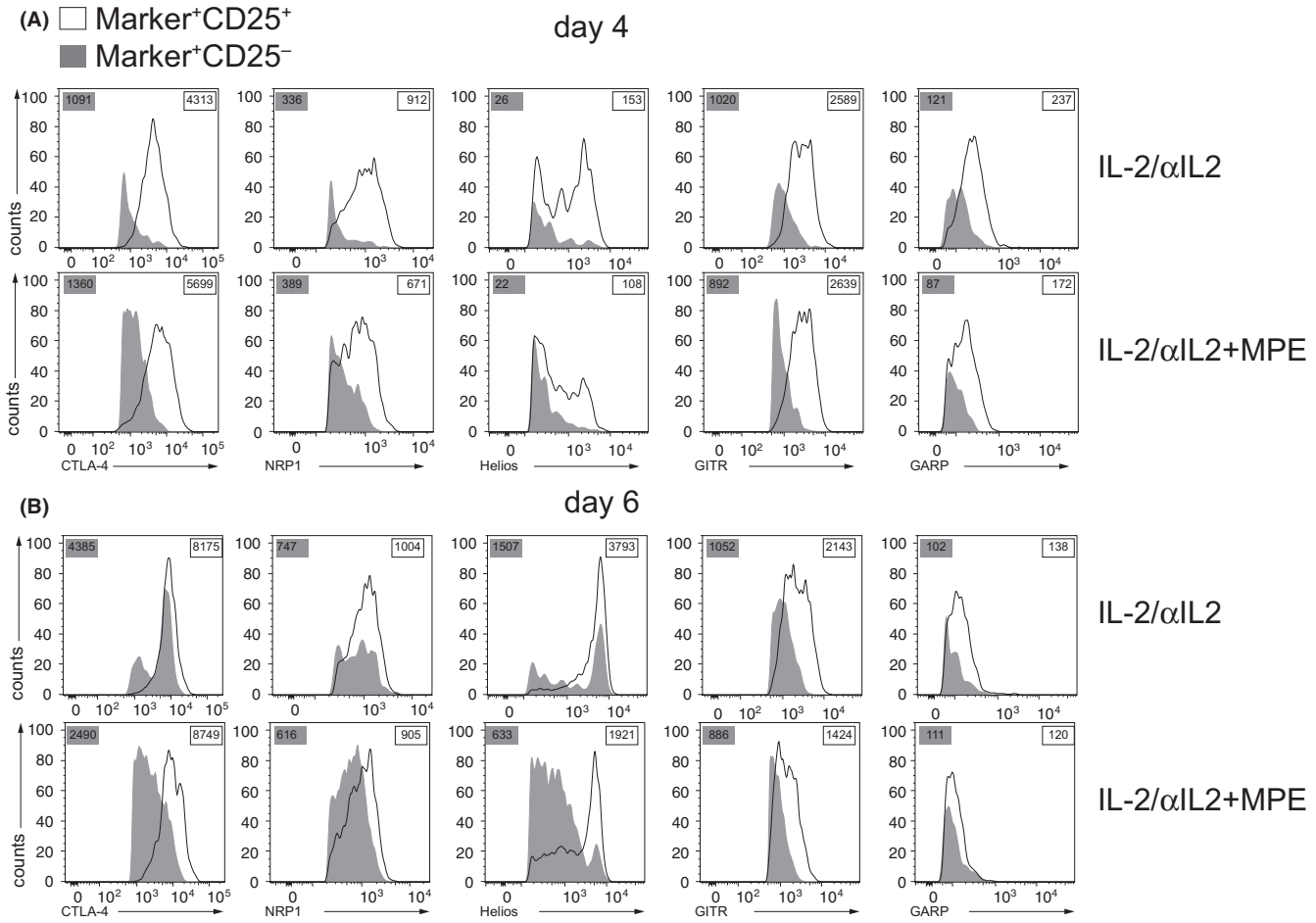


FIGURE 4 Decreased expression of Treg markers on $CD3^+CD4^+CD25^-$ cells compared to $CD3^+CD4^+CD25^+$ cells. Changes in Treg marker expression of (A) day 4 and (B) day 6 $CD3^+CD4^+CD25^-$ (gray) cells and $CD3^+CD4^+CD25^+$ (white) are shown as representative histograms for two independent experiments, each with n = 3 mice per group. Values in gray boxes indicate the geometric mean fluorescence intensity of $CD3^+CD4^+CD25^-$ in contrast to values in white boxes for $CD3^+CD4^+CD25^+$ cells

cells as determined by flow cytometry (Figure S8A) and morphologically (Figure S8B). In line, mice in which Treg numbers had been increased by virtue of IL-2/αIL-2 complexes but not PBS-treated control mice presented with a significantly mitigated hyperreactivity response to allergen challenge (Figure 6A). Interestingly, also those mice which had been systemically exposed to allergen, ie, in the presence or absence of co-administered IL-2/αIL-2 complexes, were protected from allergen re-exposure since they did not display increased AHR upon challenge. This finding might be explained by high-dose tolerance induction as shown previously during rush immunotherapy.[31] In order to correlate the observed reduction of AHR in mice treated with IL-2/αIL-2 complexes in the absence or presence of TCR ligation (*ie*, upon co-administration of allergen) with possible changes in the cytokine signatures of Th cells, cytokine levels determined upon polyclonal (Figure 6B-H) and allergen-specific (Figure 6I-O) re-stimulation were analyzed in lung single cell suspensions on day 24. Notably, only the IL-2/αIL-2 complex-treated mice, but not mice treated with allergen in the presence or absence of IL-2/αIL-2 complexes, showed reduced levels of IL-13, IL-4, and IL-5 upon both polyclonal and allergen-specific re-stimulation of lung cells (Table S5). In contrast, the secretion of the Th1 signature cytokine IFN-γ was found to be inhibited for all but the PBS treatment groups upon polyclonal but not allergen-specific re-stimulation. IL-17 und GM-CSF were not found to be modulated.

4 | DISCUSSION

Treg cell-based suppression of allergen-specific Tconv cells is critical for the prevention and therapy of allergies.[32,33] Since allergens represent exogenous antigens, tolerance induction to them takes place in the periphery and is either Tconv intrinsic, for example, based on anergy induction which can be overcome by exogenous IL-2,[31,34] or may be established by the expansion of populations of T regulatory cells impacting on Tconv cells.[31] The current study demonstrates that treatment with IL-2/αIL-2 complexes sustainably changes the phenotype and function of peripheral Th cells, induces a wave of circulating Treg cells, which can be recalled upon allergen challenge and alleviates allergen-induced lung pathology long after

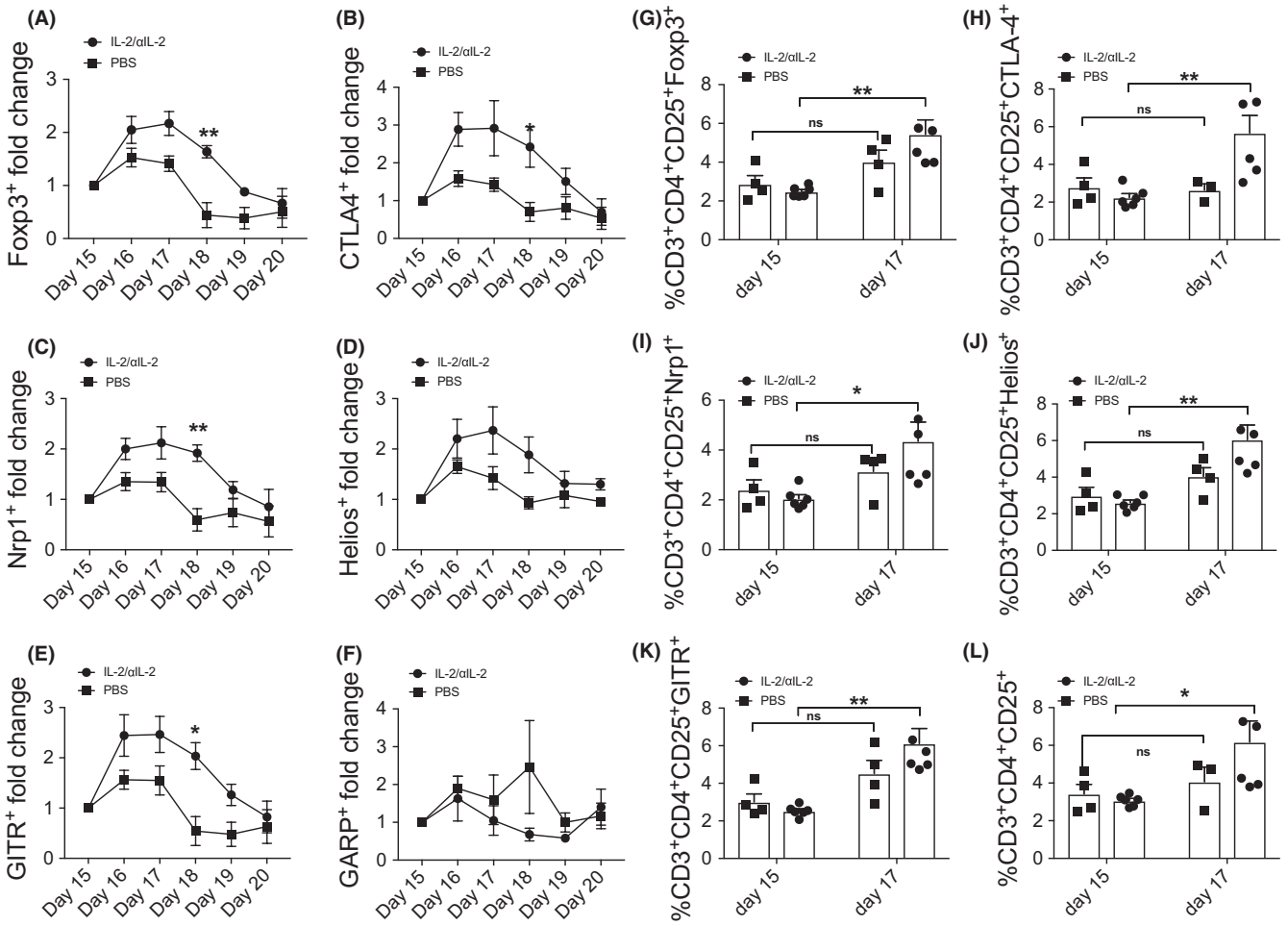


FIGURE 5 Recall Treg response in IL-2/αIL-2 complex-treated mice after allergen challenge. A-F, Fold increase and (G-L) frequency of $CD3^+CD4^+CD25^+$, $CD3^+CD4^+CD25^+Foxp3^+$, $CD3^+CD4^+CD25^+Helios^+$, $CD3^+CD4^+CD25^+GITR^+$, $CD3^+CD4^+CD25^+NRP1^+$, and $CD3^+CD4^+CD25^+CTLA4^+$ PB T cells derived from mice i.p. injected with IL-2/αIL-2 complexes after i.n. allergen challenge with MPE (1,5% (w/v), 450 μg per mouse). Data are pooled from two independently performed experiments with n = 6 mice for IL-2/αIL-2 and n = 4 mice for PBS. $^*P < .05$, $^{**}P < .01$ comparing IL-2/αIL-2 and PBS treatment group using two-way ANOVA (Sidak's multiple comparison test)

initial treatment. While it has been clearly shown that TCR signaling is not absolutely required for Treg cell division and expansion—provided that other strong Treg expansion stimuli are present, such as those delivered by IL-2R agonists or STAT5 activators[22]—our data suggest that the presence of allergen clearly alters the phenotype of expanded Treg cells, which divides the expanded $CD3^+CD4^+CD25^+$ cell population in those co-expressing or lacking other *bona fide* Treg marker molecules such as NRP1, GARP, and Foxp3 (Figure 2A and B). Moreover, we demonstrate that several Treg marker molecules lack specificity and become regularly co-expressed on Tconv when IL-2R agonists are administered together with allergen, GARP being a notable exception.

Specifically, we found that administration of IL-2/αIL-2 complexes was absolutely required for substantial expansion of PB Tregs. While administration of IL-2/αIL-2 complexes increased the fraction of $CD3^+CD4^+CD25^+Foxp3^+$ T cells among PB T cells from 2.3 ± 0.6% to 16.5 ± 3.9%, the sole administration of allergen on three consecutive days was insufficient to induce significant PB Treg cell expansion as monitored by CD25 and Foxp3 co-expression and the expression of a collection of more recently established putative Treg markers[27] (Figure 1 and Figures S2 and S3). Although concomitant TCR ligation by allergen accelerated the appearance of peak Treg levels in PB by one day, this treatment also seemed to significantly inhibit the co-expression of distinct Treg marker molecules on $CD3^+CD4^+CD25^+$ and/or $CD3^+CD4^+CD25^+Foxp3^+$ Treg cells (Figure 2 and Figure S5). For instance, the uniform expression of NRP1 on the majority of $CD3^+CD4^+CD25^+$ T cells upon treatment of mice with IL-2/αIL-2 complexes alone was clearly altered in the presence of allergen (Figure 2).

Moreover, we revealed that co-administration of allergen (TCR ligation) induced significant expression of some Treg marker molecules on Tconv cells, defined by negativity for the canonical Treg maker molecules CD25 and Foxp3 (Figure 2 and Figure S5C). For instance, a considerable fraction (26.3 ± 10.0% to 34.3 ± 10.7%) of antigen plus IL-2/αIL-2 complex-activated Tconv cells co-expressed CTLA-4. This was not entirely unexpected, since CTLA-4 has initially been characterized as a T cell activation antigen[35,36] before its role as a major co-inhibitory molecule on previously

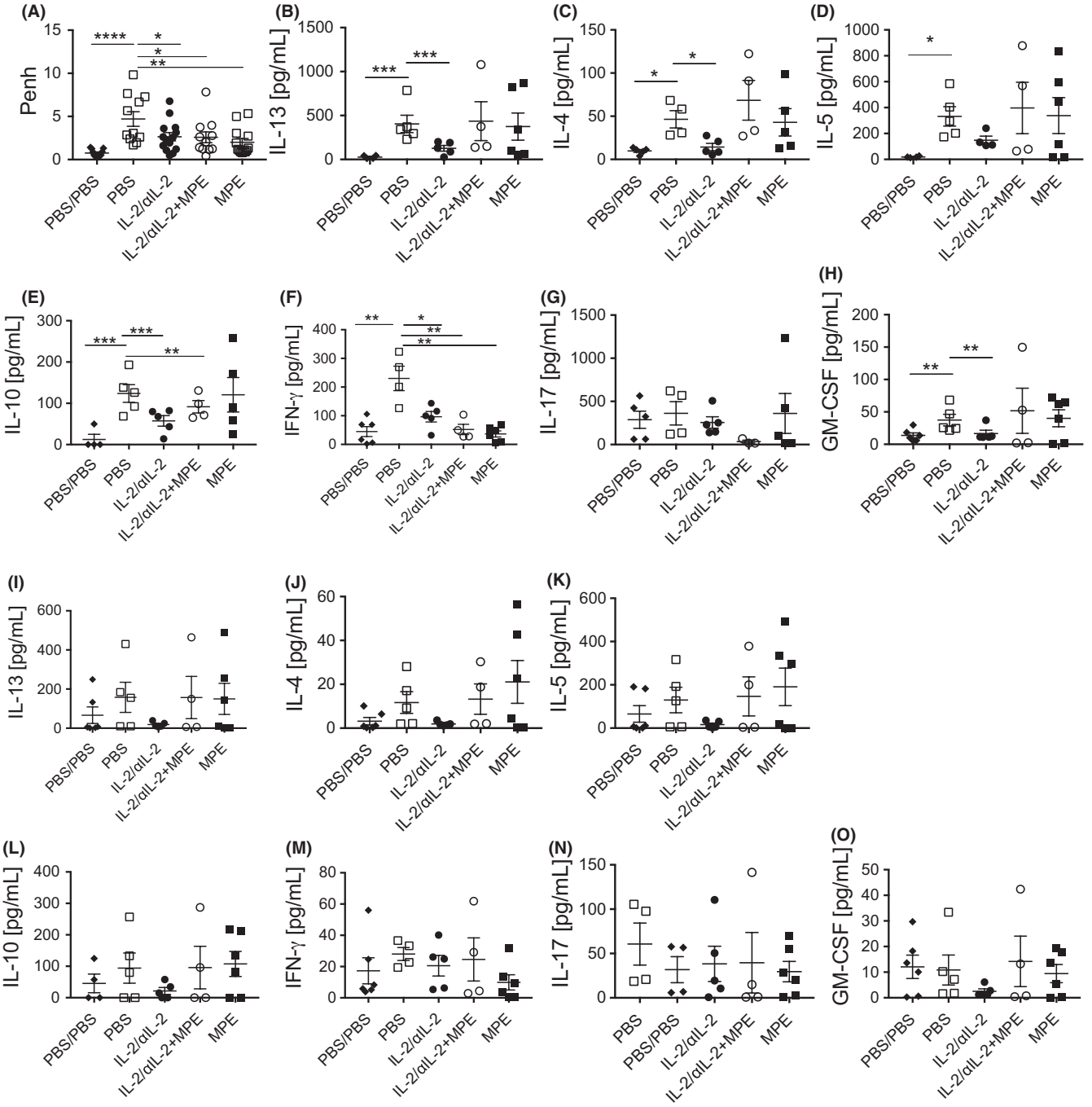


FIGURE 6 IL-2/αIL-2 complex treatment protects against allergic immune responses. A, Allergen-specific airway hyperreactivity was determined after the last challenge. Using whole-body plethysmography. Shown are mean enhanced pause (Penh) values for each mouse (individual symbols). Lung single cell suspensions were restimulated with PHA (0.15 µg/mL) (B-H) or MPE (100 µg/mL) (I-O) for 3 d, and supernatant levels of cytokines were determined using multiplex cytokine analyses. Data are pooled data of three independent experiments with a total of n = 15 mice for IL-2/αIL-2, n = 11 mice for IL-2/αIL-2 + MPE, n = 13 mice for PBS, n = 11 mice for MPE, and 11 mice for PBS/PBS shown in A. B-O, Pooled data of two independent experiments combining cytokine release data from pooled mouse lung cells data. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001 One-way ANOVA using Dunett's multiple comparison test (A) and multiple *t* test with welch correction due to the differences in variance between the study groups corrected by Bonferroni's method for multiple testing (B-O)

activated T cells was elucidated.[37,38] Surprisingly, however, under similar conditions, Tconv cells also co-expressed NRP1,[39] Helios,[40] and GITR,[41] all of which have been regarded as more or less strict Treg-restricted marker molecules so far (Figure 2). Thus, our findings suggest that a number of *bona fide* Treg marker molecules rather qualify as "activation antigens" under conditions of both TCR and IL-2R signaling. Notably, however, their expression levels on Tconv cells were generally lower when compared to the ones on $CD3^+CD4^+CD25^+$ and/or $CD3^+CD4^+Foxp3^+$ Treg cells (Figure 4 and Figure S6). Among the Treg markers under

investigation, glycoprotein-A repetitions predominant (GARP) was an exception since allergen exposure did not lead to its elevated co-expression on Tconv cells. Thus, and apart from CD25, GARP was identified as the most specific surface-expressed Treg marker analyzed in this study. Upon treatment with IL-2/αIL-2 complexes and allergen, not all of the analyzed $CD3^+CD4^+CD25^+$ T cells co-expressed GARP (Figure 2A and B). However, this was not unexpected since previous reports have shown that GARP identifies the subset of highly suppressive, activated $Foxp3^+$ Treg cells.[42,43] Apart from Treg, the type I transmembrane protein GARP, which binds and activates latent TGF-β isoforms, is typically expressed also on platelets.[42,43] Both Treg and platelets contribute to the establishment of immune tolerance[44] with missense mutations of GARP being associated with atopic dermatitis.[45]

Another intriguing finding of our study was the fact that repeated allergen exposure via the airways was able to "recall," at least partially, previously expanded Treg cells back into circulation (Figure 5 and Figure S7), while concomitant TCR ligation abrogated this phenomenon. Exclusive IL-2R agonist signaling seems to initiate sustained changes in the previously expanded Treg cell compartment, which allows for their rapid (within 3 days) recall and enhanced recirculation upon exposure to allergen. Why does co-ligation of the TCR abrogate/antagonize this recirculation/expansion capability? It is tempting to speculate that the initial presence of allergen may target Treg cells to distinct compartments within the body, which might restrict recall responses triggered by allergen challenge of the airways to compartments other than the PB. Alternatively, simultaneous TCR triggering along with IL-2R engagement might weaken the "Treg imprint," by favoring TCR- instead of IL-2R-dependent signaling pathways. Possible indications for such a mechanism are the significantly increased numbers of $CD3^+CD4^+CD25^+$ T cells which lack co-expression of the Treg marker molecules GARP, NRP1, and Foxp3. Moreover, and in light of the significant activation in the presence of antigen of Tconv, the circulatory behavior and/or expansion of Treg cells might also become influenced by the effector cytokines/receptor milieu elaborated by these cells.

Following these lines, we also observed remarkable, pretreatment-dependent differences in the T cell target populations in the lungs after allergen challenge. In fact, in lung cells of mice which had been initially treated with IL-2/αIL-2 in the absence but not in the presence of allergen, Th2 cytokine levels, including IL-13, IL-4, and IL-5, could only very modestly be recalled when compared to the PBS group, irrespective of whether re-stimulation was performed polyclonally (PHA) or allergen-specifically (MPE) (Figure 6). Similarly, sole IL-2/αIL-2 complex treatment reduced IL-10 recall responses. In contrast, especially polyclonal re-stimulation revealed a homogeneous reduction in IFN-γ production by initially IL-2/αIL-2 complex but also allergen treated mice. Thus, treatment with IL-2/αIL-2 complexes induces long-term tolerance against allergen challenge associated with consistent and significant reductions of Th2 cytokines produced by lung resident T cells. The present results corroborate our earlier findings indicating the disease alleviating potential of IL-2/αIL-2 complex-expanded Tregs in allergy.[4] The here-presented data show that treatment with IL-2/αIL-2 complexes induces long-term changes in the peripheral Treg cell compartment and the main target organ, that is, the lung. Our finding that application of antigen alone also leads to improvements of AHR are compatible with the induction of high-dose tolerance as observed during rush immunotherapy previously.[31]

A possible limitation of the current study is the fact that only PB Treg populations have been monitored, while kinetic data on Treg numbers in, for example, secondary lymphoid and target organs are missing. However, the authors have opted for the precise, time-resolved characterization of Treg expansion in PB and their marker expression profile, which would not have allowed to sample material from all organs without censoring considerably larger numbers of animals at each experimental data point.

In summary, we here show that allergen exposure alters the phenotypic composition of IL-2/αIL-2 complex-expanded Treg in PB but not tolerance induction. Moreover, we identified GARP as a further (*ie*, besides CD25) reliable surface-expressed lineage discrimination marker of IL-2/αIL-2 complex-expanded Treg cells in the presence of allergen. Finally, we provide evidence that IL-2/αIL-2 complex-expanded Treg cells can be recalled and suppress lung-specific Th2 responses long after initial treatment. Further refinement of IL-2/αIL-2 complex-based Treg treatment will help to influence diseases with aberrant/exuberant immune responses against foreign and endogenous antigens (ie, allergens and autoantigens).

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CONFLICT OF INTEREST

With regards to the authors disclosure of potential conflicts of interest, we would like to indicate that Winfried F. Pickl holds stocks of Biomay AG and receives honoraria from Novartis and Roche. All other authors have no additional financial interests.

AUTHOR CONTRIBUTIONS

WFP and CK designed research; CK, US, DT, BK, and KGS performed research and analyzed data; WFP and US supervised experiments; CK, US, BK, and WFP wrote the paper.

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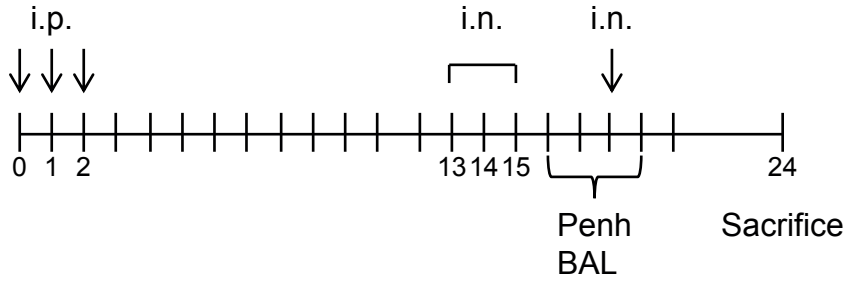
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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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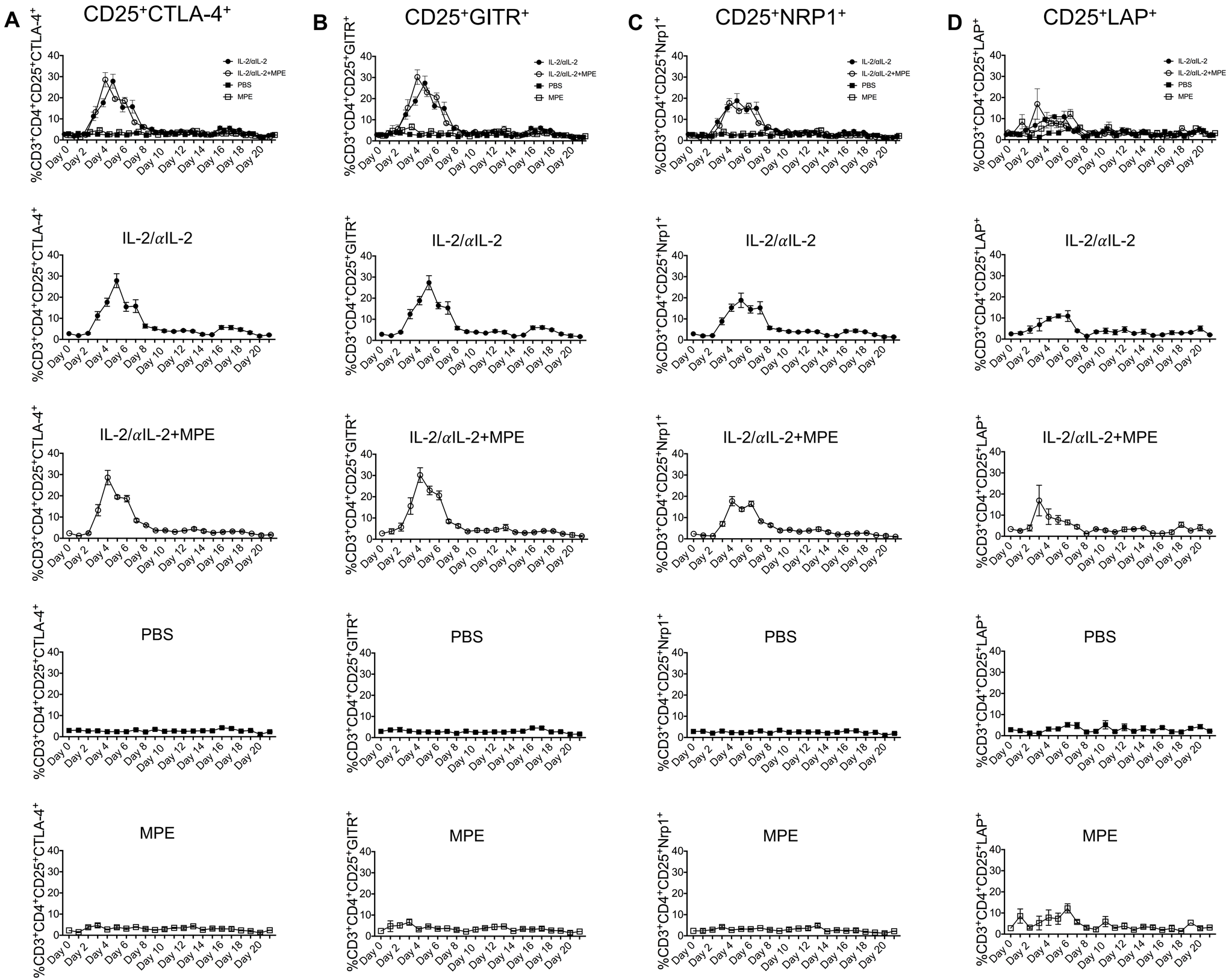
Supplemental figure 1



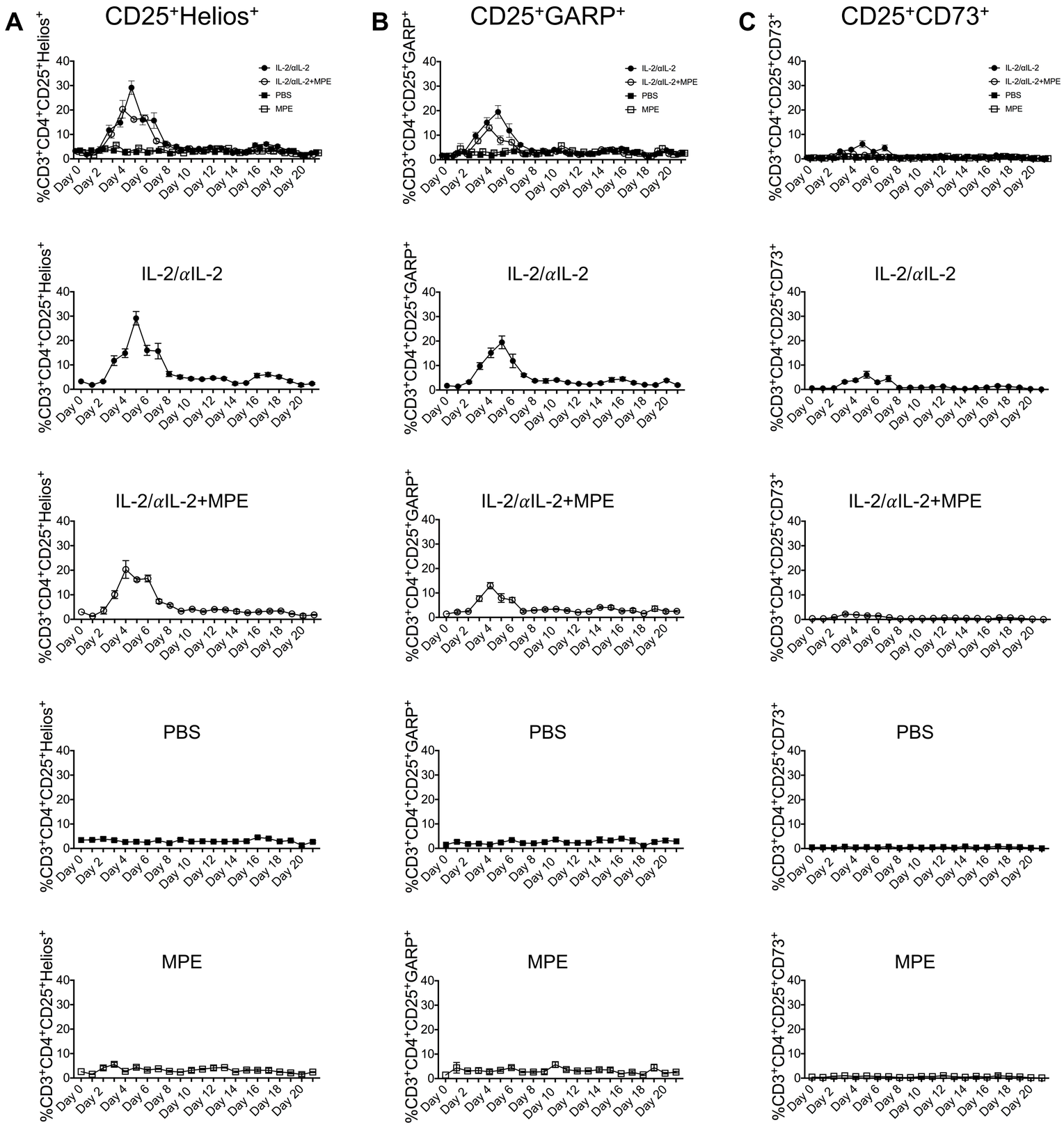
Day 0-2
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2. PBS
3. IL-2/a-IL-2
4. IL-2/a-IL-2+MPE
5. MPE

Day 13-15
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2. 450 μg MPE
3. 450 μg MPE
4. 450 μg MPE
5. 450 μg MPE

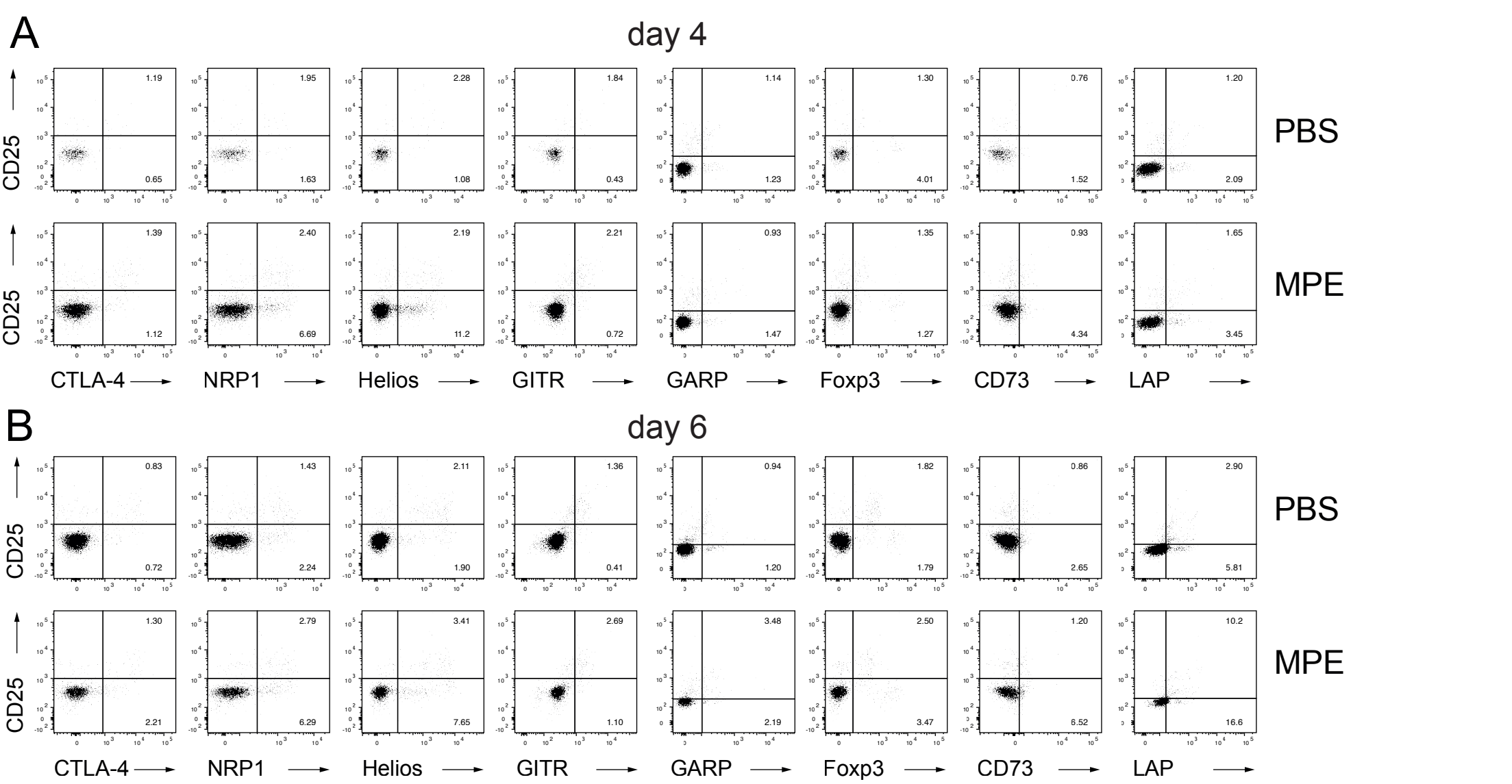
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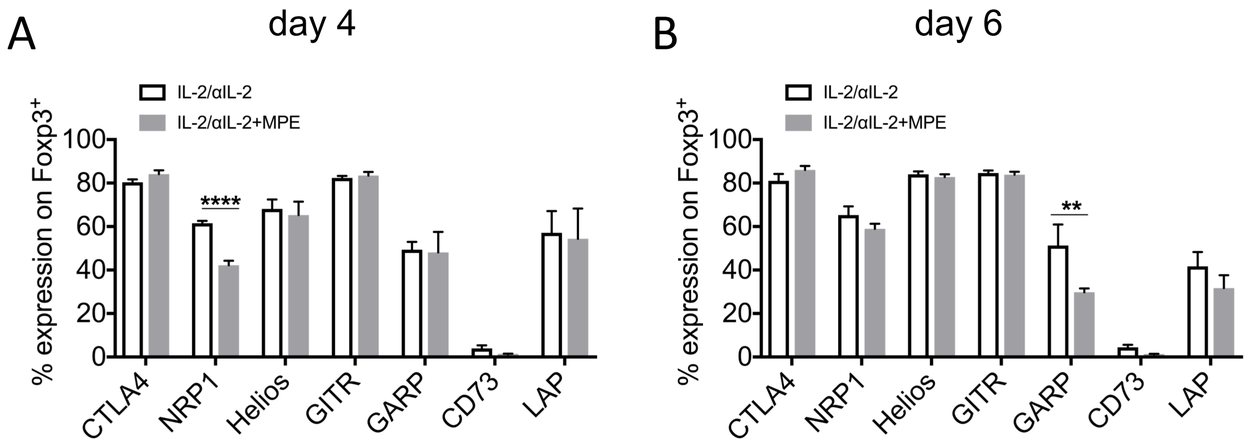
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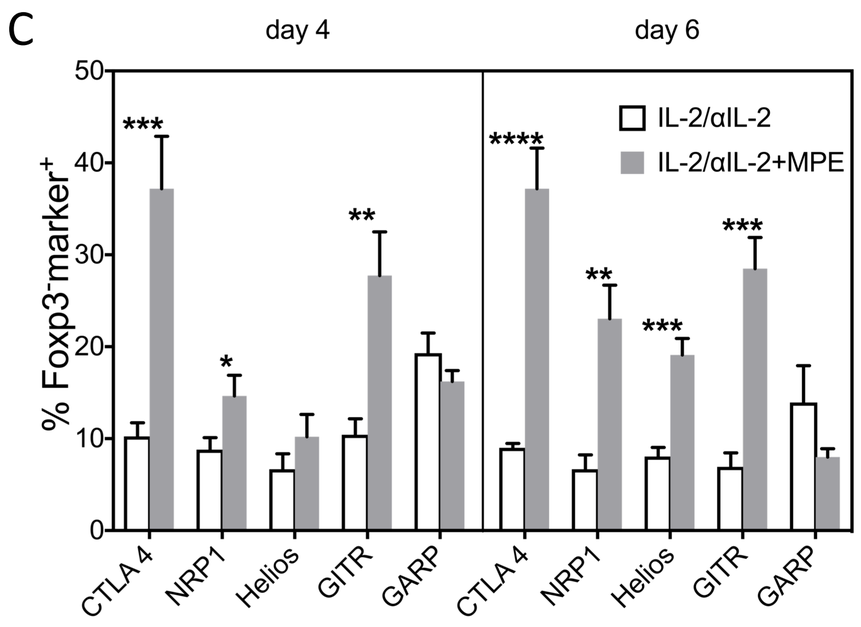


Supplemental figure 4

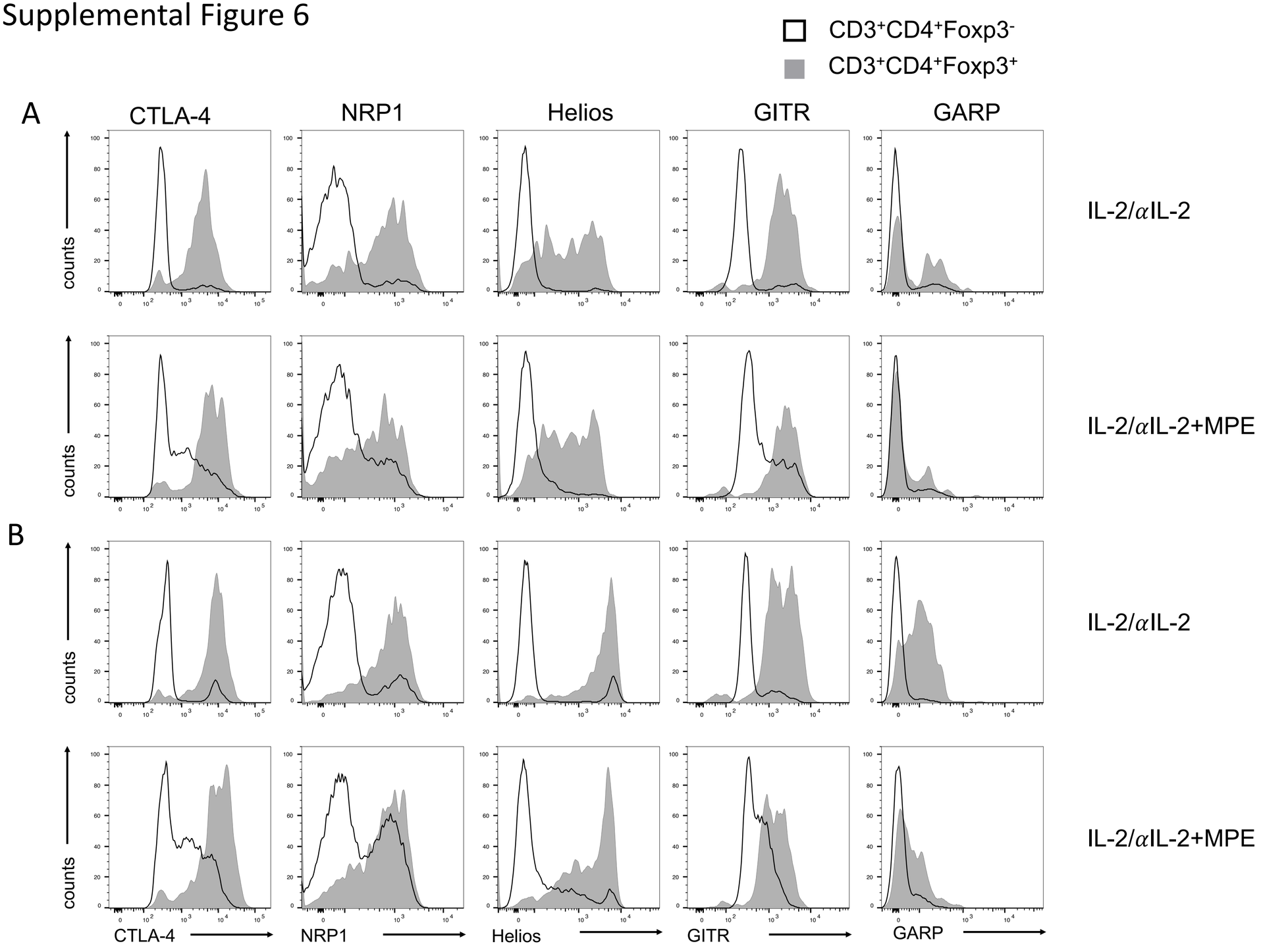


Supplemental Figure 5



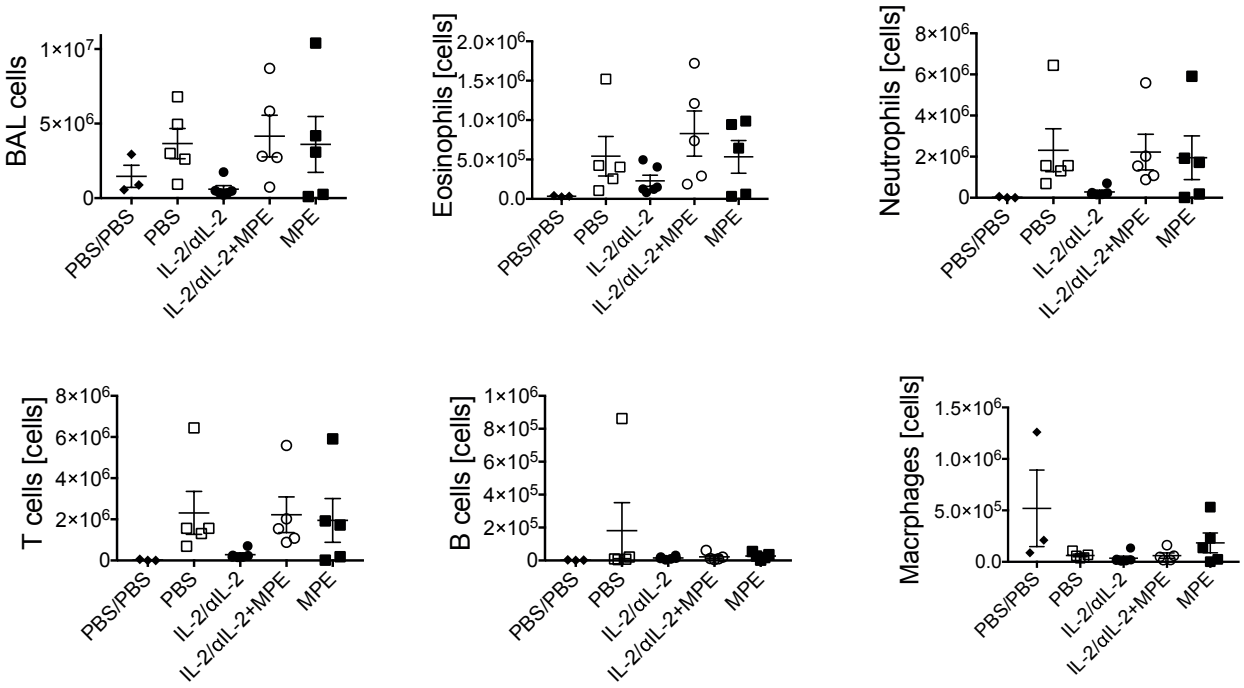


Supplemental Figure 6

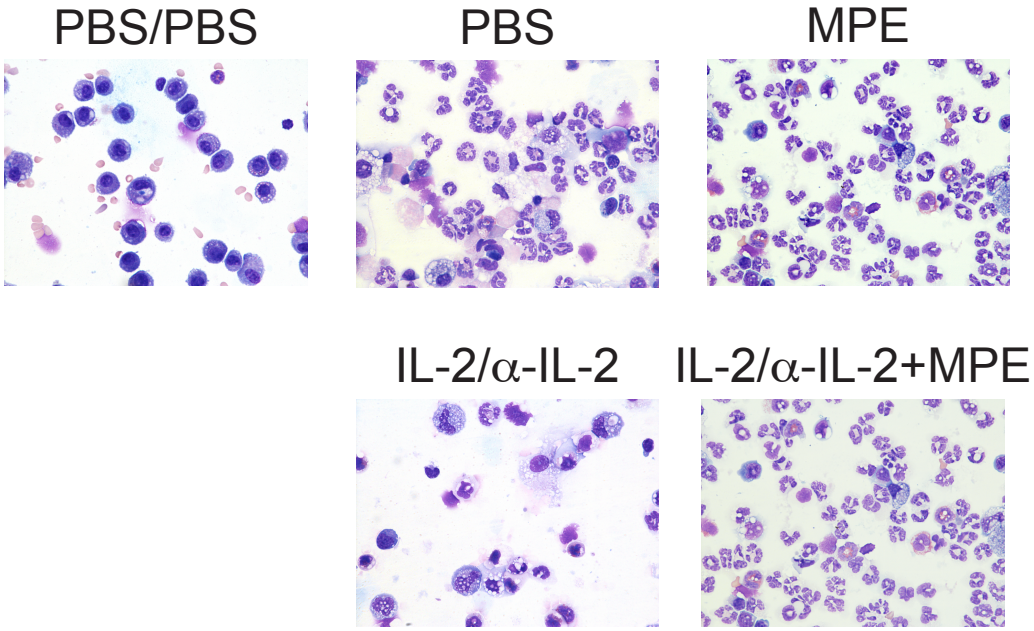


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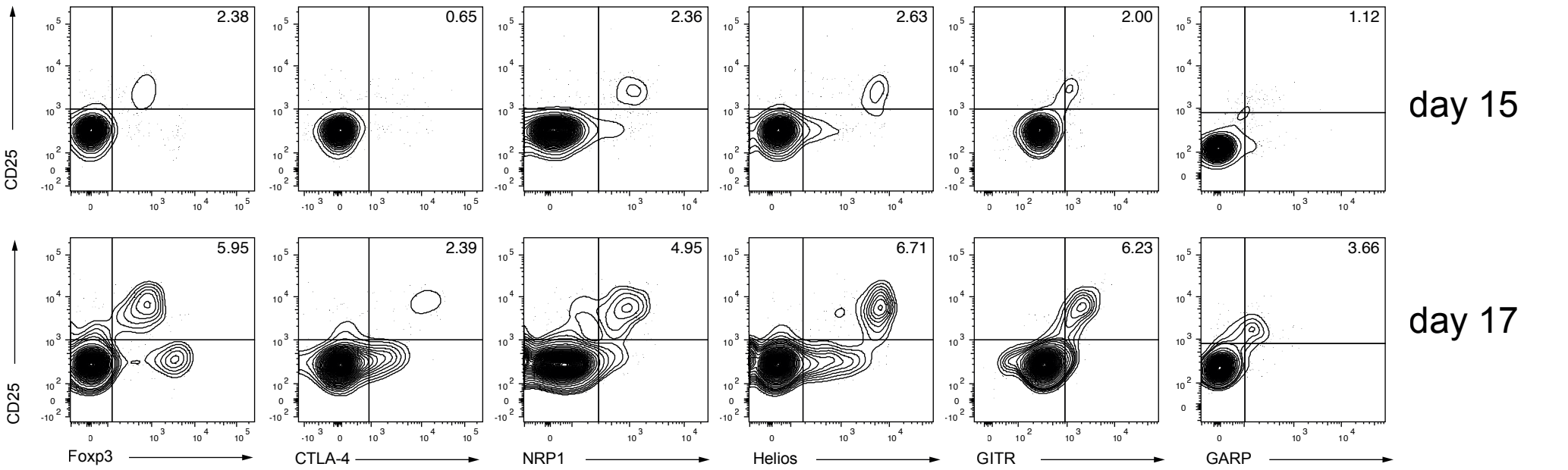
A



B



Supplemental figure 7



Supplemental Table 1. Comparisons of peak levels of $CD25^+$ and $Foxp3^+$ with baseline

Gated population	IL-2/αIL-2	IL-2/αIL-2+MPE
CD3+CD4+ $CD25^+$ (%)	5.0±1.4 vs 32.3±7.8* p<0.001	5.0±1.2 vs 34.0±9.7† p<0.001
CD3+CD4+$CD25^+Foxp3^+$(%)	2.7±1.3vs 11.8±2.9§ P<0.001	2.4±1.3 vs 11.7±2.8§ p<0.001
CD3+CD4+$CD25^+Foxp3^+$ (MFI Foxp3)	366±42 vs 569±173§ P<0.01	354±50 vs 549±262§ P<0.05
CD3+CD4+$CD25^+$ (MFI CD25)	1974±679 vs 3485±1213* p<0.05	1956±633 vs 5982±1249† p<0.001

*) day: 0 versus 5

†) day: 0 versus 4

§) day: 0 versus 6

Data show the percentage (± SD) of $CD3^+CD4^+$ cells co-expressing CD25 or Foxp3. Alternatively, the geometric mean fluorescence intensity (MFI) after the indicated days of initial (days 0-2) IL-2/αIL-2 treatment in the presence or absence of allergen application, compared to day 0 (baseline) are shown. Data were tested with an unpaired t -test for statistical significance, respective p-values are indicated.

Supplemental Table 2. Treg marker expression on $CD3^+CD4^+CD25^-$ Tconv cells

marker	day 4		day 6	
	IL-2/αIL-2+MPE vs IL-2/αIL-2		IL-2/αIL-2+MPE vs IL-2/αIL-2	
CTLA-4	26.3±10% vs 3.3±1.6%[†]	p<0.001	34.3±10.7% vs 6.0±1.4%	p<0.001
NRP1	7.3±2.7% vs 3.4±1.4%	p=0.01	21.5±9.4% vs 4.5±2.2%	p=0.001
Helios	6.6±2.6% vs 3.2±0.9%	p=0.01	16.8±3.7% vs 5.0±0.6%	p<0.001
GITR	10.5±4.0% vs 2.5±1.1%	p<0.001	11.7±6.1% vs 2.2±0.7%	p=0.004
GARP	5.2±3.2%vs 11.8±6.1%	p=0.04	13.4±7.0% vs 17.6±8.4%	p=0.4

[†] Data show percent ± SD of $CD3^+CD4^+CD25^-$ Tconv cells expressing the respective Treg marker molecule after the indicated days of initial (days 0-2) IL-2/αIL-2 treatment in the presence or absence of allergen application. Data were tested with an unpaired t -test test for statistical significance, respective p-values are indicated.

Supplemental Table 3. Treg marker expression on $CD3^+CD4^+Foxp3^-$ Tconv cells

marker	day 4		day 6	
	IL-2/αIL-2+MPE vs IL-2/αIL-2		IL-2/αIL-2+MPE vs IL-2/αIL-2	
CTLA-4	32.7±13.9% vs 10.2±3.6%[†]	p=0.001	37.2±10.8% vs 9.0±1.2%	p<0.001
NRP1	14.7±5.5% vs 8.8±3.2%	p=0.048	23.0±9.0% vs 6.7±3.8%	p=0.002
Helios	10.2±5.9% vs 6.7±4.1%	p=0.3	19.1±4.4% vs 8.0±2.5%	p<0.001
GITR	27.8±11.7% vs 10.4±4.2%	p=0.007	28.5±8.3% vs 7.0±3.7%	p<0.001
GARP	16.2±3.0% vs 19.3±5.3	p=0.24	8.0±2.2% vs 13.9±9.8%	p=0.18

[†] Data show percent ± SD of $CD3^+CD4^+Foxp3^-$ Tconv cells expressing the respective Treg marker molecule after the indicated days of initial (days 0-2) IL-2/αIL-2 treatment in the presence or absence of allergen application. Data were tested with an unpaired t-test for statistical significance, respective p-values are indicated.

Supplemental Table 4. Recall response after allergen challenge

Marker	day 15	day 17	fold-change
CD25	3.1±0.3[†]	6.2±2.7	2.0
	3.4±1.0	*4.1±1.3*	*1.2*
Foxp3	2.5±0.3	5.4±1.8	2.2
	2.9±0.9	*4.0±1.2*	*1.4*
CTLA-4	2.3±0.5	5.7±2.3	2.5
	2.8±1.0	*2.6±0.6*	*0.9*
NRP1	2.0±0.4	4.4±1.9	2.0
	2.4±0.8	*3.1±0.9*	*1.3*
Helios	2.6±0.4	6.1±2.0	2.3
	3.0±0.9	*4.0±0.9*	*1.4*
GITR	2.5±0.3	6.1±2.0	2.5
	3.0±0.8	*4.5±1.4*	*1.5*
GARP	4.1±2.3	3.0±1.2	0.7
	2.3±1.2	*3.5±2.6*	*1.6*

[†] Data show percent ± SD of $CD3^+CD4^+$ PB T cells expressing the respective Treg marker molecule after in vivo re-challenge, data of the PBS controls are shown in italics

Supplemental Table 5. Cytokine responses in PHA-restimulated lung single cell suspensions

Cytokine	PBS	IL-2/αIL-2	p-value
IL-2	45.2±15.4[†]	31.65±13.4	0.0204
IL-4	46.4±19.9	14.34±9.4	0.0452
IL-5	332±167.4	148.1±62.3	0.096
IL-10	123.8±21.3	57.39±12.9	<0.001
IL-13	404.4±219.6	127.2±73.2	<0.001
IFNγ	230.1±85.2	96.5±41.2	0.0312
IL-17	361.7±135.4	255.6±66-7	0.9056
GM-CSF	37.4±8.9	16.6±5.1	0.0384

[†] Data show cytokine levels in pg/ml ± SD. Lung cell suspensions of mice treated on days 0-2 with PBS (PBS) or IL-2/αIL-2 (IL-2/αIL-2) and i.n. challenged with MPE on days 13-15 followed by determination of AHR were restimulated on day 24 with PHA and cytokines determined after 72 hours. Data were tested by multiple T test with welch correction due to the differences in variance between the study groups and p-values were corrected according to Bonferoni's method for the number of comparisons.

Supplemental Table 6. List of monoclonal antibodies used for flow cytometry for analyses of cell surface antigens and transcription factors of peripheral blood leukocytes.

specificity	clone name	species	fluorophore	source
CD25	PC61	rat	FITC	BioLegend, San Diego, CA, USA
CD3	17A2	rat	BV785	BioLegend, San Diego, CA, USA
CD39	24DMS1	rat	Alexa Fluor 700	eBioscience, San Diego, CA, USA
CD4	GK1.5	rat	PerCP-eFluor 710	eBioscience, San Diego, CA, USA
CD4	GK1.5	rat	BV510	eBioscience, San Diego, CA, USA
CD4	17A2	rat	PE-Cy7	BioLegend, San Diego, CA, USA
CD49b	DX5	rat	BV510	BioLegend, San Diego, CA, USA
CD73	TY/11.8	rat	BV421	eBioscience, San Diego, CA, USA
CTLA-4	UC10-4B9	hamster	PE Texas Red	eBioscience, San Diego, CA, USA
Foxp3	FJK-16S	rat	APC	eBioscience, San Diego, CA, USA
Foxp3	FJK-16S	rat	PE	eBioscience, San Diego, CA, USA
GARP	YGIC86	rat	PE-Cy7	eBioscience, San Diego, CA, USA
GITR	DTA-1	rat	FITC	eBioscience, San Diego, CA, USA
Helios	22F6	hamster	PE	eBioscience, San Diego, CA, USA
IL12p35	4D10p35	rat	APC	eBioscience, San Diego, CA, USA
LAG3	C9B7W	rat	Apc-Cy7	eBioscience, San Diego, CA, USA
LAP	TW7-16B4	mouse	PerCP-Cy5.5	eBioscience, San Diego, CA, USA
NRP1	3DS304M	rat	PE Cy7	eBioscience, San Diego, CA, USA
PD-1	29F.1A12	rat	BV605	BioLegend, San Diego, CA, USA
TRBV18	BA62.6	mouse	PE	BC, Brea, CA, USA
CD3	17A	rat	FITC	BioLegend, San Diego, CA, USA
CD19	1D3	rat	PE	BD Pharmingen, Palo Alto, CA
CD11b	M1/70	rat	APC-Cy7	BioLegend, San Diego, CA, USA
CD11c	N418	hamster	PE-Cy7	BioLegend, San Diego, CA, USA
Siglec F	S17007L	rat	APC	BioLegend, San Diego, CA, USA
MHC II	M5/114.15.2	rat	BV650	BioLegend, San Diego, CA, USA
Ly6G	1A8	rat	BV605	BioLegend, San Diego, CA, USA

Table shows the specificity, clone names, species and supplier (source) of the respective monoclonal antibodies and their conjugation with the respective fluorophores used in this study.

Supplemental Table 7. List of primary and secondary antibodies used for multiplex-based cytokine determinations

specificity	clone name	species	source
IL-2	JES6-5H4 JES6-1A12	rat	eBioscience, SantaClara, CA, USA
IL-4	BVD6-24G2 11B11	rat	eBioscience, SantaClara, CA, USA
IL-5	TRFK4 TRFK5	rat	eBioscience, SantaClara, CA, USA
IL-10	JES5-2A5 JES5-16E3	rat	eBioscience, SantaClara, CA, USA
IL-13	eBio13A eBio13A	rat	eBioscience, SantaClara, CA, USA
IL-17	eBio17B7 eBio17CK15A5	rat	eBioscience, SantaClara, CA, USA
IFN-γ	R4-6A2 AN-18	rat	eBioscience, SantaClara, CA, USA
TNF-α	XT3/XT22 1F3F3D4	rat	eBioscience, SantaClara, CA, USA
GM-CSF	MP1-31G6 MP1-22E9	rat	eBioscience, SantaClara, CA, USA

Table shows the specificity, clone names, species and supplier (source) of the respective anti-cytokine antibodies for determination of cytokine levels in cell culture supernatants used in this study.

SUPPLEMENTAL INFORMATION FOR ONLINE SUPPLEMENT

Allergen alters IL-2/~IL-2-based Treg expansion but not tolerance induction in an allergen-specific mouse model

Running title: Treg expansion by IL-2/~IL-2 complexes.

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SUPPLEMENTAL MATERIALS AND METHODS

Tail vein sampling for whole blood

Heparinized venous whole blood sampled by tail vein incision, according to standardized procedures. To be able to measure single cells by flow cytometry, coagulation of the whole blood samples was performed by adding 10 µl of a heparin-solution (Natrium-Heparin, 5000I.E./mL, Gilvasan®) to the collection tubes. During blood sampling animals were fixed and the tail vein was carefully cut with a sterile razor blade. After collecting 3-4 drops of venous blood, the bleeding was stopped by compression of the incision site with a sterile swab.

Phenotypic characterization of PB Treg cells expanded in the presence of IL-2/αIL-2-complexes *in vivo*

Heparinized blood collected from individual mice and lung cell suspension were incubated for with 1 ml of a 1:10 diluted BD Pharm Lyse solution for 3 minutes, then the reaction was stopped with FACS buffer and cells were washed once by centrifugation at 500 g for 5 minutes. Cell surface antigens were stained according to standard procedures (46). Briefly, cell suspensions were incubated with 30 µl of the indicated antibodies (**Supplemental Table 6)** at RT in the dark for 30 minutes. Subsequently, cells were washed once with FACS buffer. Afterwards, cell suspensions were incubated on ice, in the dark with FIX solution (1:4) (eBioscience) for 30 minutes. Next, cell suspensions were washed once with Perm solution (1:10), incubated with the desired dilutions of antibodies recognizing intracellular antigens (diluted in Perm solution) on ice in the dark for 30 minutes. After an additional wash with Perm solution followed by one wash with FACS buffer stained cell suspensions were immediately subjected to flow cytometric acquisition. Data were recorded

exclusively from viable cells by using appropriate forward and side scatter gating. Analysis was performed on a Fortessa LSRII flow cytometer (Becton-Dickinson, Palo Alto, CA) followed by data analysis using the FlowJo v 9.3.3 software (Tree Star Inc, Ashland, OR).

Allergen challenge

TCR/DR1 mice, pre-treated with IL-2/αIL-2 complexes alone or in combination with mugwort pollen extract (MPE) and control mice pre-treated MPE or PBS were challenged i.n. either with 450 µg (1,5% (w/v) in PBS) or PBS (PBS/PBS group) on days 13, 14 and 15 after initial treatment. To facilitate administration of the extract, the mice were shortly anesthetized with isofluran (Sevofluran, AbbVie®).

Whole body plethysmography (WBP)

Lung function in the five groups of mice was analyzed upon challenge with 1% (w/v) mugwort-pollen extract solution by unrestrained whole body plethysmography (Buxco, Winchester, UK). Challenge consisted of an acclimation phase, a nebulization phase and a responding phase.

Analysis of bronchoalveolar lavage fluid (BALF) cells

BALF was collected on day 17 by flushing the airways with 1 ml of ice-cold PBS (without Ca2+ and Mg2+). Cells were collected by centrifugation at 500g for 5 min. Total leukocyte cell numbers were determined and cytospin preparations (Cytospin 4 centrifuge, Thermofisher) of BALF cells were stained using a modified Wright-Giemsa stain (HematTek Stain Pack, Siemens, Erlangen, Germany) on a Hematek slide stainer (Siemens). In parallel, BALF cells were stained with the mAb panel as described (**Supplemental Table 6**). To prevent non-specific binding to Fc receptors

the 2.4G2 blocking reagent (6 µg/ml, Becton Dickinson) was added to the mAb mix. The detailed cellular composition of BALF was determined on a Becton Dickinson Fortessa flow cytometer using FACS DIVA (Becton Dickinson) and FlowJo softwares (Treestar, Costa, Mesa, CA).

Isolation of lungs and preparation of single cell solutions

Lung homogenates were prepared according to standard procedures. Censored mice were disinfected by soaking them in 70% ethanol for one minute. Mouse lungs were obtained after placing them backsides onto a clean surface. A midline incision was performed to expose the lungs and the heart. For that purpose, the rib cage was cut and opened widely. Lungs, heart and thymus were pulled out of the thorax, the lungs were isolated accordingly. Subsequently, lungs were chopped into 5 mm pieces and incubated with digestion solution containing 1.8 µg/ml collagenase and 40 µg/ml DNase (Sigma Chemicals) at 37°C for 60 minutes. Then, cells were disaggregated and filtered through a 70 µm nylon cell strainer (Becton Dickinson). The filtrate was centrifuged at 500 x g at 4°C for 5 minutes. The cell pellet was re-suspended in ammonium-chloride lysis buffer, incubated at room temperature for 5 minutes, washed once in 1 x IMDM before subjection to functional analyses.

Cytokine measurements

Lung cell suspensions were restimulated with 0.15 µg/ml phytohaemagglutinin (PHA) or MPE (100 µg/ml) and cell culture supernatants were harvested 72 hours later for cytokine analyses using the Luminex system (Luminex 100IS, Biomedica, Vienna, Austria). **Supplemental Table 7** show the antibodies used to determine IL-2, IL-4, IL-10, IL-13, IL-17, IFN-γ, TNF- α and GM-CSF levels in cellular supernatants.

REFERENCES

1. Cossarizza A, Chang HD, Radbruch A, Akdis M, Andra I, Annunziato F, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies. Eur. J. Immunol. 2017;47(10):1584-1797.

FIGURE LEGENDS FOR ONLINE SUPPLEMENT

Figure S1. Mice were injected i.p on three consecutive days (days 0, 1 and 2) with IL-2 (1 µg) complexed to JES6-1 (5 µg) or IL-2/JES6-1 plus alum-adsorbed mugwort pollen allergen (MPE, 50 µg) and analyzed for the expression of Treg markers on peripheral blood $CD3^+CD4^+$ T cells from day 0-21. Control groups received PBS or alum-adsorbed allergen alone. Two weeks after the initial treatment, mice received three challenges with MPE extract intranasally (i.n.) on days 13-15 and subsequently airway reactivity (Penh) upon exposure to aerosolized MPE was assessed. On the day of the sacrifice (day 24), organs were isolated and immune cells stained for marker expression. Lung cells and splenocytes were restimulated in the presence of MPE and cellular proliferation and cytokine production were determined.

Figure S2. Co-expression of (**A**) CTLA-4, (**B**) GITR, (**C**) NRP1, (**D**) LAP1 on $CD3^+CD4^+CD25^+$ PB T cells. Shown are the percentages (mean±SEM) of $CD3^+CD4^+CD25^+marker^+$ PB T cells from days 0-21 derived from mice i.p. injected with IL-2/αIL-2 complexes in the absence (IL-2/αIL-2) or presence of alum-adsorbed MPE (IL-2/αIL-2+MPE). Control groups received PBS (PBS) or allergen (MPE) alone. Data are pooled from two independently performed experiments with n=6 for IL-2/αIL-2 and IL-2/αIL-2+MPE, n=5 for MPE and n=4 for PBS mice per group.

Figure S3. Co-expression of (**A**) Helios, (**B**) GARP, (**C**) CD73 on $CD3^+CD4^+CD25^+$ PB T cells. Shown are the percentages (mean±SEM) of $CD3^+CD4^+CD25^+marker^+$ PB T cells from days 0-21 derived from mice i.p. injected with IL-2/αIL-2 complexes in the absence (IL-2/αIL-2) or presence of alum-adsorbed MPE (IL-2/αIL-2+MPE). Control groups received PBS (PBS) or allergen (MPE) alone. Data are pooled from

two independently performed experiments with n=6 for IL-2/αIL-2 and IL-2/αIL-2+MPE, n=5 for MPE and n=4 for PBS mice per group.

Figure S4. Representative flow plots show CD25 versus CTLA4, NRP1, Helios, GITR, GARP, Foxp3, CD73 and LAP expression, respectively, on $CD3^+CD4^+$ PB T cells on day 4 (**A**) or day 6 (**B**) derived from mice i.p. injected with PBS or alum-adsorbed MPE. Data are representative of two independently performed experiments with n=6 mice per group.

Figure S5. Effects of IL-2/αIL-2 complexes and allergen co-exposure on Treg marker expression on $CD3^+CD4^+Foxp3^-$ and $Foxp3^+$ T cells. Changes in Treg marker expression of (**A**) day 4 and (**B**) day 6 $CD3^+CD4^+Foxp3^+$ cells. (**C**) Treg marker expression on $CD3^+CD4^+Foxp3^-$ T cells on day 4 and day 6. Shown are the percentages (mean±SEM) of $CD3^+CD4^+Foxp3^-$ cells expressing the respective markers as indicated. Data are pooled from two independently performed experiments with n=6 for IL-2/αIL-2 and IL-2/αIL-2+MPE mice per group. ****P*<0.01, ****P*<0.001, *****P* <0.0001 comparing IL-2/αIL-2 and IL-2/αIL-2+MPE treatment group (multiple t-tests assuming all rows are sampled from populations with same scatter. Holm-Sidak method was used to correct for multiple comparisons).

Figure S6. Effects of IL-2/αIL-2 complexes and allergen co-exposure on Treg marker expression on $CD3^+CD4^+Foxp3^-$ and $CD3^+CD4^+Foxp3^+$ T cells. Histogram overlays representing Treg marker expression on (**A**) day 4 and (**B**) day 6 derived from mice i.p. injected with IL-2/αIL-2 complexes in the absence (IL-2/αIL-2) or presence of alum-adsorbed MPE (IL-2/αIL-2+MPE). $CD3^+CD4^+Foxp3^-$ cells (open histograms);

$CD3^+CD4^+Foxp3^+$ T cells (grey histograms). Flow plots are representative of n=6 mice per group.

Figure S7. Recall Treg response in IL-2/αIL-2 complex-treated mice after allergen challenge**.** Shown are representative contour plots of PB (day 15 and day 17) of mice pre-treated with IL-2/αIL-2 complexes (i.p., days 0-2) and subsequently i.n. allergen-challenged with MPE (days 13-15). Numbers in the upper left quadrant indicate the fraction of $CD3^+CD4^+CD25^+marker^+$ T cells. Flow plots are representative of n=6 mice.

Figure S8. BAL cell analyses of differentially pre-treated mice**. (A)** Shown are absolute BAL cell numbers and numbers of eosinophils, neutrophils, T cells, B cells and macrophages of mice treated as indicated determined by flow cytometry. (**B)** Shown are representative cytospin preparations of the respective treatment groups. Shown is the summary (A) or representative cytospins (B) of 6 mice (except 5 for IL-2/aIL-2 plus MPE, MPE, PBS and 3 for PBS/PBS).