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This information is current as of October 14, 2016.

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J Immunol 2016; 197:1567-1576; Prepublished online 20 July 2016;
doi: 10.4049/jimmunol.1600508
<http://www.jimmunol.org/content/197/5/1567>

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In Vivo Expansion of Endogenous Regulatory T Cell Populations Induces Long-Term Suppression of Contact Hypersensitivity

Asmaa El Beidaq,* Christopher W. M. Link,* Katharina Hofmann,* Britta Frehse,* Karin Hartmann,[†] Katja Bieber,[‡] Stefan F. Martin,[§] Ralf J. Ludwig,^{†,‡} and Rudolf A. Manz*

Contact hypersensitivity (CHS) of murine skin serves as a model of allergic contact dermatitis. Hapten-specific CD8 T cells and neutrophils represent the major effector cells driving this inflammatory reaction whereas Foxp3⁺ regulatory T cells (Tregs) control the severity of inflammation. However, whether in vivo expansion of endogenous Tregs can downregulate CHS-mediated inflammation remains to be elucidated. In this study, we addressed this issue by using injection of an IL-2/anti-IL-2 mAb JES6-1 complex (IL-2/JES6-1) as a means of Treg induction in 2,4,6-trinitrochlorobenzene-induced CHS. IL-2/JES6-1 injection before or after hapten sensitization led to a considerable reduction of skin inflammation, even when rechallenged up to 3 wk after the last treatment. Conversely, Treg depletion re-established the CHS response in IL-2/JES6-1-treated mice. IL-2/JES6-1 injection resulted in increased frequencies of natural and peripheral Tregs in spleen and draining lymph nodes (LNs), elevated IL-10 and TGF- β production by CD4 T cells, reduced CD86 expression by dendritic cells, and led to lower numbers of hapten-specific IFN- γ -producing CD8 T effector cells in LNs. Neutrophil and CD8 T cell infiltration was reduced in inflamed ear tissue, whereas CTLA-4⁺Foxp3⁺ Treg frequencies were augmented. Adoptive transfer of LN cells of sensitized mice into recipients treated with IL-2/JES6-1 showed impaired CHS. Our results show that in vivo Treg expansion results in a prolonged CHS suppression, a sustained reduction of hapten-specific CD8 T cells, and a decrease in effector cell influx in inflamed tissue. *The Journal of Immunology*, 2016, 197: 1567–1576.

Allergic contact dermatitis is characterized by cutaneous lesions in response to repeated contact with chemicals or metal ions (1). The prevalence of allergic contact dermatitis is ascending worldwide, with 15–20% of the general population exhibiting this allergic disorder, rendering it a significant challenge to public health systems (2). Prevention of exposure to contact sensitizers and topical corticosteroid application remain the main treatment strategies currently used.

Contact hypersensitivity (CHS) refers to the experimental animal models of allergic contact dermatitis induced by sensitization and subsequent challenge to a specific hapten resulting in skin inflammation. CD8 T cells are the main effector cells in CHS, infiltrating inflamed skin tissue and leading to further recruitment of other leukocytes (3). In vivo depletion of CD8 T cells results in absence or decrease in CHS response (4, 5). Prototypically, CHS to 2,4,6-trinitrochlorobenzene (TNCB) triggers a Tc1-mediated allergy associated with the expansion of hapten-specific IFN- γ -producing CD8 T cells (6). Recent studies have shown that neutrophils are also critical effector cells involved in both sensitization and challenge phases (7, 8). It is suggested that neutrophils modulate CD8 T cell infiltration into inflamed tissue (9). Foxp3⁺ regulatory T cells (Tregs) are important to control inflammatory reactions (10), including CHS (11, 12). Adoptive transfer of Tregs before skin challenge leads to CHS suppression and prevention of effector cell infiltration into the inflamed tissue (13). Tregs can suppress CHS through multiple mechanisms (14–17).

IL-2 plays an important role in T cell growth, activation, and differentiation. In contrast to other T cell populations, Tregs constitutively express high levels of CD25, the IL-2 receptor α subunit (18). In vivo administration of exogenous IL-2 complexed with mAbs of the anti-IL-2 clone JES6-1 induces a systemic and selective expansion of Foxp3⁺ Tregs (19, 20). Treatment with IL-2/JES6-1 was shown to suppress different inflammatory diseases (21–24). Moreover, expansion of Tregs using IL-2/JES6-1 resulted in long-term allograft survival in mice (20).

Two distinct Treg subpopulations exist: natural Tregs (nTregs) that are generated in the thymus and are present without exposure to foreign Ags, and peripheral Tregs (pTregs), which are formed after Ag contact in the periphery. Previous studies have shown that

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Received for publication March 23, 2016. Accepted for publication June 19, 2016.

This work was supported by the priority program of the University of Lübeck SPP-MIA. A.E.B., B.F., and R.A.M. were supported by the Deutsche Forschungsgemeinschaft-funded Excellence Cluster Inflammation at Interfaces. C.W.M.L. was supported by the Deutsche Forschungsgemeinschaft-funded International Research Training Group 1911 (IRTG 1911), and K. Hofmann was supported by the Deutsche Forschungsgemeinschaft-funded graduate program Research Training Group 1727 (GRK 1727).

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The online version of this article contains supplemental material.

Abbreviations used in this article: CHS, contact hypersensitivity; DC, dendritic cell; DERE, depletion of regulatory T cells; DT, diphtheria toxin; LN, lymph node; Nrp-1, neuropilin-1; nTreg, natural Treg; pTreg, peripheral Treg; TNCB, 2,4,6-trinitrochlorobenzene; Treg, regulatory T cell.

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Helios and Neuropilin-1 (Nrp-1) represent useful markers to distinguish nTregs from pTregs (25–27). Whether IL-2/JES6-1 complexes induce nTregs or pTregs or both has not been addressed so far.

In this study, we show that treatment with IL-2/JES6-1 before sensitization or before challenge led to a considerable increase of both nTreg and pTreg populations and efficiently suppressed CHS inflammatory responses. This suppression was accompanied by a significant reduction of CD8 T cell and neutrophil infiltration in the inflamed skin. As shown in depletion of Treg (DEREG) mice (28), Tregs were required for the IL-2/JES6-1-mediated suppression of CHS. Adoptive transfer experiments indicate that IL-2/JES6-1 treatment inhibits both allergic sensitization and CHS effector phase. Additionally, *in vivo* Treg expansion resulted in a decrease of hapten-specific IFN- γ ⁺ CD8 T cells in draining lymph nodes (LNs). Notably, the impairment of CHS-mediated inflammation was still detectable during a subsequent challenge 3 wk after IL-2/JES6-1 treatment, and it was not limited to the original site of inflammation.

Materials and Methods

Mice

Female C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and maintained under pathogen-free conditions. SCID beige mice and DEREG mice (28) were bred at the animal facility of the University of Lübeck. All animal studies were performed with approval from the respective authorities (Ministry of Energy, Agriculture, the Environment, and Rural Areas).

IL-2/JES6-1 complex treatment

For each injection, 1 μ g of recombinant mouse IL-2 (ImmunoTools) was complexed with 5 μ g of anti-IL-2 mAb (clone JES6-1A12, purified from hybridomas). The mixture was diluted in sterile PBS and incubated for 30 min at 37°C for i.p. delivery according to the indicated schedules.

Diphtheria toxin treatment

For Treg depletion, DEREG mice or WT controls were injected i.p. with 1 μ g of diphtheria toxin (DT; Sigma-Aldrich) in PBS 1 d before sensitization.

CHS induction

At day 0, mice were sensitized on the shaved abdomen with 100 μ l of 3% TNCB (Sigma-Aldrich, St. Louis, MO) dissolved in acetone. At day 5, ear thickness of both ears was measured using a micrometer (Mitutoyo, Leonberg, Germany), and the right ear was challenged with 20 μ l of 1% TNCB/acetone whereas the left ear was painted with acetone only and used as control. Ear swelling reaction was measured 24 h later or at the indicated time points after challenge. In the two challenges experiments, the right or left ear was painted with 20 μ l of 1% TNCB/acetone 12 d after first challenge, and ear swelling was tracked for the following 5 d. For the induction of CHS to oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one), mice were sensitized on the shaved back with 150 μ l of 3% oxazolone in ethanol 10 d before the first IL-2/JES6-1 injection.

Skin irritation

To induce Ag-nonspecific skin inflammation, 1% croton oil dissolved in 70% acetone (20 μ l) was applied onto both sides of the right ear of SCID beige mice. Twenty-four hours later, ear thickness was measured.

Adoptive transfer

Mice were treated with either IL-2/JES6-1 or PBS on days –5, –4, and –3 and sensitized to TNCB on day 0. Five days later, cell suspensions were prepared from draining LNs and injected i.v. (2×10^7 cells/mouse) into recipient mice, which were challenged 1 h later.

Hapten-specific CD8 T cell detection

Auricular and axillary LNs were isolated at indicated time points and single-cell suspension was prepared, washed, and resuspended in RPMI 1640 media supplemented with 10% FCS, 1% penicillin-streptomycin, and 0.1% 2-ME. Naive splenic cells were used as source for APCs, treated with mitomycin C for 30 min, washed and modified with 3 mM

2,4,6-trinitrobenzenesulfonic acid (Sigma-Aldrich) for 7 min at 37°C, and resuspended in the same media. In a 48-well plate, 10^7 LN cells were mixed with 10^7 2,4,6-trinitrobenzenesulfonic acid-modified splenic cells and incubated at 37°C for a total of 5 h. Brefeldin A was added for the last 4 h. Cells were harvested and stained for IFN- γ .

Cell isolation from ear tissue

The challenged ear was split into dorsal and ventral halves using forceps, chopped into small pieces, and incubated in 1.2 mg/ml Dispase (Roche) for 1 h at 37°C. Ear skin pieces were then digested using 1 mg/ml collagenase A and 60 μ g/ml DNase I (Roche) for 1 h at 37°C. Tissues were passed through a 70- μ m cell strainer and washed in PBS-BSA (0.5%). Isolated cells were surface stained for flow cytometry.

H&E staining

Ear tissue samples were paraffin embedded and immediately frozen using liquid nitrogen. Five-micrometer tissue sections were prepared and stained with H&E.

In vivo migration of dendritic cells

Mice were sensitized by applying 20 μ l of 0.5% FITC (Sigma-Aldrich) dissolved in acetone/dibutyl phthalate (1:1) on both sides of the ear. Auricular LNs were removed and FITC⁺CD11c⁺ cells were assessed using flow cytometry.

Flow cytometry

Spleen, auricular, and axillary LNs were harvested, and single-cell suspensions were prepared and filtered through a 70- μ m cell strainer, washed, and resuspended in buffer with Fc γ receptor blocking Abs for 5 min. For surface staining, eFluor 450-labeled anti-CD4 (RM4-5; eBioscience), Alexa Fluor 700-conjugated anti-CD8 (53-6.7; in-house production), Alexa Fluor 700-conjugated anti-CD11b (M1/70; in-house production), allophycocyanin-conjugated anti-Ly6G (1A8; BioLegend), PE-Cy7-conjugated anti-Nrp1 (3E12; BioLegend), PerCP-eFluor 710-conjugated anti-LAP1 (TW7-16B4; eBioscience), and anti-CXCR4 BV421 (L276F12; BioLegend) Abs were used. For intracellular cytokine staining, cells were fixed and permeabilized (Fix/Perm kit; BioLegend) and stained with FITC-conjugated anti-IL-10 (JES5-16E3; BioLegend) and allophycocyanin-conjugated anti-IFN- γ (XMGI.2; BioLegend). Intracellular staining with PE-conjugated anti-Foxp3 (FJK-16s; eBioscience), allophycocyanin-conjugated CTLA-4 (UC10-4B9; BioLegend), and Helios-Alexa Fluor 488 (22F6; BioLegend) was performed with a Foxp3 Fix/Perm buffer set (BioLegend) according to the manufacturer's protocol. Stained cells were detected in the BD LSR II flow cytometer and analyzed using FlowJo software version 10 (Tree Star).

Statistical analysis

Data analysis was performed using an unpaired *t* test in GraphPad Prism 6 software. A *p* value <0.05 was considered significant.

Results

IL-2/JES6-1 treatment before sensitization to TNCB expands both nTregs and pTregs and efficiently suppresses CHS

Depletion of Tregs results in enhanced and sustained ear swelling following CHS induction, suggesting that these cells exhibit the capacity to suppress CHS-mediated inflammation (29). In this study, we first tested the effect of Treg expansion by means of treatment with IL-2/JES6-1 immune complexes on the sensitization phase. To reach maximal Treg induction at the time of sensitization, mice were treated with IL-2/JES6-1 before sensitization to TNCB. Later, this treatment regimen is referred to as IL-2/JES6-1 (A) (Fig. 1A). Foxp3⁺ Treg frequencies and numbers were increased in spleen and LNs on day 0 in IL-2/JES6-1-treated mice compared with PBS-treated controls (Fig. 1B, 1C). This expansion was no longer observed on day 6, which is in accordance with previous findings showing that IL-2/JES6-1 treatment induces a rapid and systemic, but only transient, Treg expansion that vanishes within few days (20). However, it had remained unclear whether this treatment selectively affects nTregs or pTregs or both. To address this issue, Treg subsets were distinguished by Helios and Nrp-1

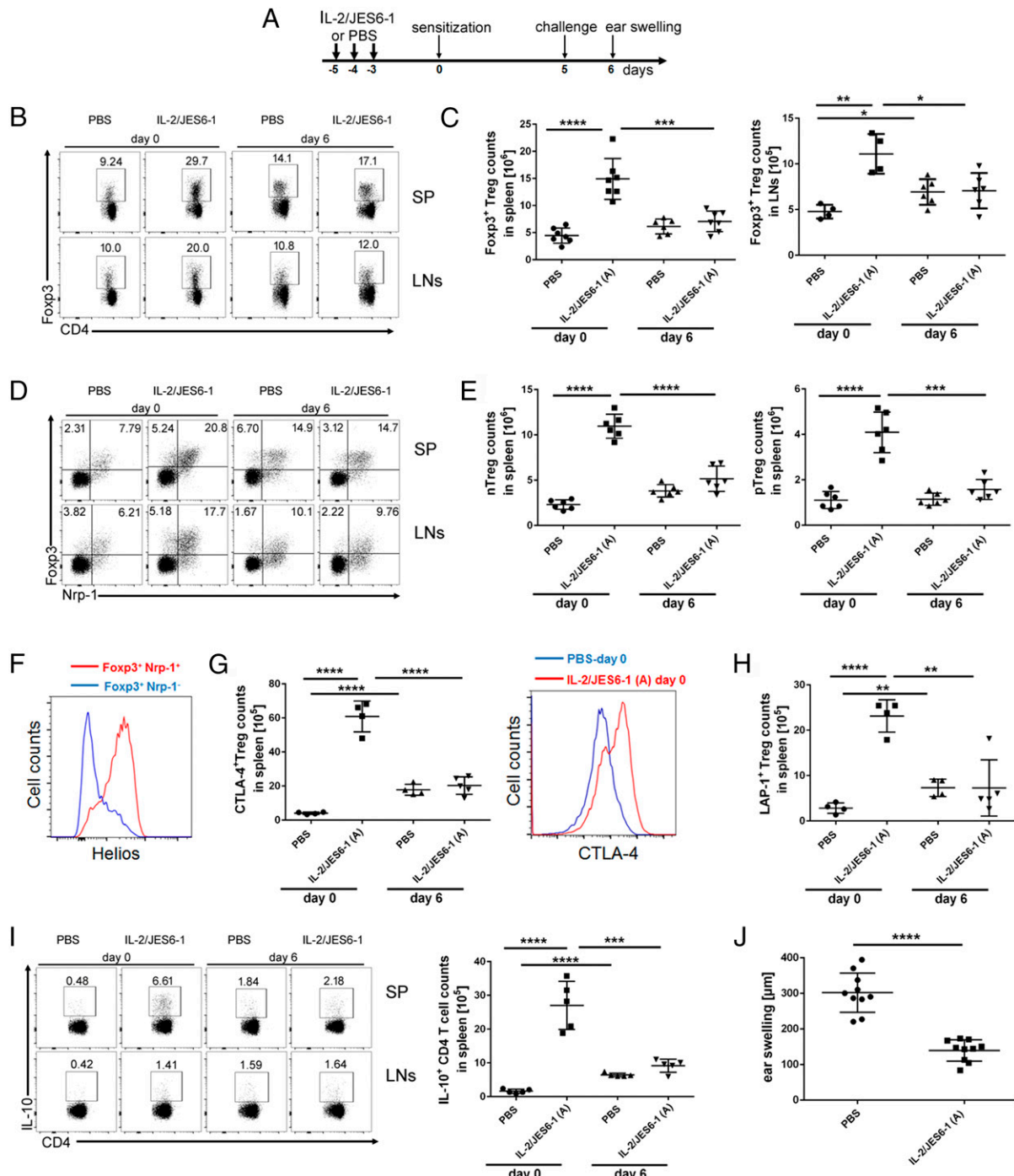


FIGURE 1. IL-2/JES6-1 treatment before sensitization leads to nTreg and pTreg induction and suppression of CHS. **(A)** Mice received injections of IL-2/JES6-1 and were sensitized and challenged with TNCB as indicated in the scheme. **(B)** and **(C)** FACS analysis and numbers of Foxp3⁺ Tregs at days 0 and 6 after sensitization [IL-2/JES6-1 **(A)** indicates IL-2/JES6-1 treated before sensitization; PBS indicates control group]. **(D)** and **(E)** Frequency and cell counts of nTregs and pTregs in spleen and LNs, nTregs, and pTregs were identified as Foxp3⁺Nrp-1⁺ T cells and Foxp3⁺Nrp-1⁻ T cells, respectively. **(F)** Helios expression by Foxp3⁺Nrp-1⁺ and Foxp3⁺Nrp-1⁻ Tregs. **(G)** CTLA-4 expression by Foxp3⁺ Tregs and their numbers in spleen. **(H)** Cell counts of TGF-β⁺ (LAP-1⁺) Tregs in spleen. **(I)** Intracellular staining for IL-10 expression by CD4⁺ T cells and absolute numbers of IL-10⁺ CD4⁺ T cells. **(J)** Mean of ear swelling 24 h after challenge. All representative flow cytometry dot plots are gated on CD4⁺ T cells. Data are shown as means ± SD and are compiled from at least two independent experiments ($n = 4$ –5 mice per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

expression (25–27). Both nTregs and pTregs were expanded in spleen and LNs upon IL-2/JES6-1 treatment (Fig. 1D, 1E). Nrp-1 expression by nTregs correlated with Helios expression (Fig. 1F). Furthermore, IL-2/JES6-1 treatment led to upregulation of CTLA-4 and TGF-β in Foxp3⁺ Tregs (Fig. 1G, 1H) and increased percentage and absolute number of IL-10-expressing CD4⁺ T cells, particularly in spleen (Fig. 1I). Finally, a 60–70% reduction of

ear swelling was observed in mice treated with IL-2/JES6-1 (Fig. 1J).

Treg expansion during the priming but before the challenge phase results in CHS suppression

We next sought to examine the impact of the IL-2/JES6-1 treatment on Tregs and CHS when injected during days 0–3 following

priming (Fig. 2A). This treatment regimen leads to maximal Treg expansion at the time of the challenge (20), but it may also affect the priming. This treatment regimen is referred to as IL-2/JES6-1 (B). Again, the ear swelling was reduced in the IL-2/JES6-1 group (Fig. 2B). nTreg and pTreg numbers were still increased at day 6 upon IL-2/JES6-1 treatment (Fig. 2C). Likewise, IL-10⁺ CD4 T cell numbers were increased (Fig. 2D). There was no significant difference in TGF- β ⁺ Treg counts between IL-2/JES6-1 (B) and PBS-treated groups (Fig. 2E), with those cells mainly resembling nTregs (Supplemental Fig. 1A). CTLA-4 expression was also up-regulated on Tregs in IL-2/JES6-1-treated mice (Fig. 2F). Interestingly, CTLA-4⁺ Tregs observed following IL-2/JES6-1 treatment were mostly pTregs (Supplemental Fig. 1B).

Reduced neutrophil and CD8 T cell infiltration into the inflamed tissue and increased Treg frequencies upon IL-2/JES6-1 treatment

Histological analysis of ear sections showed a general decrease in cell infiltration in groups treated with IL-2/JES6-1 either before sensitization or before challenge compared with the PBS controls (Fig. 3A), these results of which confirm the anti-inflammatory effects of the IL-2/JES6-1 treatment. In addition to CD8 T cells, neutrophils represent potent effector cells during both sensitization and challenge in CHS (7, 8). To test the effect of IL-2/JES6-1 treatment on neutrophil and CD8 T cell infiltration, we analyzed neutrophils and CD8 T cells in ear tissue after challenge. Groups treated with IL-2/JES6-1 showed reduced neutrophil frequencies

24 h after challenge (Fig. 3B). CHS induction as such did not alter neutrophil frequencies in blood (data not shown). However, when mice were treated before sensitization with IL-2/JES6-1, neutrophil frequencies were reduced in this tissue. Interestingly, the remaining neutrophils showed increased CXCR4 expression (Supplemental Fig. 2A, 2B). This chemokine receptor mediates neutrophil retention in the bone marrow (30). In ears, Foxp3⁺ Tregs were increased in both groups that received IL-2/JES6-1 treatment (Fig. 3C, 3D), whereas CD8 T cell frequencies were reduced (Fig. 3E). These data show that IL-2/JES6-1 treatment affects the accumulation of effector cells, but they support the presence of suppressor cells in inflamed tissue. To test whether IL-2/JES6-1 can affect neutrophils directly, or via T cells, lymphocyte-deficient SCID mice were used. In the absence of lymphocytes, induction of nonspecific skin irritation by croton oil showed similar neutrophil infiltration in both IL-2/JES6-1-treated mice and PBS controls (Fig. 3F). Ear swelling was also similar between these two groups (Supplemental Fig. 2C), indicating that the inhibitory effect of IL-2/JES6-1 on neutrophil recruitment depends on the presence of lymphocytes.

Dendritic cells (DCs) are responsible for hapten uptake, transport, and T cell activation in LNs. To examine the effect of IL-2/JES6-1 on Ag transport by DCs during the sensitization phase, ear skin was sensitized by FITC and auricular LNs were analyzed. Flow cytometric analysis showed that FITC⁺CD11c⁺ DC cell frequencies were not altered by IL-2/JES6-1 treatment either at 24 or 48 h after FITC sensitization (Supplemental Fig. 2D). However, CD86 expression

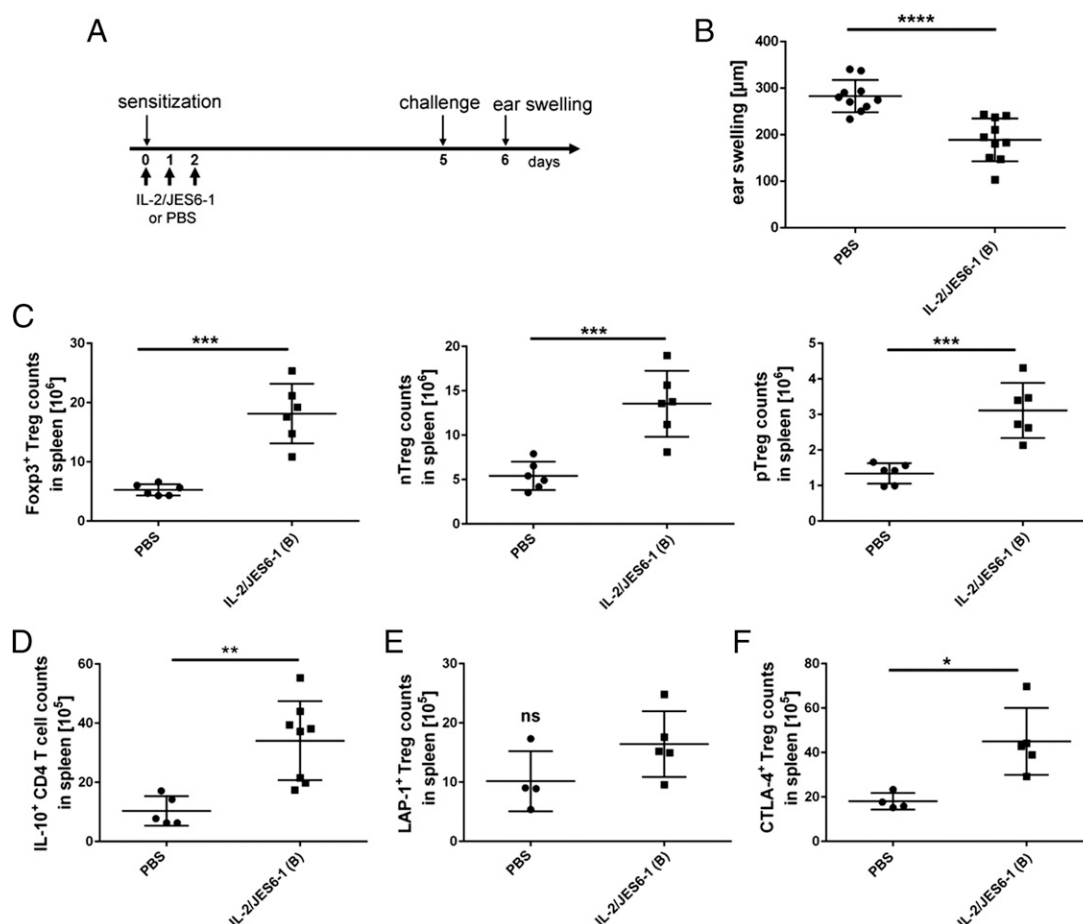


FIGURE 2. IL-2/JES6-1 treatment during sensitization but before challenge efficiently suppresses CHS. (A) Scheme of treatment schedule. (B) Mean of ear swelling 24 h after challenge. (C) Foxp3⁺ Treg numbers on day 6. (D) IL-10⁺ CD4 T cell counts in spleen on day 6. (E) TGF- β ⁺ Treg numbers. (F) Cell numbers of CTLA-4⁺ Tregs in spleen on day 6. Data are shown as means $\pm$ SD and are compiled from at least two independent experiments ($n = 4$ –5 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

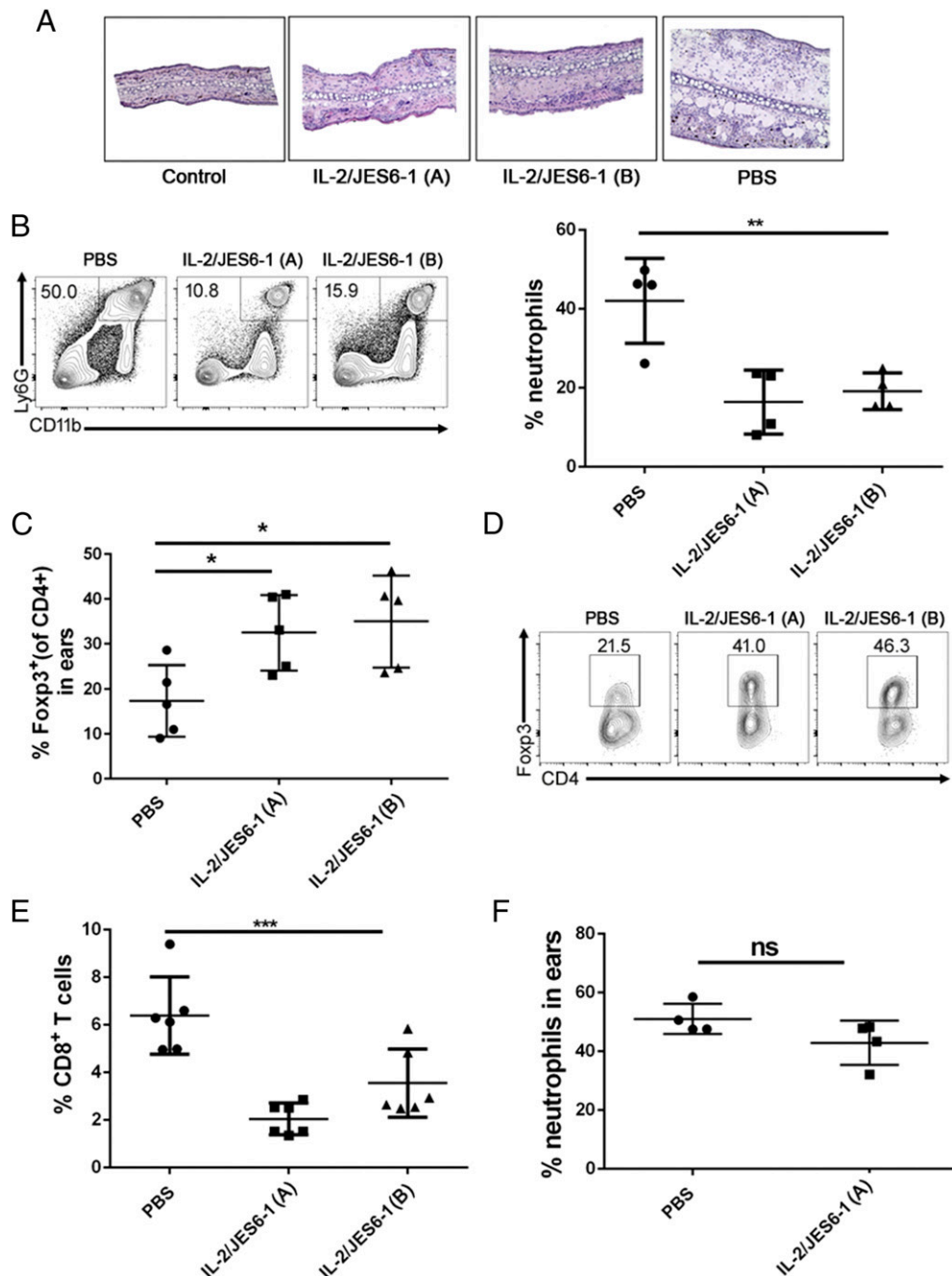


FIGURE 3. Reduced effector cell and increased Treg frequencies in inflamed ear tissue upon IL-2/JES6-1 treatment. Tissue sections were prepared from inflamed ears. Single cells were isolated and analyzed by flow cytometry. **(A)** Representative histological sections of 5 μ m after H&E staining. Original magnification $\times 200$. **(B)** Frequency of neutrophils in inflamed ear 24 h after challenge (gated on single cells). **(C and D)** Frequency of Foxp3⁺ Tregs in ear tissue (gated on CD4⁺ T cells). **(E)** CD8⁺ T cell frequency in inflamed ear tissue. **(F)** Neutrophil frequencies in irritated ear skin of SCID mice. Results are shown as mean $\pm$ SD and representative of one out of two independent experiments ($n = 4$ –6 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

by FITC-bearing DCs was downregulated 48 h after sensitization in IL-2/JES6-1-treated mice, whereas CD80 and CD83 expression remained similar between the two groups (Supplemental Fig. 2E and data not shown). These data suggest that IL-2/JES6-1 treatment does not affect DC migration but modulates DC maturation, which is important for T cell priming in the LNs.

Foxp3⁺ Tregs mediate the IL-2/JES6-1-induced CHS suppression

To assess the role of Foxp3⁺ Tregs in TNCB-induced CHS suppression after IL-2/JES6-1 treatment, DREG mice were analyzed. These mice express a DT receptor under the control of the

Foxp3 promoter, allowing effective and selective Treg depletion (28). DREG mice were treated with IL-2/JES6-1 before sensitization, and Foxp3⁺ Tregs were depleted 1 d before sensitization (Fig. 4A). Shortly after DT injection, Tregs were depleted from blood (Supplemental Fig. 3A). Treg frequencies were still reduced even 7 d later in LNs (Fig. 4B). The CHS response was completely restored in Foxp3⁺ Treg-depleted mice, neutrophil infiltration into the inflamed ear tissue was similar to PBS-treated controls, and Treg frequencies were decreased (Fig. 4C, 4D). Interestingly, all Foxp3⁺ Tregs found in the inflamed ear tissue were CTLA-4⁺ (Supplemental Fig. 3B). This molecule has been shown to be very important for Treg-mediated suppression of effector T cells (31).

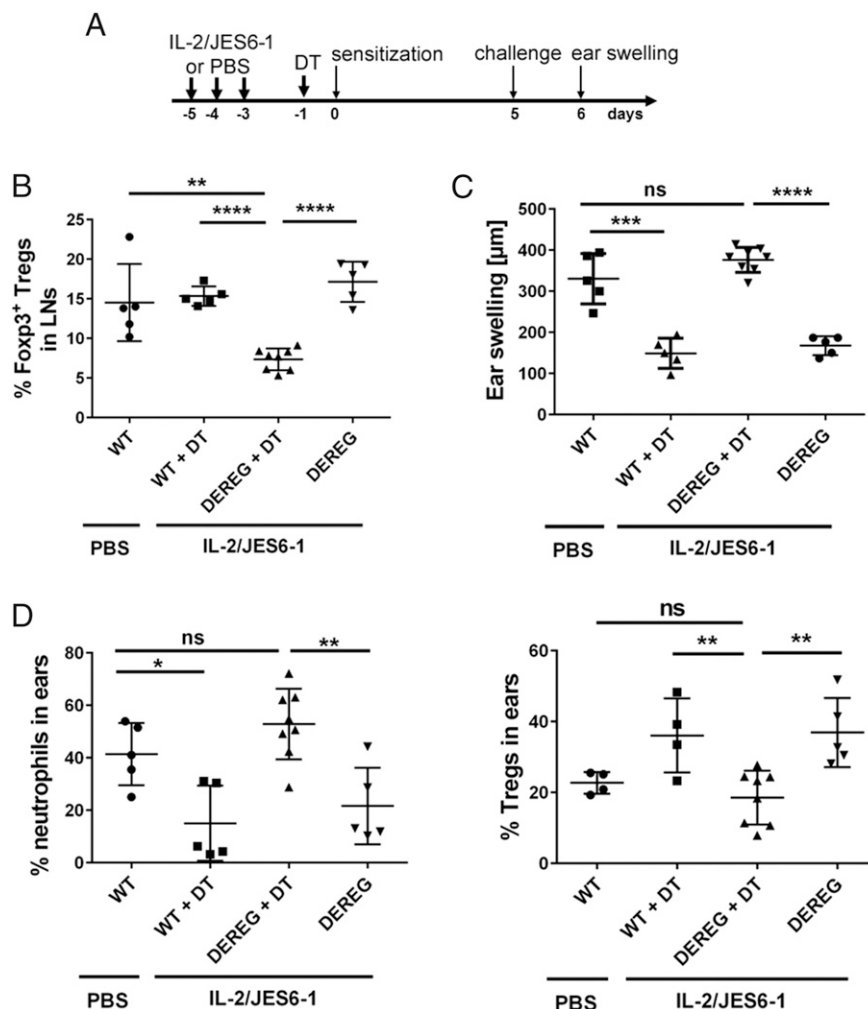


FIGURE 4. Treg depletion restores the CHS response. **(A)** DEREG mice were treated as indicated in the scheme. **(B)** Frequency of Foxp3^+ Tregs in LNs on day 6. **(C)** Mean of ear swelling 24 h after challenge. **(D)** Frequencies of neutrophils and Foxp3^+ Tregs in inflamed ear tissue on day 6. Data are depicted as mean $\pm$ SD ($n = 5$ –8 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

These observations demonstrate that Tregs are essential for IL-2/JES6-1-mediated suppression of CHS.

IL-2/JES6-1 treatment has multiple effects on CHS development

To further investigate whether IL-2/JES6-1 can restrain the priming of effector cells in LNs during sensitization and/or the effector functions after challenge, adoptive transfer of LN cells from sensitized mice was used as a passive model for CHS. Donor mice were treated with IL-2/JES6-1 or PBS before sensitization to TNCB, and 5 d later skin-draining LN cells were isolated and subsequently injected i.v. into recipient mice. Before challenge, recipients were either treated with IL-2/JES6-1 or PBS. Ear swelling measurement revealed a reduction in the CHS response in mice that received LN cells from IL-2/JES6-1-treated donors. CHS was also suppressed in recipients treated with IL-2/JES6-1 before challenge (Fig. 5). These results show that Foxp3^+ Treg expansion via IL-2/JES6-1 treatment can suppress CHS reactions through two mechanisms: inhibition of the effector cell priming during sensitization, and suppression of the effector phase after challenge.

CHS suppression by Foxp3^+ Treg expansion is long-lasting and not restricted to the inflamed tissue

Next, the duration of the immunosuppressive effect of IL-2/JES6-1 treatment was investigated. Mice were treated with IL-2/JES6-1 either before sensitization or before challenge and CHS was induced. PBS groups received three injections on days -5 , -4 , and -3 . Once the ear swelling was resolved 12 d after the first challenge, the

same ear received a second challenge (Fig. 6A). In control groups, the second challenge induced a more pronounced CHS than after the first challenge. Groups treated with IL-2/JES6-1 showed impaired CHS compared with PBS-treated controls for at least 5 d after the second challenge, that is, 22 d after the last injection of IL-2/JES6-1 (Fig. 6B). Hence, the therapeutic effect of the IL-2/JES6-1-expanded Tregs is not limited to the acute treatment period, but it can last for weeks, at least.

To address whether this long-lasting effect is limited to the site of original inflammation, in the next experiments the first challenge was performed on the right ear whereas the second challenge was done on the left ear, which was not challenged before. As indicated by the time course of the ear swelling, IL-2/JES6-1 also suppressed the inflammation on the ear that had not previously yielded an inflammatory response (Fig. 6C).

To determine whether IL-2/JES6-1 treatment during sensitization to TNCB will also suppress CHS to a different allergen, at day -15 mice were first sensitized to oxazolone, and 15 d later to TNCB. At days -5 , -4 , and -3 , that is, when sensitization to oxazolone was already established, but before sensitization to TNCB, mice were treated with IL-2/JES6-1. According to the results described above, this treatment regimen leads to the maximum expansion of Treg populations at the time of the TNCB sensitization. Subsequently, the right ear was challenged with TNCB and the left with oxazolone. Once the ear swelling was resolved 12 d after these first challenges, each ear received a second challenge with the same Ag. Notably, CHS response to TNCB was suppressed after the second challenge, whereas there

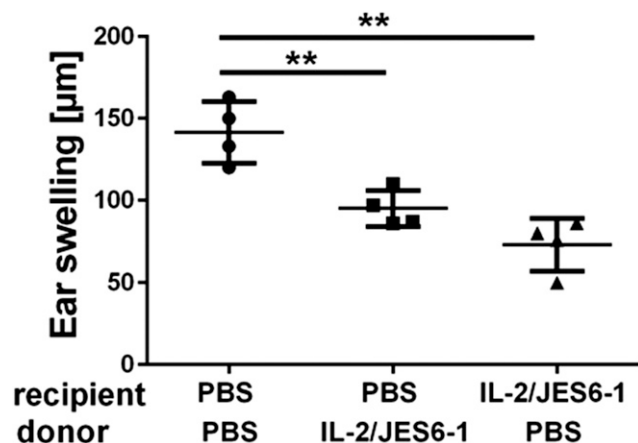


FIGURE 5. IL-2/JES6-1 treatment suppresses sensitization and challenge phases of CHS. Donor mice were treated with IL-2/JES6-1 or PBS and sensitized with TNCB. Inguinal and axillary LN cells were removed after 5 d and 2×10^7 cells were i.v. injected into recipient mice, which were challenged 1 h after the adoptive transfer. Recipients received IL-2/JES6-1 injections on 3 consecutive days, and cell transfer was performed 3 d after the last injection. The ear swelling 24 h after challenge is presented as mean $\pm$ SD ($n = 4$ mice/group).

was no significant difference in the ear swelling in response to oxazolone between IL-2/JES6-1-treated mice and PBS controls (Fig. 6D).

In conclusion, IL-2/JES6-1 does not mediate a general immune suppression, but it selectively downmodulates the allergic reaction

to haptens administered at the time of the treatment. This allergen-specific downmodulation of CHS is long-lasting and not limited to previously inflamed tissues.

In vivo Treg expansion results in prolonged reduction of hapten-specific IFN- γ^+ CD8 T cell frequencies, in favor of increased populations of Foxp3 $^+$ and TGF- β -producing Tregs

IFN- γ -producing CD8 T cells are considered to be the main effector cells in the CHS inflammatory response (32, 33), which makes the establishment of reliable and precise methods to detect hapten-reactive CD8 T cells very essential. A common method used for this purpose is the ELISPOT for detection of IFN- γ -secreting T cells upon 24 h restimulation by the respective hapten. This short-term stimulation results in selective cytokine secretion by hapten-specific cells, which can be detected by ELISPOT. To allow further analysis of the hapten-specific cells, we modified this assay by reducing the in vitro restimulation time to 5 h and substitution of the ELISPOT assay by flow cytometric analysis of IFN- γ expression. Compared to controls, IL-2/JES6-1-treated groups showed a considerable reduction of hapten-reactive IFN- γ^+ CD8 T cells (Fig. 7A, Supplemental Fig. 3C). This reduction was still detectable at day 12 after the second challenge (Fig. 7B). In contrast, total IFN- γ^+ CD8 T cells were not altered in all groups (Fig. 7B), indicating that IL-2/JES6-1 treatment acts specifically on hapten-specific IFN- γ -producing CD8 T cells without affecting total IFN- γ production in the system. As shown in DEREG mice, the IL-2/JES6-1-induced reduction of hapten-specific IFN- γ^+ CD8 T cells was dependent on Foxp3 Tregs (Supplemental Fig. 3D). nTreg and pTreg frequencies were increased even 17 d after

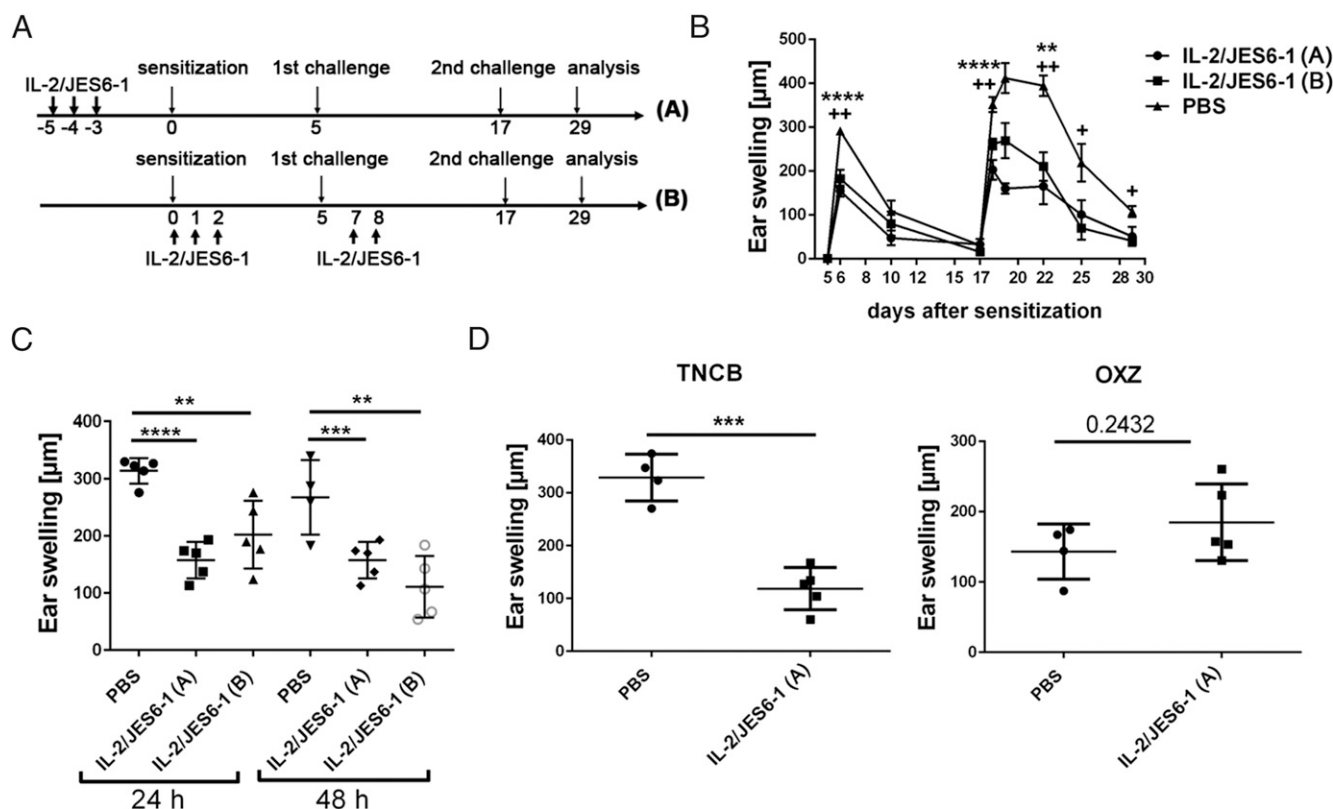


FIGURE 6. CHS suppression by IL-2/JES6-1 is long-term, Ag specific, and nonrestricted to the original inflammation site. **(A)** Scheme of treatment regimens A and B for IL-2/JES6-1 injections and TNCB challenge. First challenge with TNCB was done at the left ear. **(B)** Kinetics of ear swelling after first and second challenge of the same ear. **(C)** Ear swelling of left ear following the second challenge. **(D)** In addition to TNCB, mice were sensitized to oxazolone (OXZ) at day -15 using the IL-2/JES6-1 (A) treatment regimen. Mean of ear swelling 24 h after the second challenge. The right ear was challenged with TNCB and the left ear with OXZ. Data are shown as mean $\pm$ SEM and representative of one of two independent experiments ($n = 5$). For p values, * reflects IL-2/JES6-1 (A) versus PBS and + reflects IL-2/JES6-1 (B) versus PBS. ** $p < 0.05$, *** $p < 0.01$, **** $p < 0.001$.

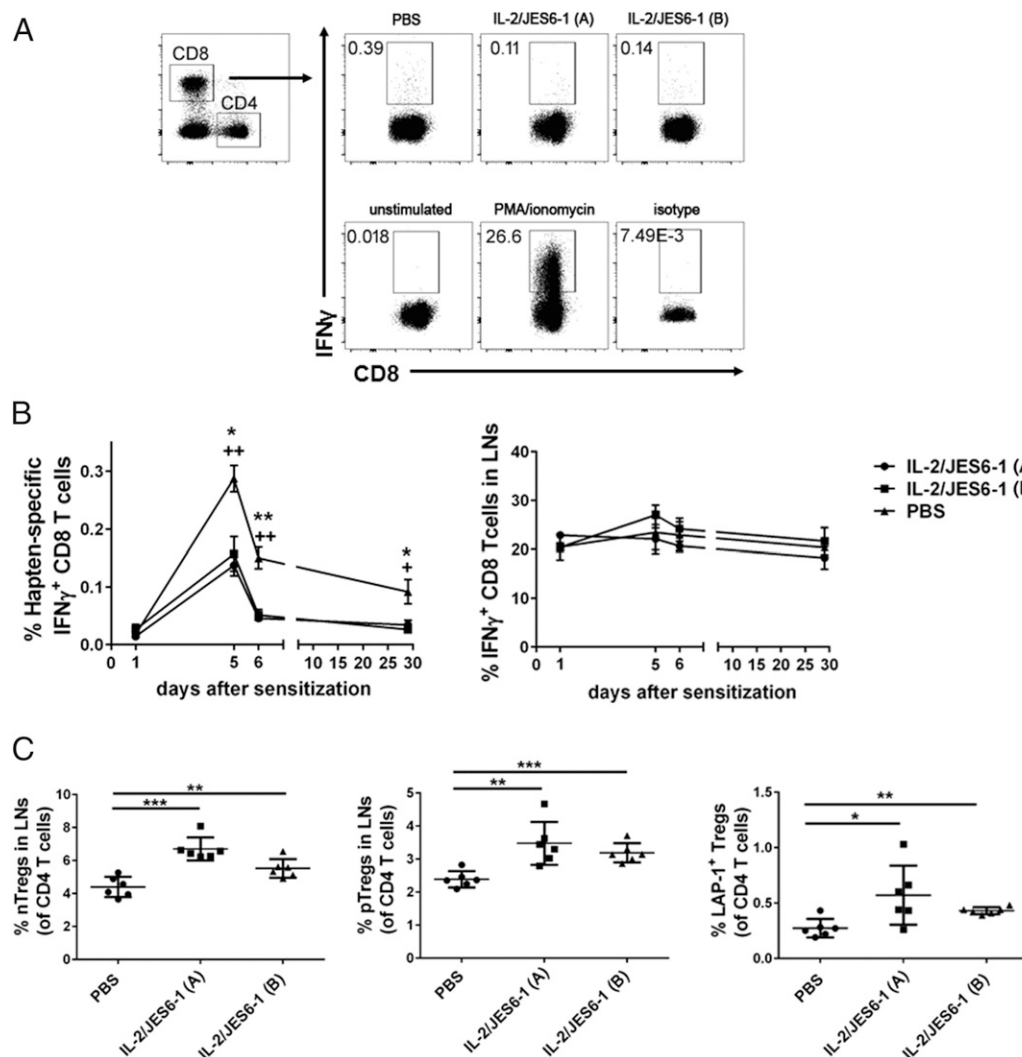


FIGURE 7. Effect of IL-2/JES6-1 treatment on T cell subsets. Auricular and axillary LN cells were isolated and analyzed for hapten-specific CD8 T cells and for Foxp3 and TGF- β expression by Tregs. **(A)** Gating strategy for hapten-specific IFN- γ ⁺ CD8 T cells after 5 h restimulation with hapten-loaded APCs (left). Intracellular cytokine staining (right). **(B)** Hapten-specific (left) and total (right) IFN- γ ⁺ CD8 T cell frequencies in skin LNs. **(C)** Frequencies of nTregs, pTregs, and LAP-1⁺ Tregs in LNs analyzed 5 d after the second challenge. Results shown are mean $\pm$ SD and representative of one out of two experiments ($n = 6$ mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

the first challenge, that is, 32 d after the last IL-2/JES6-1 injection, and this increase was associated with higher frequencies of TGF- β ⁺Foxp3⁺ Tregs (Fig. 7C). In conclusion, although IL-2/JES6-1 treatment alone induces a transient increase in Treg populations, if combined with allergic challenges, it leads to a re-establishment of expanded Treg populations much later. Also, it results in long-lasting suppression of CD8 T effector cells.

Discussion

In this study, we have demonstrated that expansion of Foxp3⁺ Treg populations by IL-2/JES6-1 leads to increased numbers of both nTregs and pTregs in a model of Tc1-mediated CHS. Tregs were required in the IL-2/JES6-1-mediated suppression of skin inflammation and hapten-specific IFN- γ ⁺ CD8 T cell response, as well as reduction of neutrophil influx into the affected skin. IL-2/JES6-1 treatment during sensitization resulted in downregulation of CD86 on hapten-bearing DCs and could inhibit effector cell priming in LNs during sensitization, but independent of the priming, it also affected the effector phase. IL-2/JES6-1 treatment showed long-lasting effects, that is, it attenuated the inflammatory reaction during repeated challenges.

Previously, IL-2/JES6-1 treatment was shown to induce Treg expansion and improvement of multiple diseases with different pathogenic profiles, for example, Th2-driven allergic asthma (21). It also renders mice resistant to autoimmune encephalomyelitis (20). Likewise, IL-2/JES6-1 inhibited development and progression of atherosclerosis (22, 24). In the present study, we have tested the effect of IL-2/JES6-1 treatment on Tc1-induced CHS where CD8 T cells are crucial effector cells and detected reduced ear swelling responses. In addition to the effects on hapten-reactive CD8 T cells, neutrophil accumulation in affected skin tissue was also reduced. Furthermore, ear swelling was still reduced even upon a second challenge in IL-2/JES6-1-treated mice. This long-lasting suppressive effect was still observed when the second challenge was applied at a previously unchallenged ear, implying that the beneficial effect of IL-2/JES6-1 treatment is not restricted to the site of original inflammation.

CHS is a DC-dependent skin inflammation in which DCs play an important role as APCs. During the sensitization phase, they are responsible for Ag-specific T cell priming in LNs (34). Tregs can downregulate the expression of CD80 and CD86 on DCs (35, 36). These molecules are critical for Ag presentation, costimulation,

and proper activation of effector T cells. Previous studies have shown that CTLA-4 plays an important role in the suppressive functions of Tregs, and its blockade leads to an abrogation of Treg functions (31, 37). Our data demonstrate that CTLA-4⁺Foxp3⁺ Treg numbers were higher in IL-2/JES6-1-treated mice, and these cells also show an upregulated CTLA-4 expression compared with PBS controls. Tregs can attenuate T cell priming through inhibition of proper DC–T cell interactions (38). CTLA-4-expressing Tregs can capture and degrade CD86 from the APCs, resulting in impaired costimulation via CD28 (39). In the present study, DCs downregulated CD86 expression after sensitization in IL-2/JES6-1-treated mice. Moreover, challenge of mice that received LN cells from sensitized mice previously treated with IL-2/JES6-1 failed to induce a normal CHS response. Taken together, these data indicate that IL-2/JES6-1-induced Treg populations impair T cell priming in LNs by restraining hapten presentation by DCs during sensitization.

Recent experimental evidence showed that neutrophils infiltrate the affected skin within 24 h following sensitization and upon challenge (8). In the present study, reduced neutrophil frequencies were observed in blood and inflamed skin of IL-2/JES6-1-treated mice. Lymphocyte-derived IL-10 can block neutrophil migration and distribution within tissues (40). Given that increased IL-10⁺ CD4 T populations were detected following IL-2/JES6-1 treatment, it is possible that IL-10 could play a role in blocking neutrophil influx into skin tissue after this treatment. Of note, IL-2/JES6-1 treatment did not affect neutrophil influx in the absence of lymphocytes in SCID mice during unspecific croton oil-mediated skin irritation. These data are in accordance with the idea that IL-2/JES6-1 does not directly act on neutrophils, but mediates its suppressive effects on these cells through induction of Tregs. Foxp3⁺ Tregs exhibit the capacity of blocking neutrophil accumulation in the skin via downregulation of their chemoattractants CXCL1 and CXCL2 (41), mainly produced by keratinocytes (42). Hence, it is possible that Tregs control neutrophil accumulation within the inflamed skin. In accordance with this idea, increased Treg frequencies were detected in IL-2/JES6-1-treated mice not only in LNs but also in inflamed ears. In this tissue, Tregs may affect neutrophil accumulation either via IL-10 production or by downregulation of CXCL1 and CXCL2.

Interestingly, IL-2/JES6-1 injection reduced neutrophil frequencies in blood. The reduced release of neutrophils from bone marrow into the blood may contribute to this effect. This hypothesis is supported by the observation that IL-2/JES6-1 treatment led to the upregulation of CXCR4 expression on neutrophils. This chemokine receptor was shown to play a pivotal role for the retention of neutrophils in the bone marrow and controlling their emigration into the periphery (30). Additionally, increased neutrophil apoptosis could also contribute to the observed reduction of neutrophil frequencies in blood. It was shown before that Tregs can promote neutrophil apoptosis via production of IL-10 and TGF- β (43, 44). In conclusion, both reduced neutrophil release from bone marrow and Treg-induced apoptosis may contribute to the reduction of neutrophil frequencies in blood, observed following IL-2/JES6-1 treatment.

Diminished populations of hapten-specific CD8 T cells were detected in LNs of IL-2/JES6-1-treated mice. Our data indicate that impaired DC function contributes to this effect. Nevertheless, reduction of neutrophil frequencies might also be involved in this process. It had been previously shown that depletion of neutrophils can result in impaired priming of hapten-specific T cells during sensitization (9), leading to the hypothesis that the IL-2/JES6-1-mediated decrease of neutrophil populations may contribute to the reduction of hapten-specific CD8 T cell frequencies. Additionally, Tregs have been shown to be able to directly suppress CD8

T effector cells via T cell–T cell interaction (45). In accordance with this idea, our results show impaired CHS following adoptive transfer of effector LN cells into IL-2/JES6-1-treated recipients, compared with PBS-treated recipients. In conclusion, Tregs induced by IL-2/JES6-1 may prevent the expansion of CD8 T effector cells either directly via T cell–T cell contact or indirectly via CD86 downregulation on DCs and reduction of neutrophil populations.

Short-term IL-2/JES6-1 treatment led to a long-lasting and allergen-specific suppression of the CHS reaction. Possibly, this effect could be mediated by a reduction of the allergen-specific CD8 T cell memory population. CD8 T cell memory is seeded by a fraction of the CD8 T effector cell population (46). Hence, the impaired DC function and reduced priming of CD8 T effector cells observed following IL-2/JES6-1 treatment could suppress the generation of CD8 T cell memory.

Recent findings have demonstrated that Tregs can mediate the quiescence of memory CD8 T cells through a CTLA-4-dependent mechanism (47). It is possible that such Treg-mediated control of CD8 memory reactivation also contributes to the long-lasting suppression of CHS.

Our findings indicate that IL-2/JES6-1 treatment similarly expands nTreg and pTreg populations. It has been shown previously that injections of IL-2/JES6-1 in the presence of Ag lead to the migration of Ag-specific Tregs to the site of Ag localization (23). Moreover, a recent study reported that IL-2 is required to generate a population of induced memory Tregs upon immunization that are maintained for >40 d (48). Hence, it is possible that IL-2/JES6-1 treatment induces the formation of memory Tregs, which could contribute to the observed long-lasting and Ag-specific CHS suppression. It had been shown that Tregs can downregulate CD8 T effector cell functions by direct cell–cell contact (49). However, such an interaction could hardly explain long-lasting suppression that is limited to allergic reactions to Ags present during the original treatment. Instead, such an Ag-specific effect may require the interaction of Ag-presenting DCs, Ag-specific Tregs, and Ag-specific effector cells. Only such an interaction could favor the activation and action of Ag-specific Tregs versus nonspecific Treg-mediated suppression.

It was shown that indeed DCs can form clusters with Tregs and CD4 T effector cells at the same time (50). However, whether similar clusters between Ag-presenting DCs, Tregs, and CD8 T effector cells are formed in our experimental setting, as well as their possible contribution to IL-2/JES6-1 mediated suppression of contact allergy, needs to be further elucidated.

Taken together, the data presented in this study indicate that IL-2/JES6-1 treatment results in the expansion of nTreg and pTreg populations that control CHS via multiple mechanisms during the sensitization and challenge phases.

Acknowledgments

We thank Kathleen Kurwahn for excellent technical assistance.

Disclosures

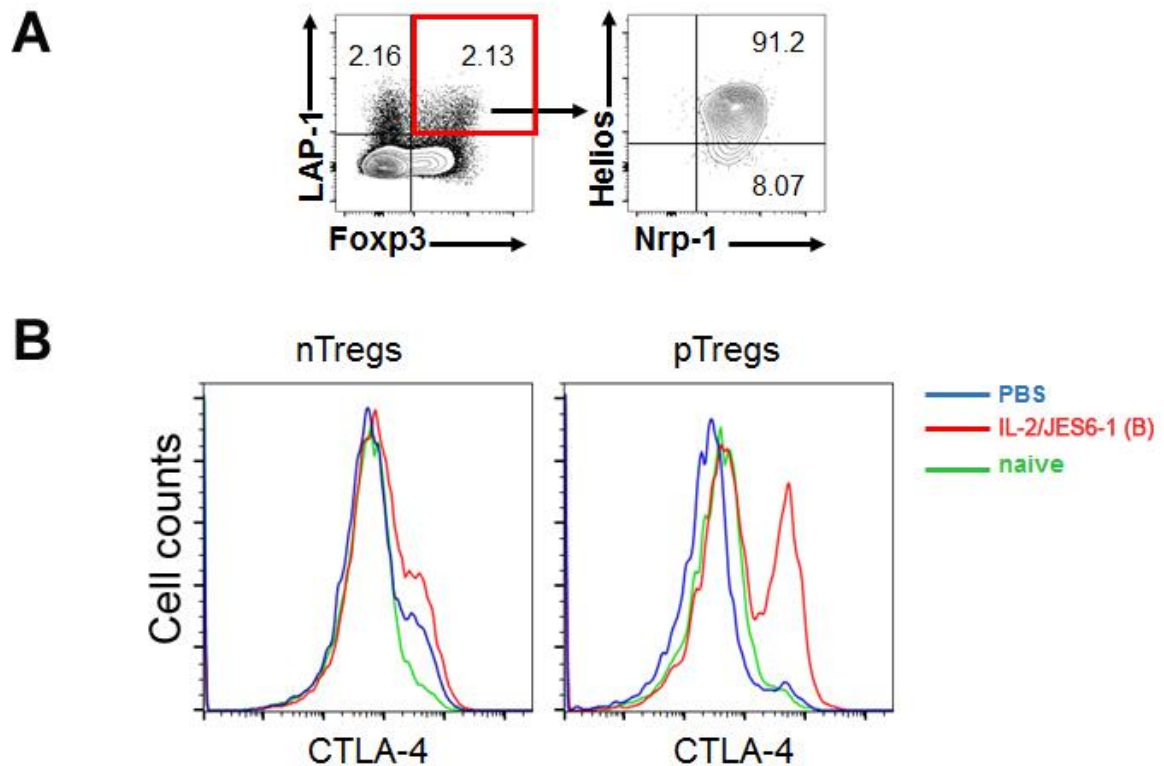
The authors have no conflicts of interest.

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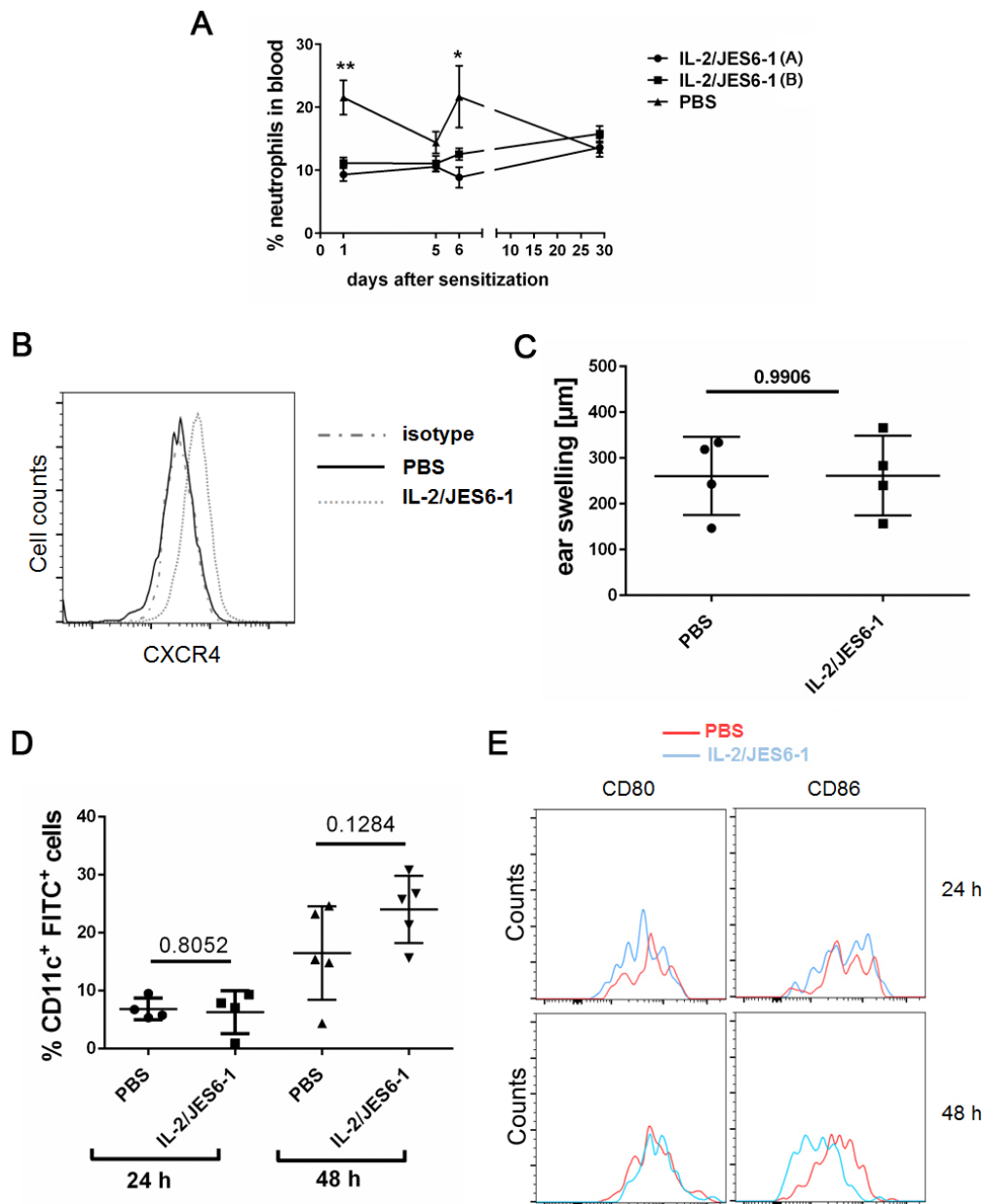
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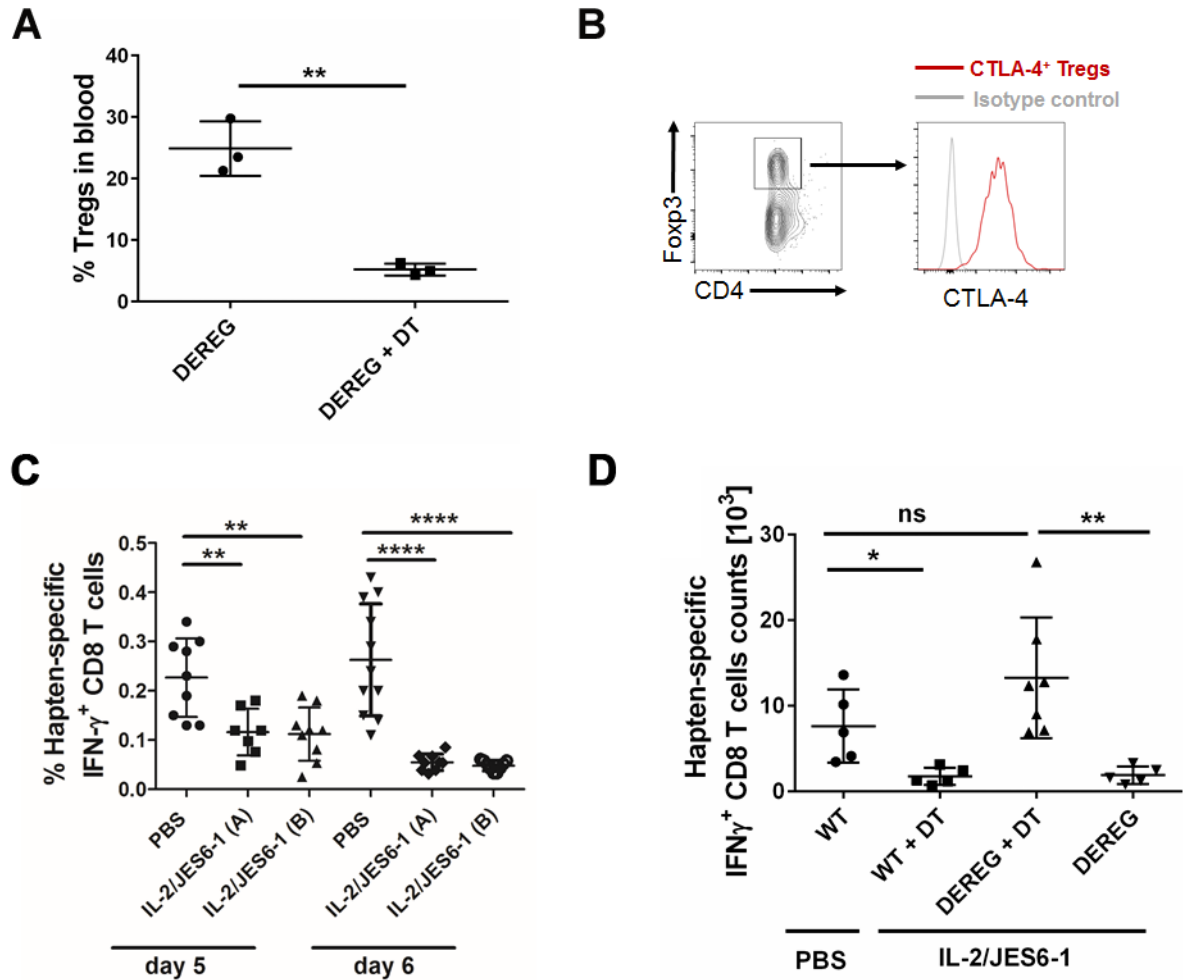
Supplemental material



Supplemental figure 1. Characterization of Treg populations following IL-2/JES6-1 treatment. IL-2/JES6-1 was injected before challenge and LN cells were analyzed. (A) Gating strategy to show that Foxp3⁺ Tregs are mainly Nrp-1⁺ and Helios⁺ (quadrants were set according to isotype controls). Dot plot of the left image was gated on CD4 T cells. (B) CTLA-4 expression by nTregs and pTregs.



Supplemental figure 2. Effect of IL-2/JES6-1 treatment on neutrophils and DCs. (A) Kinetics of Ly6G^{hi} CD11b⁺ neutrophil frequencies in blood of wild type mice upon sensitization on day 0 and challenge on day 5. (B) CXCR4 expression on blood neutrophils one day after sensitization upon IL-2/JES6-1 (A) treatment. (C) SCID beige mice were treated at days -5, -4, and -3 with IL-2/JES6-1 or PBS, and skin irritation was induced using croton oil at day 0. Data indicate ear swelling at 24 h after challenge. (D, E) Wild type mice were sensitized with FITC and LNs were harvested 24 h and 48 h later. Gated on CD11c⁺ MHCII^{hi} DCs, FITC⁺ DC were analyzed. Data are presented as mean $\pm$ SD. *P < 0.05, **P < 0.01.



Supplemental figure 3. Effect of Treg depletion on CHS suppression by IL-2/JES6-1 treatment. DEREG mice were treated as shown in Fig. 4A. (A) Frequency of Foxp3⁺ Tregs in blood one day after DT injection. (B) Gating strategy used to show that all infiltrated Tregs in inflamed ear are CTLA-4⁺. Dot plots of the left image were gated on CD4 T cells. (C) Frequencies of hapten-specific IFN- γ ⁺ CD8 T cells in LNs at days 5 and 6. (D) Cell numbers of hapten-specific IFN- γ ⁺ CD8 T cells in LNs one day after challenge. Data are presented as mean $\pm$ SD.