

Crosstalk of regulatory T cells and tolerogenic dendritic cells prevents contact allergy in subjects with low zone tolerance

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Background: Allergic contact dermatitis is one of the most common occupational diseases. A main protective mechanism in those who do not develop allergic contact dermatitis is tolerance induction by repeated exposure to low doses of contact allergen, which is termed low zone tolerance (LZT). The mechanisms that determine the tolerance induction in subjects with LZT are still elusive.

Objective: We performed analysis of the role of CD4⁺CD25⁺ forkhead box protein 3 (FOXP3)-positive regulatory T (Treg) cells and dendritic cells (DCs) in mice with LZT.

Methods: Mechanisms of tolerance induction were analyzed in a murine model of LZT by using FOXP3 and IL-10 reporter mice, as well as mice that allow the selective depletion of Treg cells or DCs.

Results: Depletion of CD4⁺CD25⁺FOXP3⁺ Treg cells during tolerance induction completely abolishes the development of LZT, resulting in a pronounced contact hypersensitivity response. Adoptive transfer experiments, depletion studies, and use of cell type-specific deficient mice revealed that IL-10 production is critical for the suppressor function of Treg cells in mice with LZT and that tolerogenic CD8⁺CD11c⁺ DCs located in the skin-draining lymph nodes are essential for LZT. In the absence of Treg cells, DCs did not develop tolerogenic functions, indicating that activated IL-10⁺ Treg cells might imprint the tolerogenic DC phenotype. Cell communication analysis

revealed that the education of tolerogenic DCs might involve a direct interaction with Treg cells mediated by gap junctions. Subsequently, induction of tolerogenic CD11c⁺ DCs leads to the generation of hapten-specific CD8⁺ Treg cells, which protect against contact hypersensitivity.

Conclusions: Our data demonstrate critical interactions between CD4⁺CD25⁺FOXP3⁺ Treg cells and tolerogenic CD8⁺CD11c⁺ DCs during the induction of LZT. (J Allergy Clin Immunol 2012;130:781-97.)

Key words: Tolerance, low zone tolerance, contact hypersensitivity, regulatory T cells, dendritic cells, hapten, IL-10, contact allergen, allergic contact dermatitis

Allergic contact dermatitis, also referred to as contact hypersensitivity (CHS), is a major health issue and one of the most common occupational diseases in Western countries, with great socioeconomic effect.¹ CHS is an allergic cutaneous inflammation mediated by CD8⁺ effector T (T_C1) cells and regulated by CD4⁺ T cells.^{2,3} Allergy prevention through induction of specific tolerance to allergens might be an approach to improve patient outcomes. Induction of specific tolerance to contact allergens has been studied extensively in various animal models, including low zone tolerance (LZT), in which repeated epicutaneous applications of low doses of contact allergens prevent the development of CHS.⁴⁻⁶ LZT is regarded as a relevant model of naturally occurring tolerance to contact allergens because LZT is achieved by the physiologic uptake of very small amounts of allergens through the skin.^{5,7-10} It was reported that LZT is mediated by a population of CD8⁺ regulatory T (Treg) cells in a hapten-specific manner.^{5,8} However, the precise immune mechanisms and signals involved in the generation of CD8⁺ Treg cells of subjects with LZT remain unknown. CD4⁺CD25⁺ forkhead box protein 3 (FOXP3)-positive Treg cells are naturally present in the immune system. Their dysfunction causes fatal autoimmune diseases, immunopathology, and allergy.^{11,12} Furthermore, mutations in human FOXP3 are responsible for the immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, providing unequivocal evidence that developmental or functional anomalies of natural Treg cells cause autoimmune diseases and allergies in human subjects.¹³ In addition to thymic generation of natural Treg cells, naive T cells in the periphery can upregulate FOXP3 expression and consequently acquire a Treg cell phenotype and function to control aberrant or excessive immune responses, such as allergies.^{14,15}

In our study we demonstrate that IL-10-producing CD4⁺CD25⁺FOXP3⁺ Treg cells are critically involved in the development of LZT. In the absence of Treg cells, lymph node (LN) CD11c⁺ dendritic cells (DCs) do not become tolerogenic and do not induce hapten-specific CD8⁺ Treg cells of subjects with LZT.

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Abbreviations used

AOO:	Acetone/olive oil
CD62L:	CD62 ligand
CHS:	Contact hypersensitivity
CTLA-4:	Cytotoxic T lymphocyte-associated antigen 4
DC:	Dendritic cell
DNFB:	1-Fluoro-2,4-dinitrobenzene
DT:	Diphtheria toxin
FOXP3:	Forkhead box protein 3
GFP:	Green fluorescent protein
GITR:	Glucocorticoid-induced TNF receptor family-related gene
ICOS:	Inducible costimulator
LN:	Lymph node
LZT:	Low zone tolerance
TNCB:	2,4,6-Trinitro-1-chlorobenzene
Treg:	Regulatory T
WT:	Wild-type

METHODS**Tolerance induction**

Groups of transgenic mice, wild-type (WT) mice, or both were painted up to 10 times with tolerizing doses (0.01% of 1-fluoro-2,4-dinitrobenzene [DNFB] and 0.45 or 4.5 μ g of 2,4,6-trinitro-1-chlorobenzene [TNCB] in 15 μ L of acetone/olive oil [AOO; 3:1 vol/vol]) or AOO alone as a control onto shaved areas. Subsequently, CHS was induced by sensitizing mice with an epicutaneous application (1% DNFB or 450 μ g of TNCB in 15 μ L of AOO) on the abdomen. Challenge was performed by painting 0.1% DNFB or 45 μ g of TNCB onto the dorsal side of the right ear at day 16 or as indicated. In Treg cell-depletion experiments modified protocols were used as indicated (PC61 mice, see Fig E1, A, in this article's Online Repository at www.jacionline.org; DERE mice, see Fig E2, A, in this article's Online Repository at www.jacionline.org) with respect to recovery of Treg cell numbers. The increase in ear thickness was measured after 24 hours with an engineer's micrometer (Oditest; Kroeplin, Schüchtern, Germany).

Depletion of CD11c⁺ DCs and tolerance induction in CD11c-DTR mice

Transgenic CD11c-DTR mice were injected intraperitoneally with 4 ng of diphtheria toxin (DT)/g of body weight at days 0 and 1. PBS-treated groups served as controls. At days 1 and 2, the mice were tolerized twice a day with 4.5 μ g TNCB. At day 8, mice were sensitized by epicutaneous application of 450 μ g of TNCB in 15 μ L AOO. Challenge was performed by painting 45 μ g of TNCB onto the dorsal side of the right ear at day 12.

Depletion of CD25⁺ T cells with anti-CD25 antibody

WT mice were depleted of CD25⁺ T cells by using an anti-CD25 mAb (clone PC61; 75 μ g per mouse) administered through intraperitoneal injection at days -1 and 0 followed by tolerization. Control mice were injected intraperitoneally with equivalent amounts of control antibody (IgG 72137; Jackson ImmunoResearch Laboratories, Suffolk, United Kingdom). Sensitization was performed after recovery of Treg cells (detected by using flow cytometry), and 5 days later, a challenge reaction was elicited (see Fig E1).

Depletion of FOXP3⁺ Treg cells and tolerance induction in DERE mice

Foxp3-DTR-enhanced green fluorescent protein (GFP) bac transgenic mice (DERE mice) were injected intraperitoneally with DT (1 μ g) on day 0. At days -1 and 2, mice were tolerized twice a day with 4.5 μ g TNCB. At day 7, after recovery of Treg cells, mice were sensitized by epicutaneous application of 450 μ g TNCB in 15 μ L AOO. Challenge was performed by painting 45 μ g of TNCB onto the dorsal side of the ear at day 12 (see Fig E2).

Statistical significance

The statistical significance of differences between experimental groups was evaluated by applying the Wilcoxon-Mann-Whitney 2-sample test or the student *t* test with the Statview 5.0 software package (SAS Institute, Cary, NC). Differences in *P* values of less than .05 were considered significant.

All animal studies were approved by the Animal Care Committee of Rhineland-Palatinate and the Regierungspräsidium Freiburg and supervised by the Animal Protection Representatives of the University Medical Center Mainz and the University Freiburg Medical Center.

For description of mice, reagents, and antibodies; isolation of LN-related CD8⁺ T cells, CD4⁺ T cells, CD4⁺CD25⁺ T cells, and CD11c⁺ DCs; and adoptive transfer, cell culture, haptenation, ELISA, ELISpot, and IL-10 transcription *in vivo*, see the **Methods** section in this article's Online Repository at www.jacionline.org.

RESULTS**CD4⁺CD25⁺FOXP3⁺ Treg cells are pivotal for LZT induction**

CD4⁺CD25⁺FOXP3⁺ Treg cells play a critical role in multiple immune responses, including allergic reactions.¹⁶ Therefore we analyzed the potential role of Treg cells in LZT induction. For this purpose, we depleted Treg cells in the induction phase of LZT using an anti-CD25 mAb (PC61; see Fig E1, A).¹⁷ The efficacy of depletion and the recovery of Treg cells before sensitization were assessed by means of flow cytometry (see Fig E1, B). Control tolerized mice exhibited typical properties of LZT, including an inhibited ear-swelling response and a reduced TNCB-specific T_H1 T-cell activation compared with those seen in sensitized mice (Fig 1, A-C). In contrast, in Treg cell-depleted tolerized mice the development of LZT was completely abrogated (Fig 1, A-C). Here we observed a pronounced allergic skin reaction (Fig 1, A), an enhanced T-cell proliferation, and CD8⁺ T cell-related IFN- γ and IL-2 release after hapten-specific restimulation (Fig 1, B and C).

The fact that CD25 is also upregulated on activated T cells and that CD25⁺ subsets of Treg cells exist¹⁸⁻²⁰ prompted us to additionally analyze the role of Treg cells in mice with LZT by using DERE mice, which allow the selective depletion of FOXP3⁺ Treg cells (see Fig E2).²¹ In line with our experiments with anti-CD25, depletion of FOXP3⁺ Treg cells during induction of tolerance in DERE mice resulted in the complete abrogation of LZT, as demonstrated by a significant increase in the allergic response *in vivo*, enhanced hapten-specific T-cell proliferation, and significantly enhanced T_H1 cytokine production (IFN- γ and IL-2; Fig 1, D-F).

Treg cells are activated by low doses of contact allergen but operate in an allergen-nonspecific manner

Next we evaluated the potential of Treg cells to transfer LZT and the allergen specificity of their suppressor function. For this purpose, CD4⁺CD25⁺ T cells from TNCB-tolerized mice were adoptively transferred into naive mice before sensitization. The recipients were either sensitized and challenged with TNCB or a second unrelated hapten, DNFB, respectively. Notably, regardless of the allergen (TNCB or DNFB) used for sensitization/challenge, Treg cells generated by exposure to low-dose TNCB induced typical features of LZT in recipient mice (Fig 2, A-C), indicating antigen-nonspecific activity of Treg cells in mice with LZT. Transfer of nonactivated Treg cells did not induce tolerance but resulted in typical features of CHS, indicating that activation

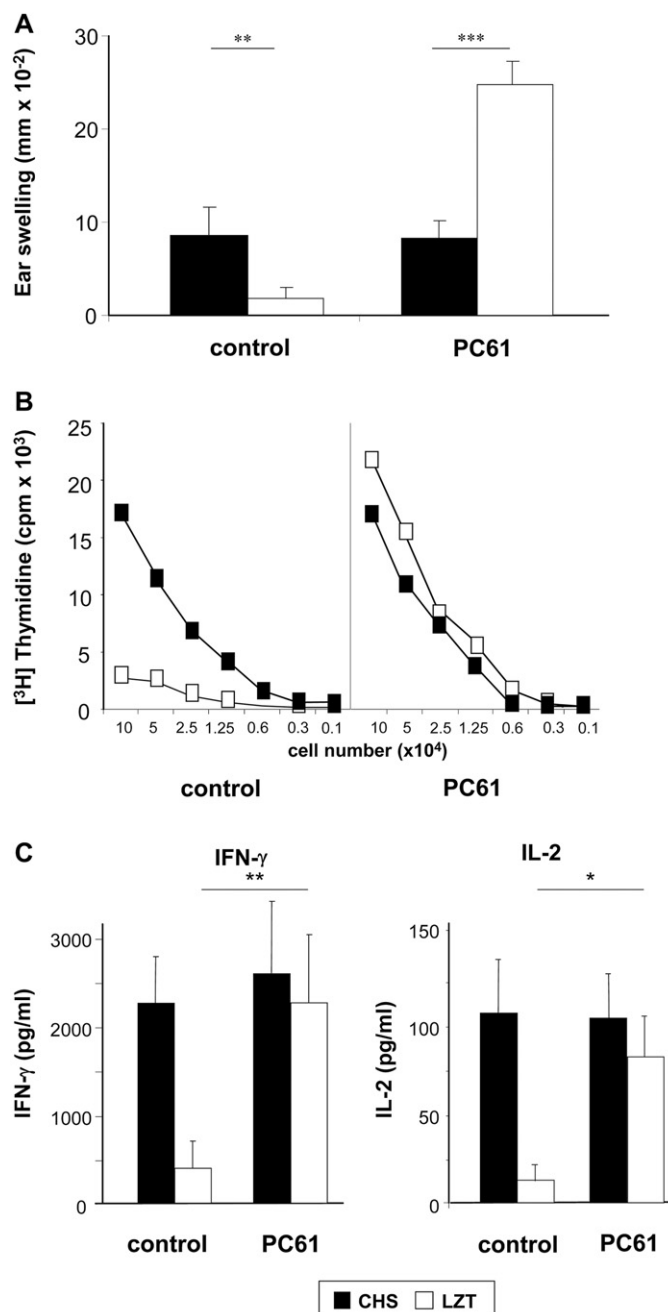


FIG 1. Pivotal role of CD4⁺CD25⁺FOXP3⁺ Treg cells for the development of LZT. **A-C**, Mice were treated with the anti-CD25 mAb (PC61) or a control antibody. Solvent (CHS) or TNCB (LZT) was applied epicutaneously 10 times. After recovery of CD4⁺CD25⁺FOXP3⁺ Treg cells (see Fig E1), animals were sensitized and challenged as described. **D-F**, DEREG mice were treated with DT for Treg cell depletion. Solvent (CHS) or tolerizing doses (LZT) of the hapten TNCB were applied epicutaneously, and subsequently, mice were sensitized and challenged (see Fig E2). Fig 1, **A** and **D**, The ear-swelling response was assessed after challenge (mean $\pm$ SD). Fig 1, **B**, **C**, **E**, and **F**, Skin-draining LNs were obtained for T-cell analysis. Fig 1, **B** and **E**, T-cell proliferation was depicted (mean value of triplicates in counts per minute) after restimulation. Data represent one of 4 (Fig 1, **A-C**) and one of 3 (Fig 1, **D-E**) independent experiments with similar results. Pooled data of release of IFN- γ and IL-2 by T cells of 4 (Fig 1, **C**) or 3 (Fig 1, **F**) independent experiments are shown (means $\pm$ SDs). * P < .05, ** P < .01, and *** P < .001.

of Treg cells by low doses of allergens is critical for their suppressor function (Fig 2, **D** and **E**).

As previously shown, LZT is a hapten-specific immune reaction mediated by CD8⁺ Treg cells.^{5,8} Adoptive transfer experiments of CD8⁺ Treg cells and subsequent sensitization with

TNCB or a second unrelated hapten revealed that, in contrast to CD4⁺CD25⁺ T cells, CD8⁺ Treg cells of mice with LZT were allergen specific (Fig 2, **F**). Phenotypic analysis (cytotoxic T lymphocyte-associated antigen 4 [CTLA-4], glucocorticoid-induced TNF receptor family-related gene [GITR], inducible costimulator

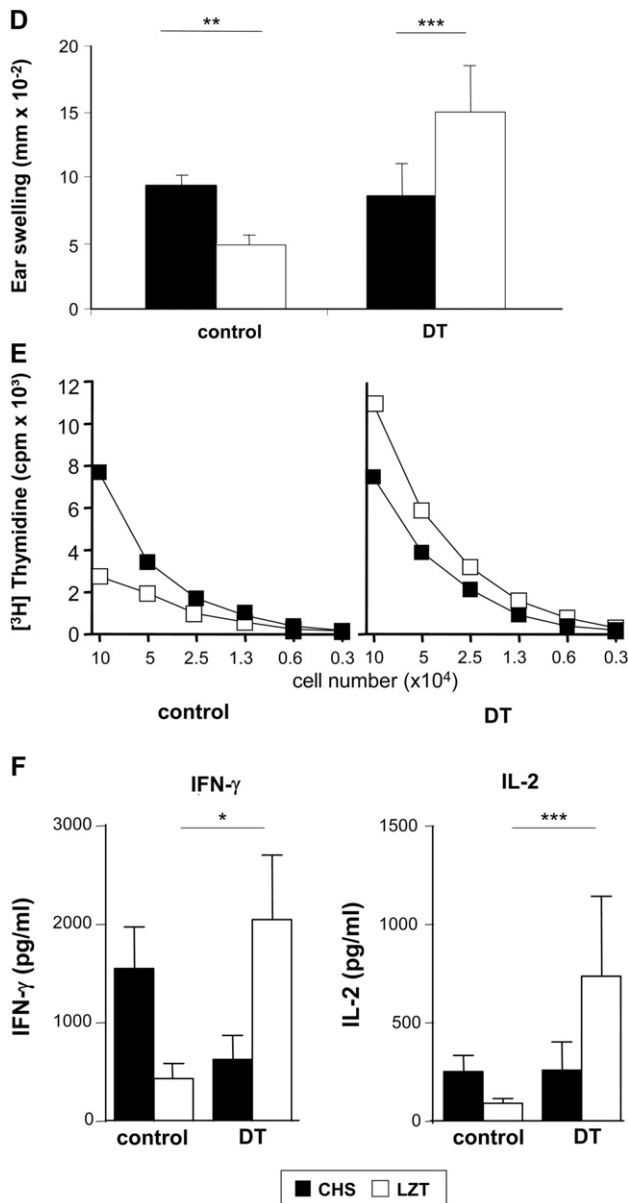


FIG 1. (Continued)

[ICOS], CD25, CD28, CD38, CD44, CD62 ligand [CD62L], CD69, CD103, CD127, CD134, and CD162) of CD8⁺ T cells obtained after LZT demonstrated no differences compared with control (solvent treatment) CD8⁺ T cells (data not shown). Moreover, we did not observe an induction of FOXP3 expression in CD8⁺ T cells after tolerization compared with that seen in control cells (data not shown).

Induction of a CCR7⁺ Treg cell subset during induction of tolerance

Analysis of the numbers of CD4⁺CD25⁺FOXP3⁺ Treg cells in draining LNs revealed no changes in the percentages at any phase of LZT (after tolerization, sensitization, and challenge) compared with percentages seen in control mice (see Fig E3 in this article's Online Repository at www.jacionline.org).

To identify an activated subpopulation of Treg cells induced by the LZT protocol, we analyzed the expression of a panel of molecules considered to be involved in T-cell/Treg cell activation (CD25, CD44, CD62L, CD69, CD103, CD127, CD154, CCR7, CTLA-4, ICOS, programmed death 1 [PD-1], and GITR).^{11,15,22,23} Enhanced numbers of CCR7⁺CD4⁺CD25⁺FOXP3⁺ Treg cells and an increased surface expression of CCR7 and CD103 were found on CD4⁺CD25^{high} FOXP3 Treg cells of draining LN after tolerization and sensitization compared with solvent-treated control animals (Fig 3, A and B).

To evaluate the function of CCR7⁺ Treg cells in mice with LZT, we performed adoptive transfer experiments with CD4⁺CD25⁺ Treg cells from CCR7^{-/-} mice after tolerization. Compared with injection of WT CD4⁺CD25⁺ Treg cells (Fig 2) and LZT control mice, transfer of CCR7^{-/-} Treg cells did not induce an LZT response in the recipients, but the mice showed a robust CHS reaction (as demonstrated by increased ear swelling [Fig 3, C] and hapten-specific T cell activation [data not shown]).

CD4⁺CD25⁺ Treg cells are critical for the generation of CD8⁺ Treg cells in mice with LZT

In previous transfer experiments it was shown that tolerance can be transferred by regulatory CD8⁺ T cells as important mediators of LZT.^{5,7,8} However, CD8⁺ T cells obtained after tolerance induction in the absence of Treg cells failed to transfer the tolerance, as demonstrated by the development of a strong CHS immune response (increased ear swelling, T-cell activation, and T_H1 cytokine pattern; Fig 4).

Pivotal role for tolerogenic CD11c⁺ DCs during induction of LZT

In former studies we showed that epidermal Langerhans cells, dermal DCs, and B cells are dispensable for tolerance induction.^{7,9} To further address the role of DCs in mice with LZT, we used a model of CD11c-DTR mice in which DT treatment allowed a significant reduction in CD11c⁺ DC numbers limited to the phase of tolerance induction (see Fig E4, A and B, in this article's Online Repository at www.jacionline.org). No alteration in the numbers of Treg cells was found after DC depletion (see Fig E4, C). Transient depletion of CD11c⁺ DCs during tolerance induction resulted in a complete inhibition of tolerance, whereas CHS development was normal (Fig 5). Notably, we observed a complete restoration of LZT after reconstitution of the mice with CD11c⁺ DCs after injection of DT but before tolerization (Fig 5).

Analysis of LN CD11c⁺ DCs did not reveal any differences in the number of CD11c⁺ MHC class II⁺ DCs during LZT or Treg cell depletion (see Fig E5, A, in this article's Online Repository at www.jacionline.org). However, analysis of a panel of surface markers considered to contribute to DC/T-cell interactions (CCR7, CD103, CD80, CD86, CD273, CD274, CD275, CD276, B7-H4, and DEC-205) demonstrated a significantly reduced expression of the costimulatory molecule CD80 (see Fig E5, B) but did not reveal any alteration of other parameters, including CCR7 on CD11c⁺ MHC class II^{high} DCs (data not shown). Notably, Treg cell depletion restored CD80 expression levels, suggesting the induction of a tolerogenic phenotype of DCs by Treg cells (see Fig E5, B).

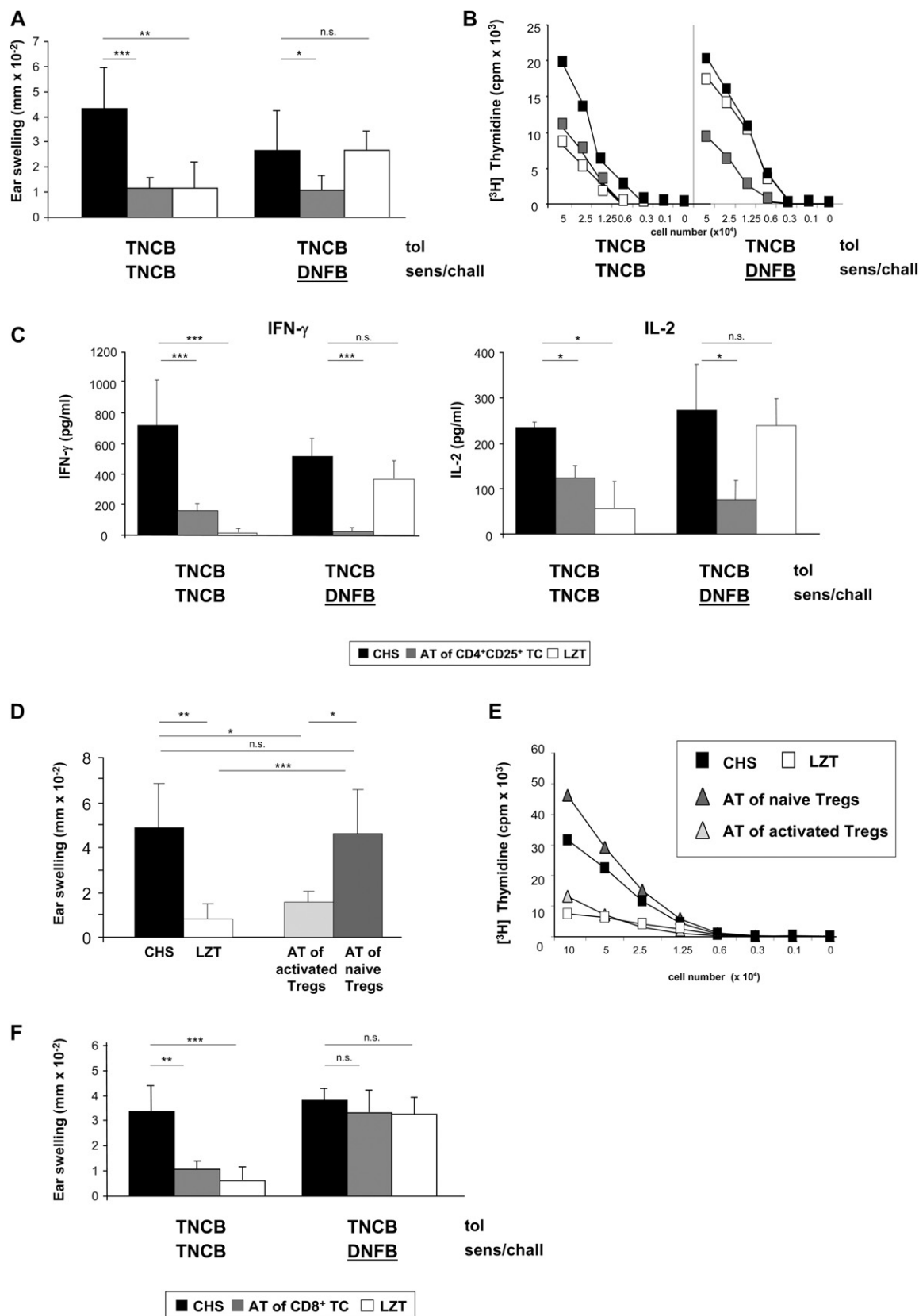


FIG 2. CD4⁺CD25⁺ Treg cells activated by low amounts of contact allergen operate in an allergen-nonspecific manner in contrast to regulatory CD8⁺ T cells. **A-C**, After tolerization with TNCB, CD4⁺CD25⁺ T cells were purified from LN cells and adoptively transferred (AT) into naive WT mice. Tolerized mice (LZT) and mice with CHS without cell transfer served as control animals. Subsequently, recipients and

We confirmed our results using adoptive transfer experiments with CD11c⁺ DCs obtained from LNs of tolerized mice that resulted in tolerance induction in the recipients (Fig 6, A and B). To better characterize the role and subset of the relevant CD11c⁺ DC population, we performed experiments using Batf3^{-/-} mice that lack CD8⁺CD11c⁺ DCs of lymphoid tissues known to be involved in peripheral tolerance.^{24,25} In our hands Batf3^{-/-} mice displayed a normal CHS response, as demonstrated previously,²⁶ indicating that CD8⁺CD11c⁺ DCs are dispensable for this skin inflammatory immune response. However, these mice completely failed to have LZT, as shown by a conspicuous CHS reaction (enhanced ear swelling and increased T-cell response), demonstrating that CD8⁺CD11c⁺ DCs are critically involved in tolerance to contact allergens (Fig 6, C-E).

CD4⁺CD25⁺ Treg cells are required for imprinting the tolerogenic phenotype of CD11c⁺ DCs

It was previously shown that crosstalk between DCs and Treg cells contribute to the initiation and maintenance of tolerance.²⁷ Therefore we analyzed the interplay between Treg cells and CD11c⁺ DCs in mice with LZT. In these experiments CD11c⁺ DCs were obtained after tolerization in the absence of Treg cells and transferred into naive mice. Under these conditions, the recipients did not have LZT demonstrated by a strong allergic skin reaction *in vivo* and enhanced T-cell proliferation (Fig 7, A and B).

As demonstrated, CD4⁺CD25⁺ Treg cells are critical for the generation of the CD8⁺ Treg cells of mice with LZT (Fig 4), and LZT can be transferred by CD8⁺ Treg cells.^{5,8} On the basis of these results, we addressed the question of whether tolerogenic DCs are the link between CD4⁺CD25⁺ Treg cells and hapten-specific CD8⁺ T cells. Transfer of CD8⁺ T cells, which were obtained from tolerized and CD11c-depleted mice, did not induce a tolerance reaction in subsequently sensitized recipients (Fig 7, C and D).

These results suggest that interactions between activated Treg cells and LN CD11c⁺ DCs imprinted a tolerogenic phenotype of the antigen-presenting cell, which in turn exhibit the capacity to induce the development of CD8⁺ Treg cells of mice with LZT. To formally prove a direct crosstalk between CD4⁺CD25⁺ Treg cells and CD11c⁺ DCs, we took advantage of the knowledge that Treg cells and DCs interact through gap junctions.²⁸ Thus we used the dye calcein, which spreads from donor to host cells through gap junctions.^{29,30} Adoptive transfer experiments of calcein-labeled CD4⁺CD25⁺ Treg cells and subsequent flow

cytometric analysis of LN-related immune cells revealed a higher percentage of calcein-positive CD11c⁺ DCs after tolerization compared with that seen in control cells (Fig 7, E), demonstrating a direct interaction between CD4⁺CD25⁺ Treg cells and CD11c⁺ DCs. Other immune cell populations (B cells and CD4⁺ and CD8⁺ T cells) in the skin-draining LNs did not exhibit any difference in the calcein uptake between tolerized and control animals (see Fig E6, B-D, in this article's Online Repository at www.jacionline.org). Transferred calcein-high CD4⁺CD25⁺ Treg cells are depicted in the right gates (see Fig E6, A).

In addition, we performed transfer experiments of CD45.2 CD11c⁺ DCs after tolerization (or solvent treatment as controls) into naive CD45.1 WT mice. After sensitization, LNs were obtained, and immunohistochemistry of tissue sections was performed to analyze the crosstalk between tolerogenic DCs and FOXP3⁺ Treg cells. Here we found Treg cell/DC clusters with significantly higher numbers of recipient FOXP3⁺ T cells in contact with transferred tolerogenic DCs obtained after tolerization compared with those seen in DCs injected after solvent control (Fig 7, F).

IL-10 is critical for the tolerogenic function of CD4⁺CD25⁺ Treg cells

Several mechanisms, including the release of IL-10, were reported to contribute to the regulatory activity of Treg cells.^{8,15,31} ELISpot analysis clearly demonstrated that CD4⁺CD25⁺ T cells obtained after tolerance induction released significant amounts of IL-10 *in vitro* after restimulation (Fig 8, A).

To evaluate whether these results were reflected by IL-10 production of Treg cells *in vivo*, we used IL-10 transcriptional reporter mice (Vert-X) in which IL-10-producing cells are identified by means of GFP expression.^{32,33} Tolerized mice (LZT) displayed significantly enhanced numbers of CD4⁺CD25⁺ IL-10-producing T cells after induction of tolerance and subsequent sensitization (Fig 8, B).

To formally prove the functional relevance of IL-10-producing CD4⁺CD25⁺ T cells during LZT, T cell-deficient Rag1^{-/-} mice were reconstituted with CD4⁺ and CD8⁺ T cells from naive WT and IL-10^{-/-} mice before tolerance induction. As previously shown, in contrast to WT mice IL-10^{-/-} mice did not have a tolerance reaction (Fig 8, C).⁸ By using crossover transfer experiments of CD4⁺ T cells and CD8⁺ T cells obtained from IL-10^{-/-} and WT mice, respectively, CD4⁺ T cell-derived IL-10 was determined to be critical for LZT. However, reconstitution of Rag1^{-/-} mice with CD4⁺ T cells depleted of CD25⁺ Treg cells did not induce LZT in the recipients, clearly indicating the

control groups were sensitized and challenged with either TNCB or DNFB, respectively. Fig 2, A, The ear-swelling response was assessed after challenge (mean $\pm$ SD). Fig 2, B, T-cell proliferation is detected after restimulation (mean value of triplicates in counts per minute). One representative of 4 experiments is shown in Fig 2, A and B. Fig 2, C, Release of IFN- γ and IL-2 by T cells was assessed (means $\pm$ SD). Pooled data of 4 experiments are depicted. D and E, CD4⁺CD25⁺ T cells were purified from LN cells from solvent-treated (naive) or tolerized (activated) mice and adoptively transferred into naive animals. Tolerized (LZT) and solvent-treated (CHS) mice without cell transfer served as control animals. Recipients and control groups were sensitized and challenged. Fig 2, D, Ear thickness (mean $\pm$ SD) was assessed. E, T-cell proliferation is depicted as the mean counts per minute of triplicates after hapten-specific restimulation. One representative experiment of 3 is demonstrated in Fig 3, D and E. F, After tolerization with TNCB, CD8⁺ T cells were purified from LN cells and adoptively transferred (AT) into naive WT mice. Tolerized (LZT) mice and mice with CHS without cell transfer served as control animals. Subsequently, recipients and control groups were sensitized and challenged with either TNCB or DNFB, respectively. Increase in ear thickness (mean $\pm$ SD) is detected. One representative of 3 experiments is shown. * P < .05, ** P < .01, and *** P < .001. n.s., Not significant. tol, Tolerized; sens/chall, sensitized/challenged.

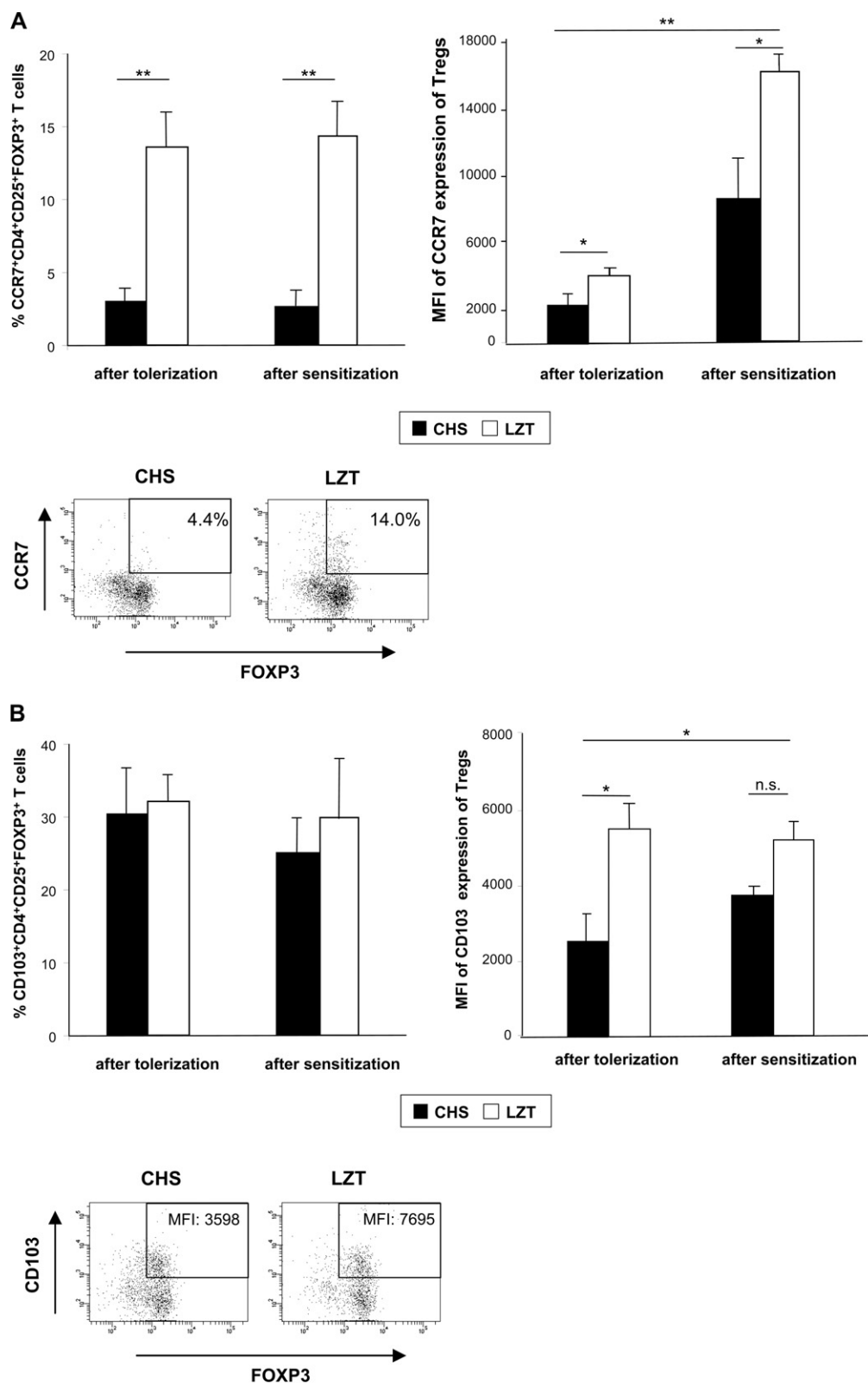


FIG 3. Induction of an activated phenotype of CD4⁺CD25⁺FOXP3⁺ Treg cells during LZT. Mice were tolerized or solvent treated, and thereafter, some groups were sensitized. Flow cytometric analysis of CD4⁺CD25⁺FOXP3⁺ Treg cells of LN cells was performed. Percentages $\pm$ SD (left panel) and mean fluorescence intensity (MFI $\pm$ SD, right panel) of CCR7⁺ (A) or CD103⁺ (B) Treg cells (gated on CD4⁺CD25⁺FOXP3⁺ T cells) were analyzed after induction of tolerance or solvent treatment (24 hours after the last application of last tolerizing dose) or after sensitization (day 5 after sensitization). Pooled data of 4 experiments and 1 representative experiment after tolerization are shown. C, After tolerization (LZT) or solvent treatment (CHS),

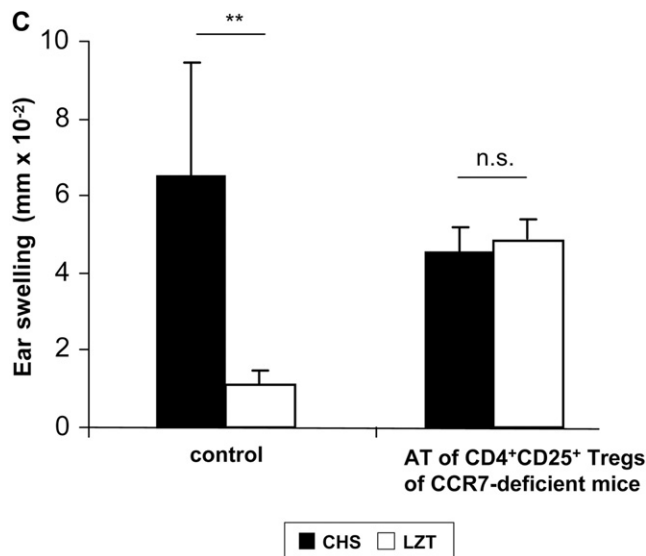


FIG 3. (Continued)

functional relevance of IL-10–producing CD4⁺CD25⁺ Treg cells during the induction of LZT.

To exclude a general deficiency in the suppressor function of Treg cells in IL-10–deficient mice, we additionally inhibited the effect of IL-10 during the induction of tolerance through the use of an IL-10 receptor blocking antibody. As depicted in Fig 8, D, the development of LZT was significantly prevented by blocking IL-10 activity *in vivo*, indicating a pivotal role for IL-10 during the induction of LZT.

DISCUSSION

In this study we demonstrated the pivotal role for CD4⁺CD25⁺FOXP3⁺ Treg cells, which protect against allergic skin reactions in mice with LZT. Activation of Treg cells with low amounts of contact allergen results in IL-10 production, interaction with LN-related CD11c⁺ DCs, and induction of a tolerogenic DC phenotype (Fig 9). This process was followed by the induction of CD8⁺ Treg cells that inhibit the development of T_C1-mediated CHS (Fig 9).

In human subjects it has been shown that CD4⁺CD25⁺ T cells of healthy nonallergic subjects regulate the activation of both naive and nickel-specific T cells.^{34,35} In comparison, CD4⁺CD25⁺ T cells obtained from subjects with nickel allergy exhibited a limited or absent capacity to control CD4⁺ or CD8⁺ nickel-specific T cells. These data indicate that CD4⁺CD25⁺ cells are critically involved in the regulation of hapten-specific immune responses in human subjects as well and therefore are in line with our results in the murine model of LZT.^{34,35} In addition, LZT was not only effective in a preventive setting but also when the induction of tolerance was performed after sensitization, indicating a therapeutic potential of LZT and emphasizing the importance of this model (see Fig E7, A-C, in this article's Online Repository at www.jacionline.org).

The critical role for CD4⁺CD25⁺ Treg cells in hapten-specific immune responses was previously shown in the murine model of CHS. After depletion of Treg cells, a significant increase in skin inflammation and the CD8⁺ T_C1 immune reaction of CHS was observed.^{36,37} Similarly, the absence of CD4⁺CD25⁺ Treg cells during tolerance induction resulted in a markedly enhanced allergic skin reaction compared with that seen in control CHS mice. These results suggest that the threshold for sensitization was reduced under these conditions and that repeated applications of low amounts of allergens in combination with the subsequent sensitization induced an exaggerated allergic immune response.

It has been shown that Treg cells require activation to function as suppressor T cells. In mice with LZT, Treg cells were activated by low subimmunogenic doses of allergens. The induction of tolerance by low doses of antigens has been known for a long time, and one mechanism might be the extrathymic conversion of conventional CD4⁺ T cells into FOXP3⁺ Treg cells by minute amounts of antigens.^{38,39} In our hands suppression by activated Treg cells is allergen nonspecific, a mode of action that is reported for several models analyzing the function of Treg cells *in vivo*.⁴⁰ In contrast, regulatory CD8⁺ T cells of subjects with LZT act in a hapten-specific manner.⁵ Recently, it was reported that these CD8⁺ Treg cells interacted with killer DCs during the effector phase of LZT, resulting in DC-derived TNF production, apoptosis of effector CD8⁺ T cells of CHS, and prevention of the allergic skin response.⁴¹

ICOS-expressing, suppressive, antigen-specific FOXP3⁺ T cells were identified that control the generation of CD8⁺ effector T cells of patients with CHS.⁴² In subjects with LZT, we did not observe an increase in the number of ICOS⁺ Treg cells but found the induction of a CCR7⁺ Treg cell subset known to be critically involved in the suppressor function of Treg cells *in vivo* and in cutaneous skin inflammation.²³ Because direct analysis of LZT in CCR7^{-/-} mice was no viable approach because CCR7^{-/-} mice are known to exhibit an altered CHS reaction dependent on the protocol and hapten used, we performed transfer experiments.^{23,43} Injection of CCR7^{-/-} Treg cells after tolerization did not induce LZT in the recipients, suggesting a pivotal role for this Treg cell subpopulation in tolerance to contact allergens. This failure might be due to the absence of regulatory properties of CCR7⁺ Treg cells, which are functionally relevant for LZT, or might simply reflect the reduced capacity of CCR7^{-/-} Treg cells for LN homing. Future studies will address this issue in more detail.

FOXP3⁺ Treg cells exert suppression by contact-dependent mechanisms and mechanisms mediated by soluble factors, such as IL-10.^{15,42,44-47} In mice with LZT, an increase in IL-10⁺CD4⁺CD25⁺ Treg cell numbers was observed *in vitro* and *in vivo*. We highlighted the importance of Treg cell–derived IL-10 in mice with LZT by showing that reconstitution of T cell–deficient mice with CD4⁺IL-10⁺ T cells but not with CD4⁺IL-10⁺ T cells depleted of CD25⁺ Treg cells allows for the development of LZT. Additional mechanisms, such as adenosine-dependent egress of Treg cells or gap junction–mediated communication between Treg cells and DCs have been demonstrated to be of relevance

CD4⁺CD25⁺ T cells were purified from LN cells of CCR7^{-/-} mice and adoptively transferred (AT) into naive WT mice. Tolerized (LZT) mice and mice with CHS without cell transfer served as control animals. Subsequently, recipients and control groups were sensitized and challenged. The ear-swelling response was assessed after challenge (mean ± SD). **P* < .05 and ***P* < .01. *n.s.*, Not significant.

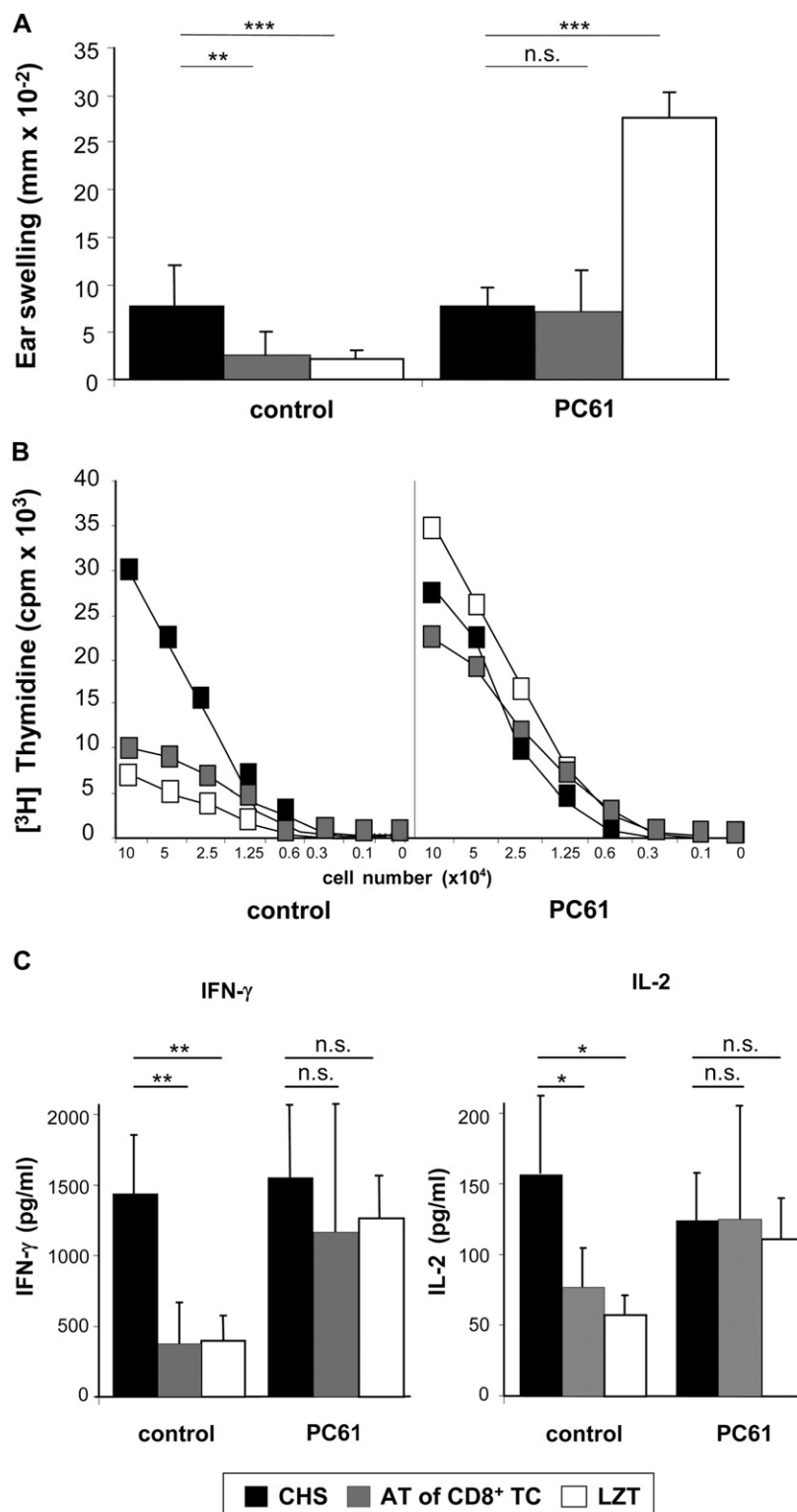


FIG 4. CD4⁺CD25⁺ Treg cells are required for generation of CD8⁺ Treg cells of mice with LZT. Depletion of CD4⁺CD25⁺FOXP3⁺ Treg cells was induced by injection of PC61 (anti-CD25 mAb; right panels, PC61). Control mice received an IgG control antibody (left panels, control). After tolerization, CD8⁺ T cells were obtained and adoptively transferred into naive WT mice (gray bars, squares). Mice without cell transfer served as control animals for CHS (black bars, squares) or LZT (white bars, squares). Recipients and control mice were sensitized and challenged. **A** and **B**, The allergic inflammatory response (Fig 4, A) and proliferation of T cells (Fig 4, B) were assessed as described in the legend for Fig 1. Fig 4, A and B, Results of one representative experiment of 3 independent experiments are shown. **C**, Release of IFN- γ and IL-2 by T cells was assessed (mean $\pm$ SD). Pooled data of 3 independent experiments are demonstrated. * P < .05, ** P < .01, and *** P < .001. n.s., Not significant.

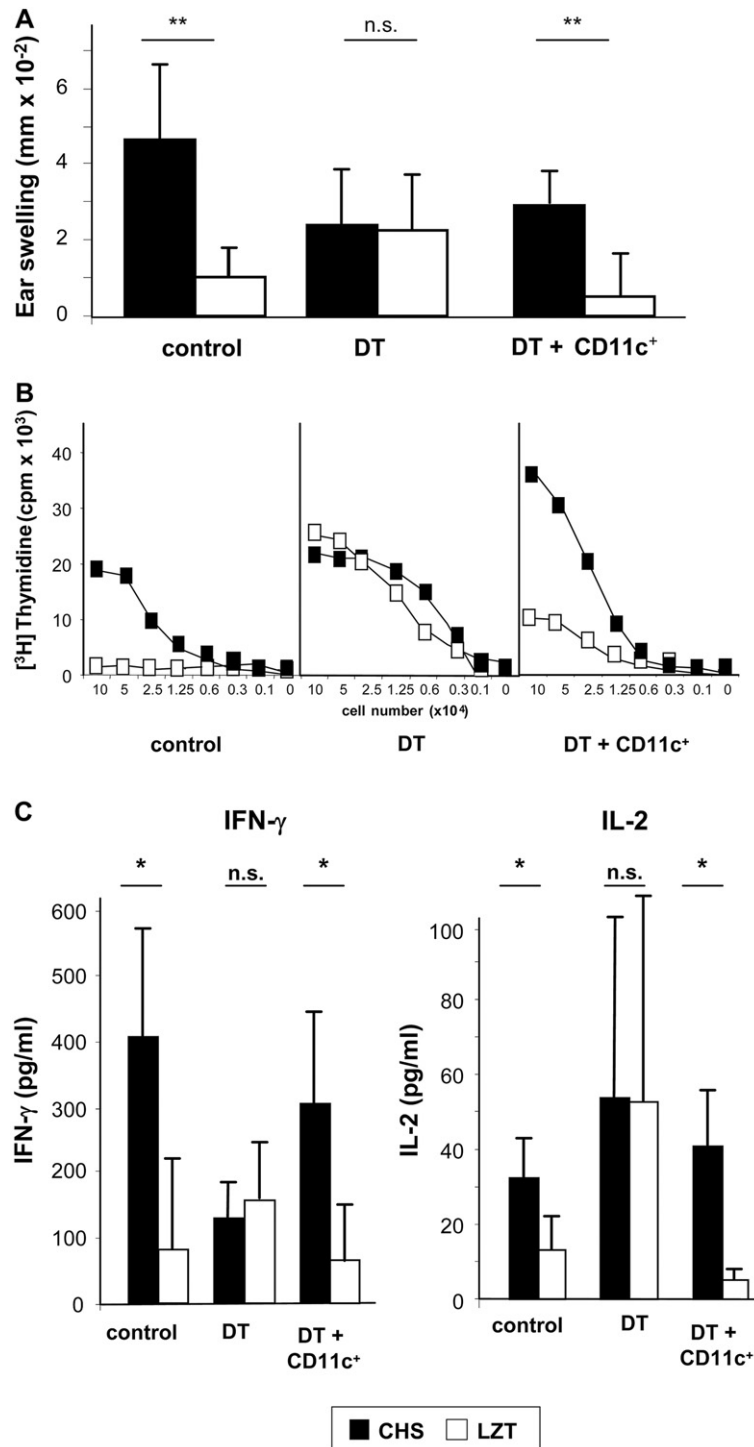


FIG 5. Tolerogenic CD11c⁺ DCs are required for tolerance induction. Depletion of CD11c⁺ DCs was induced by injection of DT into CD11c-DTR mice. PBS administration served as a control. Subsequently, mice were tolerized (LZT) or treated with solvent (CHS), and after recovery of CD11c⁺ DCs, sensitization and challenge were conducted. In some experiments mice were reconstituted with CD11c⁺ DCs after depletion of DCs and before tolerance induction. **A**, The ear-swelling response was assessed after challenge (mean $\pm$ SD). **B**, T-cell proliferation after hapten-specific restimulation was detected (mean value of triplicates in counts per minute). One representative result of 3 experiments is shown. **C**, Amounts of IFN- γ and IL-2 by T cells was detected (mean $\pm$ SD). Pooled data of 3 independent experiments are depicted. * P < .05 and ** P < .01. n.s., Not significant.

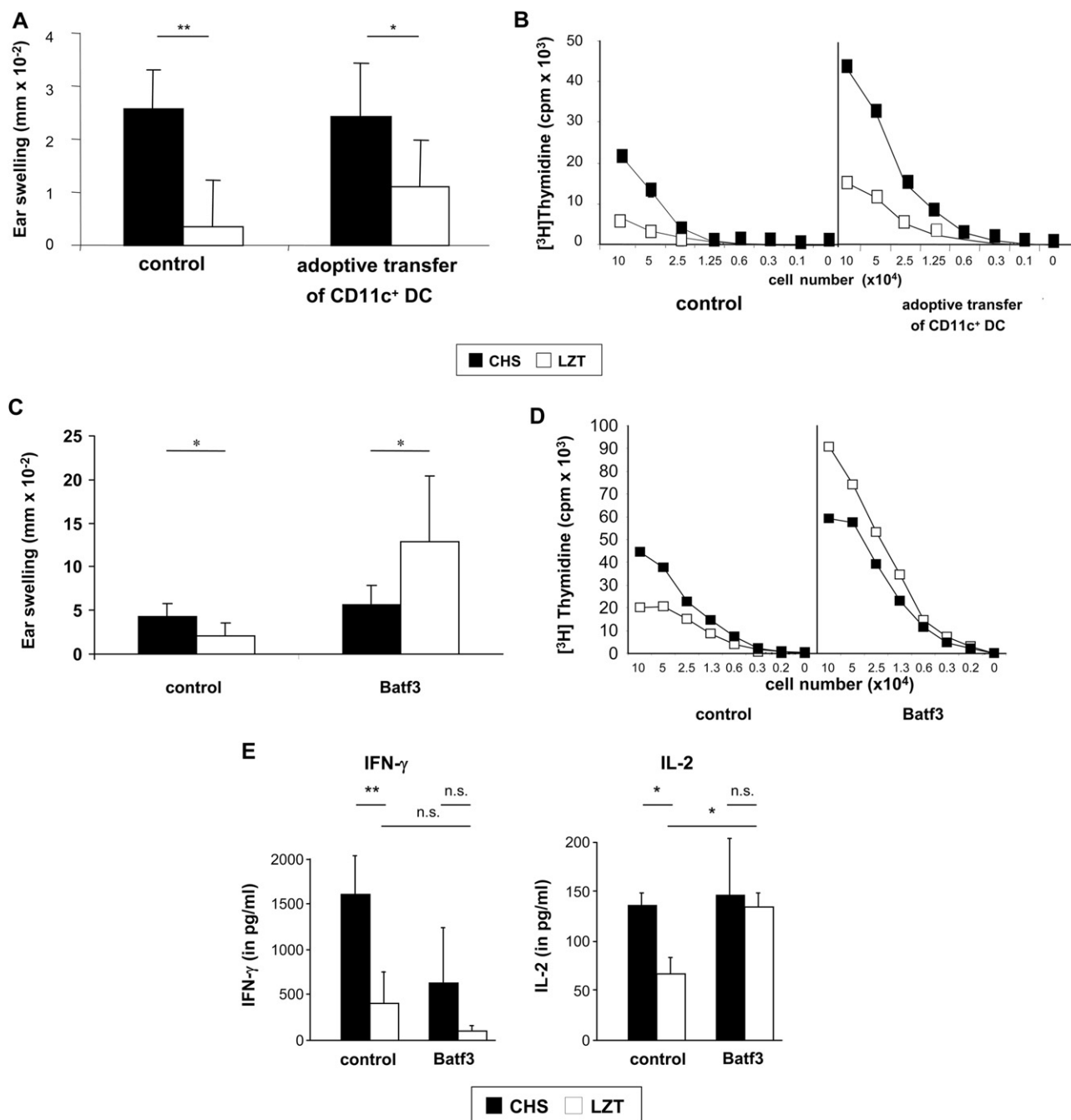


FIG 6. LZT is transferable by CD11c⁺ DCs and abrogated in the absence of CD8⁺CD11c⁺ DCs. **A** and **B**, Animals were tolerized or solvent treated. Thereafter, CD11c⁺ DCs were isolated from peripheral LNs and adoptively transferred to naive mice. Recipients and control animals (CHS and LZT without cell transfer) were sensitized and challenged. Skin inflammation (Fig 6, **A**) and T-cell proliferation (Fig 6, **B**) was assessed as described in the legend for Fig 1. One of 3 experiments with similar results is depicted. **C-E**, Solvent (CHS) or TNCB (LZT) was applied epicutaneously 10 times onto Batf3 mice lacking CD8⁺CD11c⁺ DCs. WT mice served as control animals. Subsequently, animals were sensitized and challenged, as previously described. Fig 6, **C**, The ear-swelling response was assessed after challenge (mean ± SD). Fig 6, **D**, T-cell proliferation was depicted (mean value of triplicates in counts per minute) after restimulation. Fig 6, **C** and **D**, Data represent one of 3 independent experiments with similar results. Fig 6, **E**, Pooled data of release of IFN-γ and IL-2 by T cells of 3 independent experiments are shown (means ± SD). **P* < .05 and ***P* < .01. n.s., Not significant.

for Treg cell function in hapten-specific cutaneous immune responses.^{28,48} In line with these former reports, we found a direct crosstalk between Treg cells and tolerogenic DCs mediated through gap junctions in the skin-draining LNs after tolerization.

One key mechanism of Treg cell-mediated suppression seems to be the control of DC function.⁴⁹⁻⁵² In our study the induction of a tolerogenic DC phenotype by Treg cells is finally supported by adoptive transfer experiments of DCs obtained from mice that

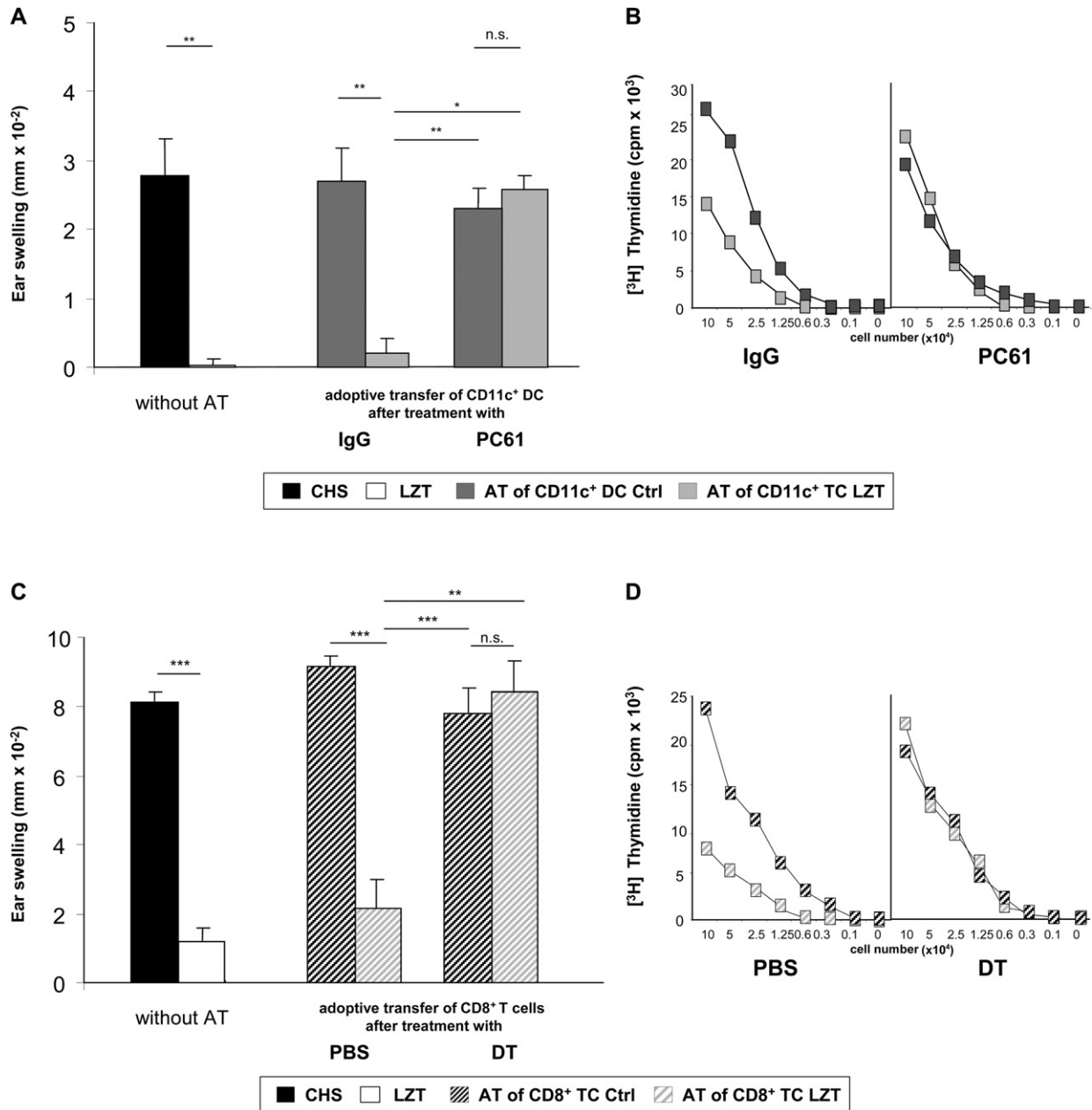


FIG 7. CD4⁺CD25⁺ Treg cells are required for activation of tolerogenic CD11c⁺ DCs followed by induction of hapten-specific regulatory CD8⁺ T cells of LZT. **A** and **B**, Mice were treated with anti-CD25 mAb to deplete Treg cells or with IgG control antibody. Subsequently, after tolerance induction (AT CD11c⁺ LZT) or solvent treatment (AT CD11c⁺ Ctrl), CD11c⁺ DC were transferred. Recipients and control animals (CHS and LZT without cell transfer) were sensitized and challenged. Fig 7, **A**, Ear thickness was assessed in mean $\pm$ SD after challenge. One representative experiment of 3 is shown. Fig 7, **B**, T-cell proliferation (mean values of triplicates in counts per minute) was detected. Results of one of 3 independent experiments are depicted. **C** and **D**, Injections of DT into CD11c-DTR mice were performed to deplete CD11c⁺ DCs. After tolerization (AT of CD8⁺ LZT) or solvent treatment (AT of CD8⁺ Ctrl), CD8⁺ T cells were obtained and injected into naive recipients. Recipients and control animals (CHS and LZT without cell transfer) were sensitized and challenged. Fig 7, **C**, Absolute increase in ear thickness ($\pm$ SD) was assessed after challenge. Fig 7, **D**, Proliferation (mean values of triplicates in counts per minute) of T cells is shown. One representative result of 3 experiments is demonstrated. **E**, CD4⁺CD25⁺ Treg cells (Ly5.2) were obtained after tolerization (LZT) or solvent treatment (CHS) and labeled with calcein. Thereafter, CD4⁺CD25⁺ Treg cells were transferred into naive Ly5.1 WT recipients that were subsequently sensitized. Skin-draining LN cells were harvested, and calcein-positive Ly5.1⁺ CD11c⁺ DCs were detected by using flow cytometry. One of 3 representative experiments is shown. **F**, After transfer of tolerogenic or control CD45.2 CD11c⁺ DCs into the recipients (CD45.1 mice), tissue sections of skin-draining LNs were stained for FOXP3 (red nuclear staining) and CD45.2 DCs (depicted in green). Interactions of FOXP3⁺ Treg cells and CD45.2 DCs in mice with LZT or with CHS of representative tissue sections (left panels) and expressed as the mean number of Treg cells per DC in 20 high-power fields (right panel) are demonstrated. Bar scale = 50 μ m. * P < .05, ** P < .01, and *** P < .001 or as indicated. n.s., Not significant.

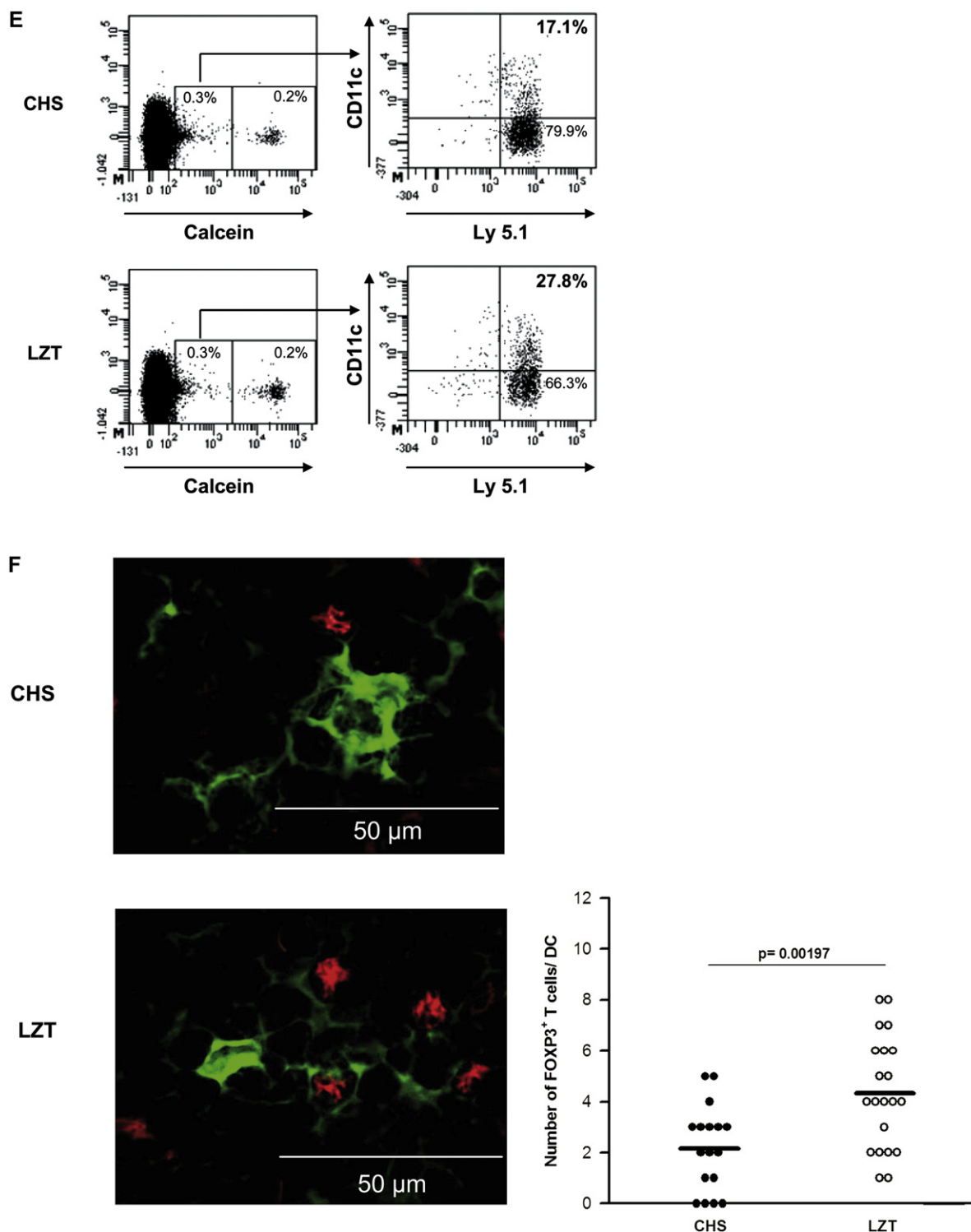
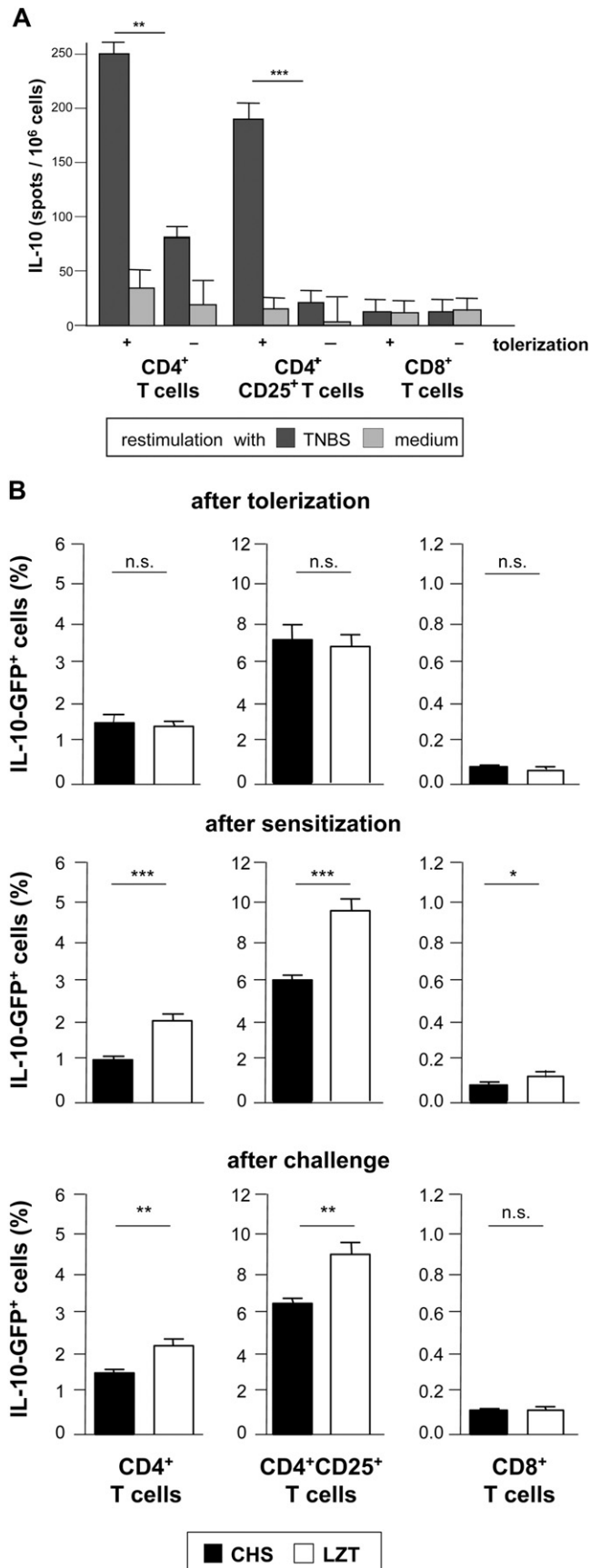


FIG 7. (Continued)

were tolerized in the absence of Treg cells (anti-CD25 treatment or DT in DEREG mice) and had lost entirely their capacity to transfer tolerance. CD8⁺CD11c⁺ DCs are capable of cross-presenting to CD8⁺ T cells, and many studies strongly implied CD8⁺ DCs as contributors to the maintenance of peripheral tolerance.^{25,53-55} These data concur with our results identifying CD8⁺CD11c⁺ DCs as the tolerogenic subpopulation in mice with LZT.

Thus crosstalk between CD4⁺CD25⁺FOXP3⁺ Treg cells and CD11c⁺ DCs after epicutaneous exposure to low amounts of allergens is likely to shift the quality of the hapten-specific immune response toward a tolerance reaction protecting against allergy (Fig 9). Subsequently, tolerogenic DCs induce CD8⁺ Treg cells of mice with LZT, preventing the development of an allergic cutaneous inflammation (Fig 9). This improved



understanding of the involved mechanisms can be used to identify novel therapeutic targets to modulate and/or prevent allergic immune responses.

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Clinical implications: LZT is a main protective mechanism for prevention of allergic contact dermatitis, and crosstalk between Treg cells and tolerogenic DC cells can be used as a target for novel therapeutic approaches.

REFERENCES

- Lushniak BD. The importance of occupational skin diseases in the United States. *Int Arch Occup Environ Health* 2003;76:325-30.
- Gocinski BL, Tigelaar RE. Roles of CD4⁺ and CD8⁺ T cells in murine contact sensitivity revealed by in vivo monoclonal antibody depletion. *J Immunol* 1990; 144:4121-8.
- Xu H, DiJulio NA, Fairchild RL. T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon gamma-producing (Tc1) effector CD8⁺ T cells and interleukin (IL) 4/IL-10-producing (Th2) negative regulatory CD4⁺ T cells. *J Exp Med* 1996; 183:1001-12.
- Elmets CA, Bergstresser PR, Tigelaar RE, Wood PJ, Streilein JW. Analysis of the mechanism of unresponsiveness produced by haptens painted on skin exposed to low dose ultraviolet radiation. *J Exp Med* 1983;158:781-94.
- Steinbrink K, Sorg C, Macher E. Low zone tolerance to contact allergens in mice: a functional role for CD8⁺ T helper type 2 cells. *J Exp Med* 1996;183: 759-68.
- Dubois B, Chapat L, Goubier A, Papiernik M, Nicolas JF, Kaiserlian D. Innate CD4⁺CD25⁺ regulatory T cells are required for oral tolerance and inhibition of CD8⁺ T cells mediating skin inflammation. *Blood* 2003;102:3295-301.
- Steinbrink K, Kolde G, Sorg C, Macher E. Induction of low zone tolerance to contact allergens in mice does not require functional Langerhans cells. *J Invest Dermatol* 1996;107:243-7.
- Maurer M, Seidel-Guyenot W, Metz M, Knop J, Steinbrink K. Critical role of IL-10 in the induction of low zone tolerance to contact allergens. *J Clin Invest* 2003; 112:432-9.

FIG 8. CD4⁺CD25⁺ Treg cell-derived IL-10 is critical during the induction phase of LZT. **A**, IL-10 release from CD4⁺, CD4⁺CD25⁺, and CD8⁺ T cells was assessed by means of ELISpot after hapten-specific restimulation. The results of one of 2 representative experiments are demonstrated, and the pooled data of 5 animals are depicted. **B**, *In vivo* IL-10 transcription of CD4⁺, CD4⁺CD25⁺, and CD8⁺ T cells from skin-draining LNs was analyzed by using IL-10 transcriptional reporter mice (Vert-X). Pooled data (n = 19 mice per group) from 4 independent experiments are depicted (percentage of positive cells ± SEM). **C**, CD4⁺ and CD8⁺ or CD4⁺ T cells depleted of CD25⁺ Treg cells, respectively, were obtained from untreated WT or IL-10^{-/-} mice for reconstitution of T cell-deficient Rag1^{-/-} mice. Subsequently, the mice were repeatedly painted with tolerizing doses of TNBS (LZT) or solvent treated (CHS), followed by sensitization and challenge. The allergic skin inflammation was assessed based on the increase in ear swelling (mean ± SD). **D**, Animals were treated with an IL-10 receptor blocking antibody (*α*IL-10R) or isotype control. Ear thickness in absolute values ± SDs was assessed 24 hours later. One of 2 representative experiments is demonstrated. *P < .05, **P < .01, and ***P < .001. n.s., Not significant.

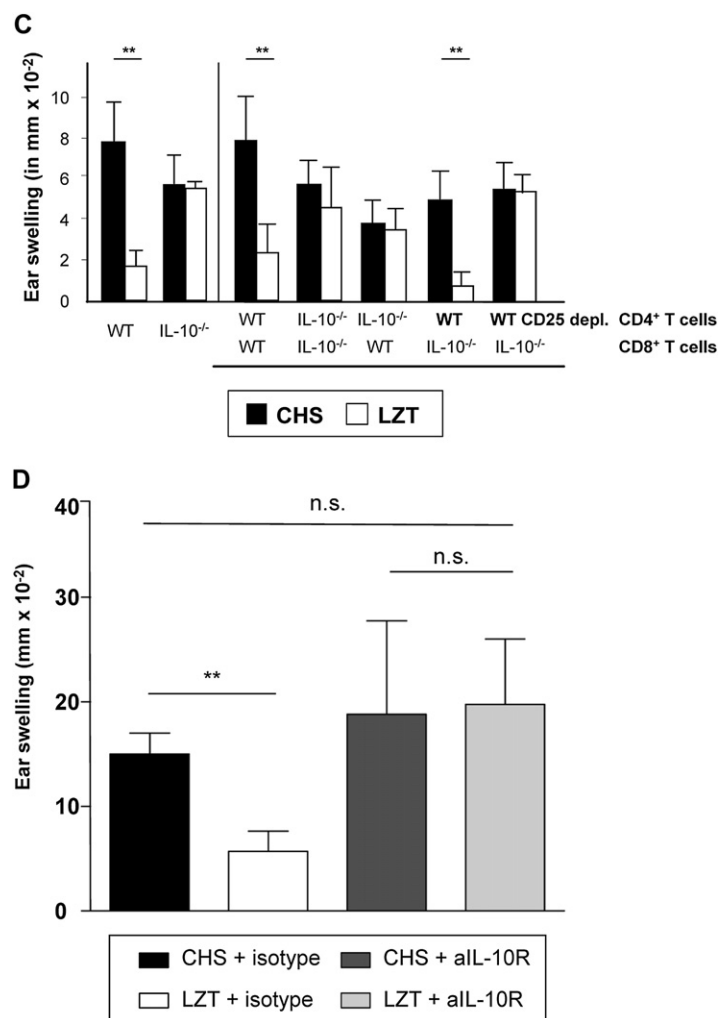


FIG 8. (Continued)

- Seidel-Guyenot W, Alt R, Perschon S, Knop J, Steinbrink K. B cells are not required for T cell priming in low zone tolerance to contact allergens and contact hypersensitivity. *Eur J Immunol* 2004;34:3082-90.
- Seidel-Guyenot W, Perschon S, Dechant N, Alt R, Knop J, Steinbrink K. Low zone tolerance induced by systemic application of allergens inhibits Tc1-mediated skin inflammation. *J Allergy Clin Immunol* 2006;117:1170-7.
- Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol* 2010;11:7-13.
- Tang Q, Bluestone JA. The Foxp3⁺ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 2008;9:239-44.
- McMurphy AN, Gillies J, Allan SE, Passerini L, Gambineri E, Roncarolo MG, et al. Point mutants of forkhead box P3 that cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked have diverse abilities to reprogram T cells into regulatory T cells. *J Allergy Clin Immunol* 2010;126:1242-51.
- Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* 2005;6:331-7.
- Shevach EM. Mechanisms of foxp3⁺ T regulatory cell-mediated suppression. *Immunity* 2009;30:636-45.
- Rolland JM, Gardner LM, O'Hehir RE. Functional regulatory T cells and allergen immunotherapy. *Curr Opin Allergy Clin Immunol* 2010;10:559-66.
- Comes A, Rosso O, Orengo AM, Di Carlo E, Sorrentino C, Meazza R, et al. CD25⁺ regulatory T cell depletion augments immunotherapy of micrometastases by an IL-21-secreting cellular vaccine. *J Immunol* 2006;176:1750-8.
- Lehmann J, Huehn J, de la Rosa M, Maszyra F, Kretschmer U, Krenn V, et al. Expression of the integrin alpha Ebeta 7 identifies unique subsets of CD25⁺ as well as CD25⁻ regulatory T cells. *Proc Natl Acad Sci U S A* 2002;99:13031-6.
- Stephens LA, Mason D. CD25 is a marker for CD4⁺ thymocytes that prevent autoimmune diabetes in rats, but peripheral T cells with this function are found in both CD25⁺ and CD25⁻ subpopulations. *J Immunol* 2000;165:3105-10.
- Wan YY, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc Natl Acad Sci U S A* 2005;102:5126-31.
- Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, et al. Selective depletion of Foxp3⁺ regulatory T cells induces a scurfy-like disease. *J Exp Med* 2007;204:57-63.
- Stephens GL, Andersson J, Shevach EM. Distinct subsets of FoxP3⁺ regulatory T cells participate in the control of immune responses. *J Immunol* 2007;178:6901-11.
- Schneider MA, Meingassner JG, Lipp M, Moore HD, Rot A. CCR7 is required for the in vivo function of CD4⁺ CD25⁺ regulatory T cells. *J Exp Med* 2007;204:735-45.
- Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, et al. Batf3 deficiency reveals a critical role for CD8alpha⁺ dendritic cells in cytotoxic T cell immunity. *Science* 2008;322:1097-100.
- Shortman K, Heath WR. The CD8⁺ dendritic cell subset. *Immunol Rev* 2010;234:18-31.
- Edelson BT, Kc W, Juang R, Kohyama M, Benoit LA, Klekotka PA, et al. Peripheral CD103⁺ dendritic cells form a unified subset developmentally related to CD8alpha⁺ conventional dendritic cells. *J Exp Med* 2010;207:823-36.
- Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol* 2007;7:610-21.
- Ring S, Karakhanova S, Johnson T, Enk AH, Mahnke K. Gap junctions between regulatory T cells and dendritic cells prevent sensitization of CD8⁺ T cells. *J Allergy Clin Immunol* 2010;125:237-46.

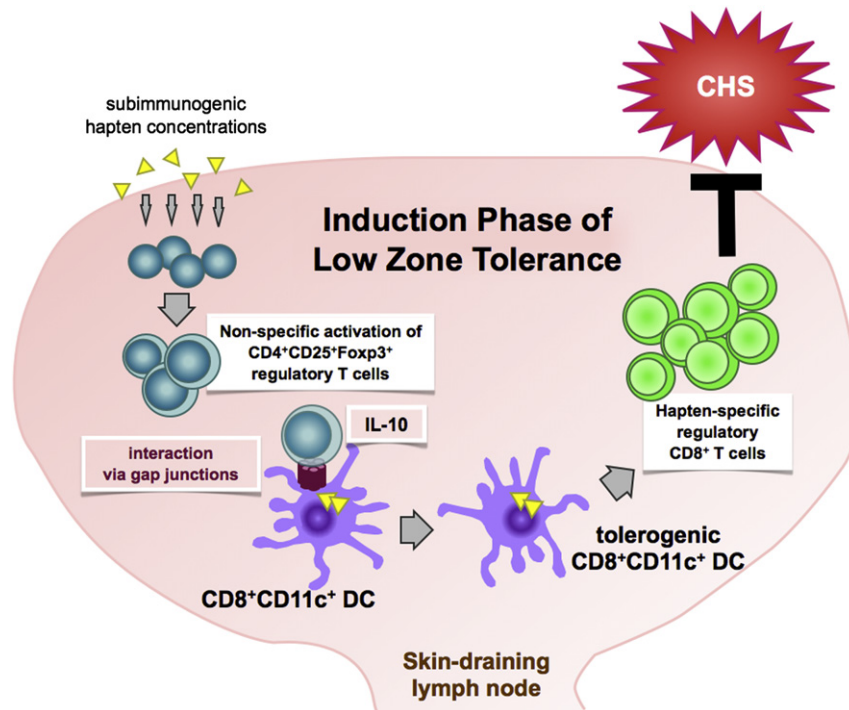


FIG 9. Immune mechanisms during the induction phase of LZT. Repeated applications of low subimmunogenic doses of contact allergens induce a non-hapten-specific activation of IL-10-producing CD4⁺CD25⁺FOXP3⁺ Treg cells that interact with LN-related CD8⁺CD11⁺ DCs. This crosstalk results in a tolerogenic phenotype of DCs that induce the generation of CD8⁺ Treg cells of mice with LZT. These CD8⁺ Treg cells of mice with LZT efficiently inhibit the development of T_C1-mediated allergic skin immune responses in a hapten-specific manner.

29. Bopp T, Becker C, Klein M, Klein-Hessling S, Palmethofer A, Serfling E, et al. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* 2007;204:1303-10.
30. Fonseca PC, Nihei OK, Savino W, Spray DC, Alves LA. Flow cytometry analysis of gap junction-mediated cell-cell communication: advantages and pitfalls. *Cytometry A* 2006;69:487-93.
31. Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* 2006;212:28-50.
32. Madan R, Demircik F, Surianarayanan S, Allen JL, Divanovic S, Trompette A, et al. Nonredundant roles for B cell-derived IL-10 in immune counter-regulation. *J Immunol* 2009;183:2312-20.
33. Sun J, Madan R, Karp CL, Braciale TJ. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. *Nat Med* 2009;15:277-84.
34. Cavani A. T regulatory cells in contact hypersensitivity. *Curr Opin Allergy Clin Immunol* 2008;8:294-8.
35. Cavani A, Nasorri F, Prezzi C, Sebastiani S, Albanesi C, Girolomoni G. Human CD4⁺ T lymphocytes with remarkable regulatory functions on dendritic cells and nickel-specific Th1 immune responses. *J Invest Dermatol* 2000;114:295-302.
36. Ikezawa Y, Nakazawa M, Tamura C, Takahashi K, Minami M, Ikezawa Z. Cyclophosphamide decreases the number, percentage and the function of CD25⁺ CD4⁺ regulatory T cells, which suppress induction of contact hypersensitivity. *J Dermatol Sci* 2005;39:105-12.
37. Kish DD, Gorbachev AV, Fairchild RL. CD8⁺ T cells produce IL-2, which is required for CD4⁺CD25⁺ T cell regulation of effector CD8⁺ T cell development for contact hypersensitivity responses. *J Leukoc Biol* 2005;78:725-35.
38. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 2005;6:1219-27.
39. O'Garra A, Vieira P. Regulatory T cells and mechanisms of immune system control. *Nat Med* 2004;10:801-5.
40. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T. Regulatory T cells: how do they suppress immune responses? *Int Immunol* 2009;21:1105-11.
41. Luckey U, Maurer M, Schmidt T, Lorenz N, Seebach B, Metz M, et al. T cell killing by tolerogenic dendritic cells protects mice from allergy. *J Clin Invest* 2011;121:3860-71.
42. Vocanson M, Rozieres A, Hennino A, Poyet G, Gaillard V, Renaudineau S, et al. Inducible costimulator (ICOS) is a marker for highly suppressive antigen-specific T cells sharing features of TH17/TH1 and regulatory T cells. *J Allergy Clin Immunol* 2010;126:280-9.
43. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional micro-environments in secondary lymphoid organs. *Cell* 1999;99:23-33.
44. Powrie F. Immune regulation in the intestine: a balancing act between effector and regulatory T cell responses. *Ann N Y Acad Sci* 2004;1029:132-41.
45. Couper KN, Blount DG, Wilson MS, Hafalla JC, Belkaid Y, Kamanaka M, et al. IL-10 from CD4⁺CD25⁺Foxp3⁺CD127⁺ adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. *PLoS Pathog* 2008;4:e1000004.
46. Maeda A, Schwarz A, Bullinger A, Morita A, Peritt D, Schwarz T. Experimental extracorporeal photopheresis inhibits the sensitization and effector phases of contact hypersensitivity via two mechanisms: generation of IL-10 and induction of regulatory T cells. *J Immunol* 2008;181:5956-62.
47. Akdis M, Burgler S, Cramer R, Eiwegger T, Fujita H, Gomez E, et al. Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. *J Allergy Clin Immunol* 2011;127:701-21.
48. Ring S, Oliver SJ, Cronstein BN, Enk AH, Mahnke K. CD4⁺CD25⁺ regulatory T cells suppress contact hypersensitivity reactions through a CD39, adenosine-dependent mechanism. *J Allergy Clin Immunol* 2009;123:1287-96.
49. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3⁺ regulatory T cell function. *Science* 2008;322:271-5.
50. Schildknecht A, Brauer S, Brenner C, Lahl K, Schild H, Sparwasser T, et al. FoxP3⁺ regulatory T cells essentially contribute to peripheral CD8⁺ T-cell tolerance induced by steady-state dendritic cells. *Proc Natl Acad Sci U S A* 2010;107:199-203.
51. Misra N, Bayry J, Lacroix-Desmazes S, Kazatchkine MD, Kaveri SV. Cutting edge: human CD4⁺CD25⁺ T cells restrain the maturation and antigen-presenting function of dendritic cells. *J Immunol* 2004;172:4676-80.

52. Liu K, Victora GD, Schwickert TA, Guermonprez P, Meredith MM, Yao K, et al. In vivo analysis of dendritic cell development and homeostasis. *Science* 2009;324:392-7.
53. Dudziak D, Kamphorst AO, Heidkamp GF, Buchholz VR, Trumpfheller C, Yamazaki S, et al. Differential antigen processing by dendritic cell subsets in vivo. *Science* 2007;315:107-11.
54. Belz GT, Behrens GM, Smith CM, Miller JF, Jones C, Lejon K, et al. The CD8alpha⁺ dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. *J Exp Med* 2002;196:1099-104.
55. Scheinecker C, McHugh R, Shevach EM, Germain RN. Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. *J Exp Med* 2002;196:1079-90.

METHODS

Mice

T and B cell-deficient ($\text{Rag1}^{-/-}$), IL-10-deficient ($\text{IL-10}^{-/-}$), CD11c-DTR, Ly5.1, and CD45.1 mice on a C57BL/6 background and WT C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, Me). C57BL/6 DREG mice (FOXP3-DTR-GFP) were kindly provided by Tim Sparwasser (Twincore), C57BL/6 Vert-X mice (IL-10 transcriptional reporter mice) by Christopher Karp (University of Cincinnati College of Medicine, Cincinnati, Ohio), $\text{CCR7}^{-/-}$ by M. Lipp (Max Delbrück Center, Berlin, Germany)^{E1} and $\text{Batf3}^{-/-}$ and control 129SvEv mice by K. Murphy (Department of Pathology and Immunology, Washington University School of Medicine, St Louis, Mo, and Howard Hughes Medical Institute, Washington University School of Medicine, St Louis, Mo) and H. C. Probst (Institute of Immunology, University Medical Center Mainz, Germany).^{E2} All murine strains were bred in the Animal Care Facility of the University of Mainz, the University Medical Center Freiburg, or both. All animal studies were approved by the Animal Care Committee of Rhineland-Palatinate and the Regierungspräsidium Freiburg and supervised by the Animal Protection Representatives of the University Medical Center Mainz and the University Freiburg Medical Center.

Reagents and antibodies

TNCB (2,4,6-trinitro-1-chlorobenzene) was purchased from VeZerf Laborsynthesen (Idar-Oberstein, Germany). 2,4-Dinitrobenzenesulfonate (DNBS), 2,4,6-trinitro-benzenesulfonic acid (TNBS), and DNFB (fluoro-2,4-dinitrobenzene) were purchased from Sigma (Taufkirchen, Germany). Antibodies against CD4, CD8, and Thy1.2 were from BD Biosciences, Heidelberg, Germany). Foxp3 and antibodies against FOXP3 (FJK-16s), CD25, CD28, CD38, CCR7, CD80, CD86, MHC class II, CD44, CD154, CD127, CD134, CD162, CTLA-4, ICOS, PD-1, CD103, CD62L, GITR, CD69, CD273-CD276, B7-H4, and IgG_{2a} (eBR2s) were from eBioscience (San Diego, Calif).

Flow cytometry and intracellular FOXP3 detection

Antibody staining and intracellular FOXP3 staining were performed according to the manufacturer's protocol. The detection of antibody was done by using flow cytometry with the BD LSR2 or BD Canto (BD Biosciences) and analyzed with DIVA6.0 software.

Preparation of LN cells, enriched CD8⁺ T cells, CD4⁺ T cells, CD4⁺CD25⁺ T cells, and CD11c⁺ DCs for cell culture and adoptive transfer

Twenty-four hours after challenge, LZT induction, or solvent treatment, auricular, cervical, and inguinal LNs were taken. LN cells (10^6 cells/200 μL) were resuspended in RPMI-1640 (BioWhittaker, Verviers, Belgium) supplemented with 2% heat-inactivated syngeneic normal murine serum. CD8⁺, CD4⁺, CD4⁺CD25⁻, and CD4⁺CD25⁺ T cells were purified ($\geq 95\%$ and $\geq 85\%$ for CD4⁺CD25⁺ T cells) with CD8a (Ly-2) or CD4 (L3T4) antibody-coated magnetic beads, respectively, or the CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (MACS System; Miltenyi Biotec, Bergisch Gladbach, Germany) or anti-CD4-biotinylated/SA-beads, anti-CD25-biotinylated/SA-PE beads, and anti-PE beads (MACS System, Miltenyi Biotec). The enriched CD4⁺ or CD4⁺CD25⁻ (2×10^7 cells), CD8⁺ (1×10^7 cells), or CD4⁺CD25⁺ Treg cells (1.5×10^6 cells) were injected intravenously into untreated $\text{Rag1}^{-/-}$ or WT mice. CD11c⁺ cells were purified ($\geq 85\%$ purity) with anti-CD11c-coated beads (MACS System, Miltenyi Biotec). After tolerance induction or solvent treatment as controls, CD11c⁺ DCs were isolated and injected intravenously ($1-2 \times 10^5$), or in some experiments CD45.2 DCs were injected subcutaneously as indicated into untreated CD45.1 recipients.

Haptenation of LN or spleen cells

LN or spleen cells (10^7 cells/mL) were incubated in 10 mmol/L 2,4,6-trinitro-benzenesulfonic acid or 1000 μg 2,4-dinitrobenzenesulfonate in HBSS (without Ca^{++} , Mg^{++} , and Phenol Red; BioWhittaker) for 10 minutes, subsequently washed 3 times in RPMI-1640, and resuspended in RPMI-1640 supplemented with 2% heat-inactivated syngeneic murine serum.

Cell culture and proliferation assay

LN cells (10^6 /200 μL per well) were resuspended after haptenation in RPMI-1640 supplemented with 2% heat-inactivated syngeneic normal murine serum and incubated in 96-well U-bottom microtiter plates at $37^\circ\text{C}/5\% \text{CO}_2$. LN cells without hapten-specific restimulation served as controls (data are not shown because nonspecific proliferation was consistently low in all experiments). After 24 hours, cultured cells were pulsed with 1 μCi of tritiated thymidine (PerkinElmer, Rodgau, Germany) for 18 hours. Incorporated radioactivity was determined by using a liquid scintillation counter (1205 Betaplate; LKB Wallac, Bromma, United Kingdom). Results are expressed as mean counts per minute of triplicate wells. For T-cell experiments, CD4⁺/CD8⁺ T cells or CD4⁺CD25⁺ T cells were finally resuspended in RPMI-1640 complete (1×10^5 cells/100 μL) stimulated with 1×10^6 (in 100 μL) hapten-modified and irradiated (3000 rad) spleen cells (highest T-cell/spleen cell ratio, 1:10), and cells were pulsed after 24 or 48 hours, as described above.

Cytokine ELISAs

Supernatants of LN cells were harvested after 24, 48, and 72 hours and kept frozen at -80°C until further use. Cytokine ELISAs for IL-2 (BD Biosciences) and IFN- γ (R&D Systems Europe, Wiesbaden, Germany) were performed according to the manufacturer's protocol.

ELISpot for IL-10 production

An ELISpot was performed according to the manufacturer's instructions (BD Biosciences) and as previously described^{E3} by using CD4⁺ CD8⁺, and CD4⁺CD25⁺ T cells separated by means of positive selection with magnetobeads, as described above.

Analysis of IL-10 transcription *in vivo*

In vivo IL-10 transcription of T cells from skin-draining LNs was analyzed at different time points by using IL-10 transcriptional reporter mice (VertX). Solvent or tolerizing doses of TNCB were applied epicutaneously 10 times, and mice were sensitized and challenged as described above. IL-10 signal (GFP) was analyzed by using flow cytometry in CD4⁺, CD4⁺CD25⁺, and CD8⁺ T cells of skin-draining LNs obtained directly after tolerization (day 11), after sensitization (day 16), and 24 hours after challenge (day 17).

Blocking of IL-10 *in vivo*

For blocking IL-10 *in vivo*, mice were injected intraperitoneally with 500 μg of anti-mouse IL-10 receptor (clone 1B1.3a; Becton Dickinson, Heidelberg, Germany) 1 day before LZT induction. Injection of equivalent amounts of rat IgG₁ (Jackson ImmunoResearch Laboratories, Suffolk, United Kingdom) served as a control.

Calcein staining and gap junction interactions

CD4⁺CD25⁺ Treg cells were obtained from skin-draining LN cells of C57BL/6 Ly5.2 WT mice after application of solvent or tolerizing doses of the hapten TNCB and purified as previously described. Treg cells (1×10^7) were incubated with 1 $\mu\text{mol/L}$ calcein AM (Invitrogen, Darmstadt, Germany) at 37°C in $5\% \text{CO}_2$ for 30 minutes.^{E4} After intensive washing, 2 to 3×10^6 Treg cells were injected intravenously into Ly5.1 recipients, which were sensitized after 24 hours. Twenty-four hours after injection and 72 hours after sensitization, skin-draining LNs were harvested, and calcein transfer was detected with BD LSR2 (BD Bioscience) and analyzed with DIVA6.0 software.

Immunofluorescence staining and analysis of Treg cell/DC interactions

Immunofluorescence of cryosections was performed with the TSA Cy3, TSA Cy5, TSA Fluorescein system (PerkinElmer) and a fluorescence microscope (Olympus IX81; Olympus, Center Valley, Pa). Tissue sections

of popliteal LNs were cut, air-dried, fixed with 4% paraformaldehyde, and stained overnight at 4°C in humidified chamber with primary antibody. The following primary antibodies were used: CD4, CD11c (both from BD Biosciences), FOXP3, and CD45.2 (both from eBioscience). Then slides were incubated at room temperature for 30 minutes with biotinylated secondary antibody (anti-Armenian hamster: Dianova, Hamburg, Germany; anti-rat: BD PharMingen). We evaluated the number of FOXP3⁺ Treg cells, which tightly interact with (injected) CD45.2 DCs in 20 high-power fields of representative histologic sections, and the mean number of Treg cells per DC was calculated for mice with LZT and mice with CHS.

REFERENCES

- E1. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 1999;99:23-33.
- E2. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, et al. Batf3 deficiency reveals a critical role for CD8alpha⁺ dendritic cells in cytotoxic T cell immunity. *Science* 2008;322:1097-100.
- E3. Maurer M, Seidel-Guyenot W, Metz M, Knop J, Steinbrink K. Critical role of IL-10 in the induction of low zone tolerance to contact allergens. *J Clin Invest* 2003;112:432-9.
- E4. Bopp T, Becker C, Klein M, Klein-Hessling S, Palmethofer A, Serfling E, et al. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* 2007;204:1303-10.

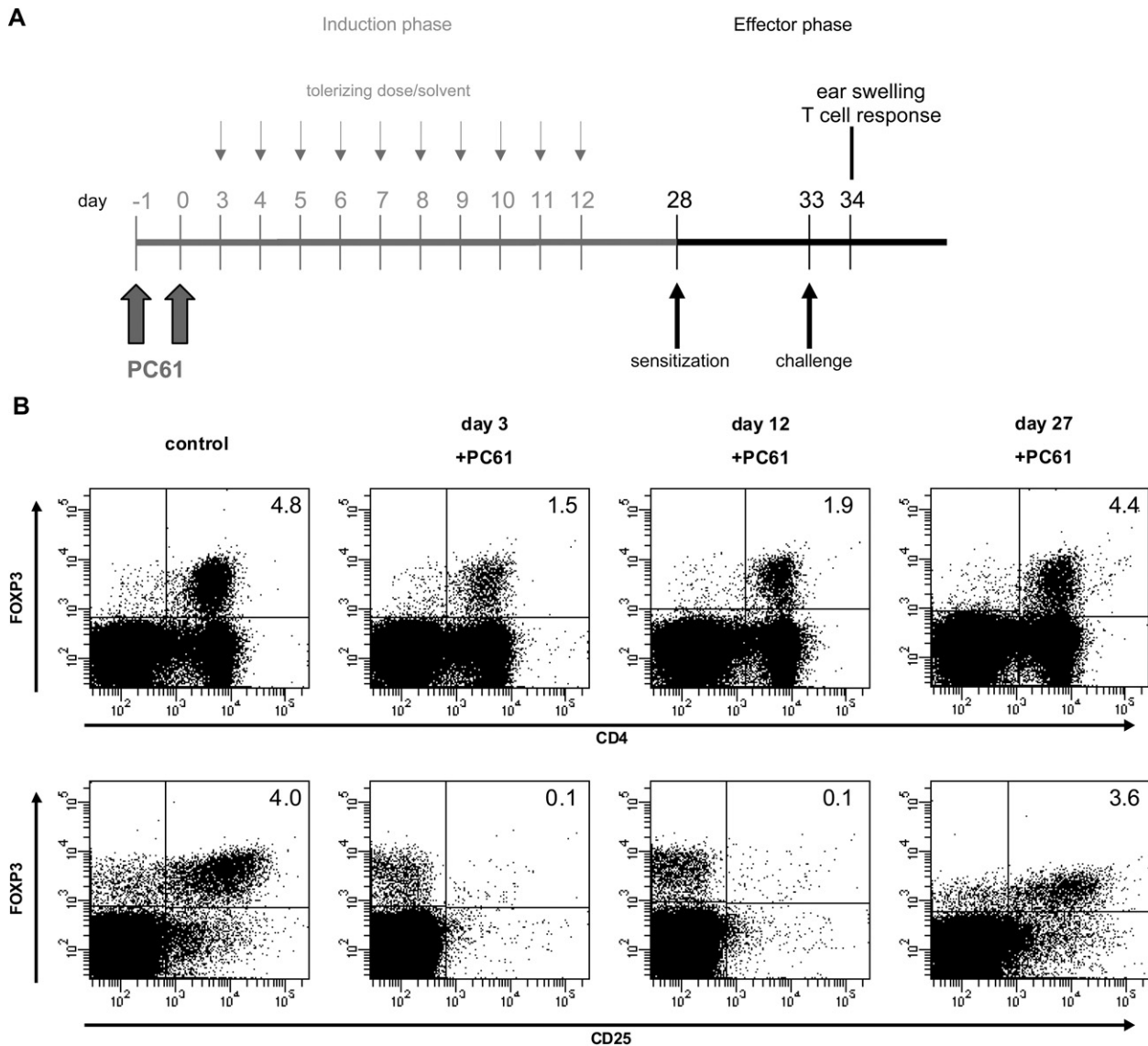


FIG E1. Evaluation of Treg cell depletion after application of an anti-CD25 mAb (PC61) during tolerization. **A**, Treatment of mice was performed as depicted. **B**, Flow cytometric analysis was conducted to analyze percentages of CD4⁺FOXP3⁺ (upper panel) and FOXP3⁺CD25⁺ (gated on CD4⁺ T cells, lower panel) Treg cells at day 0 (before treatment), days 3 and 12 (phase of tolerization), and day 27 (before sensitization) after application of the anti-CD25 mAb (PC61). Representative data of 6 individual experiments are depicted.

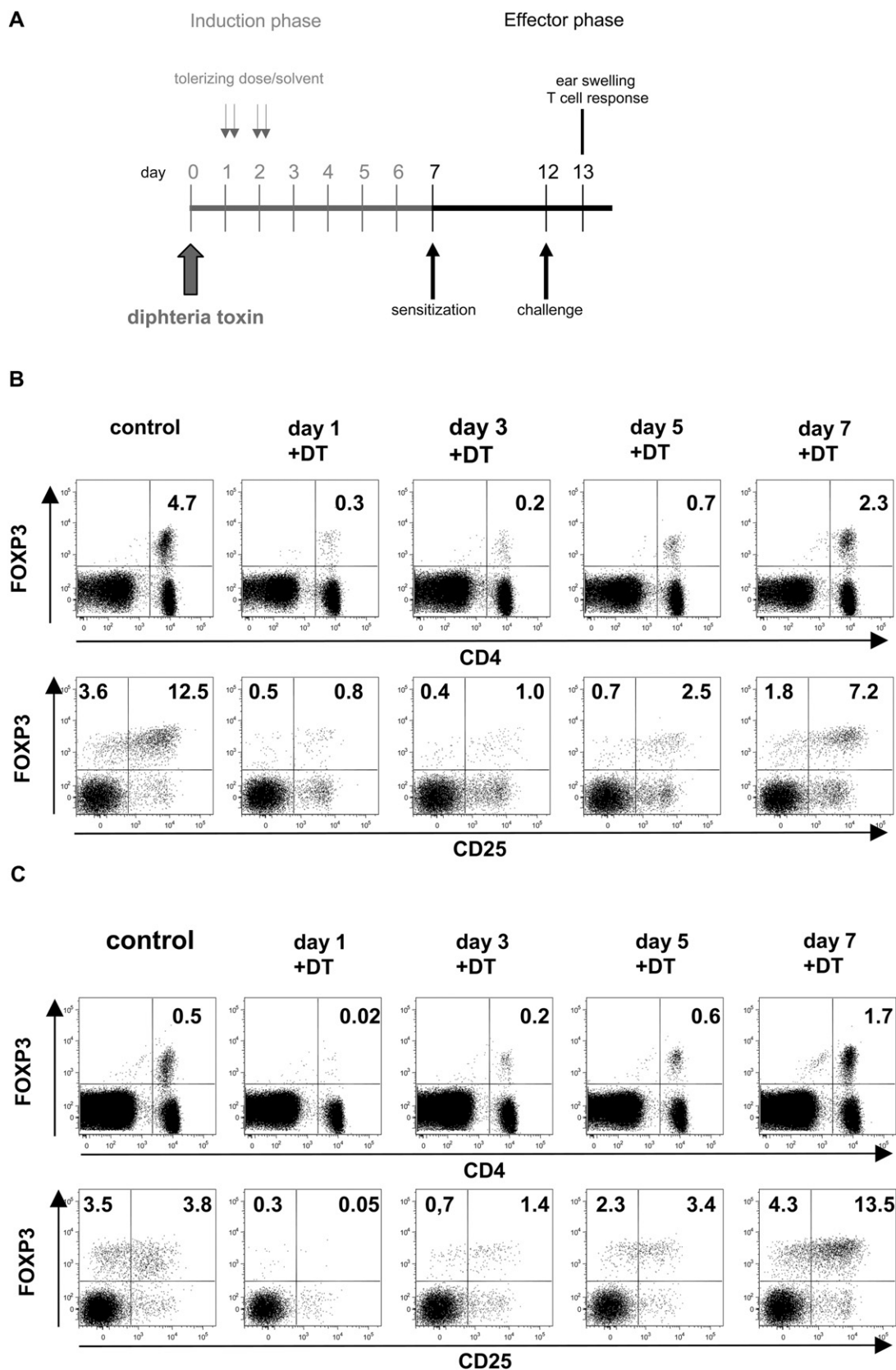
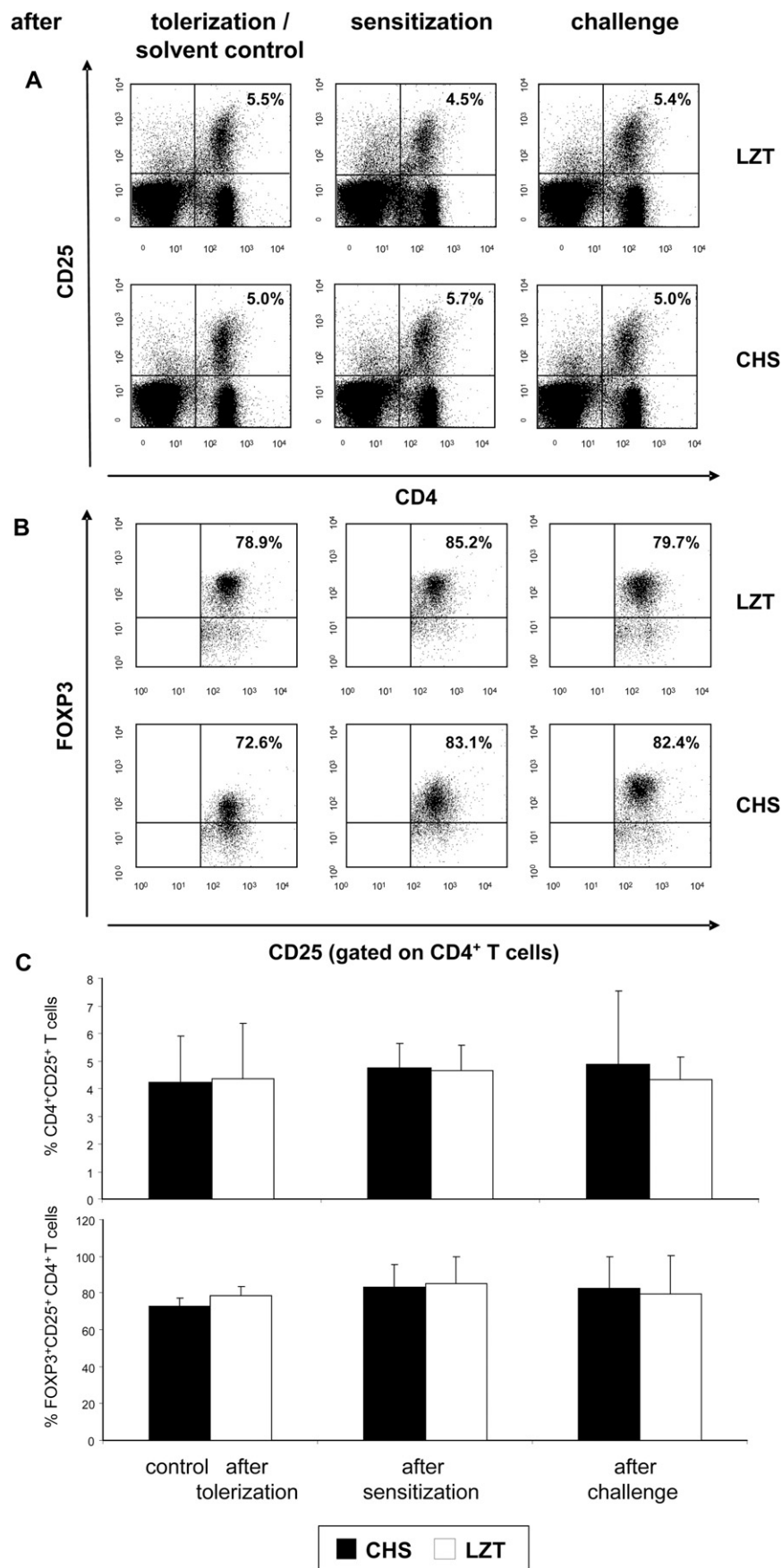


FIG E2. Efficacy of Treg cell depletion in DERE mice. **A**, Mice were injected with DT before tolerization. **B** and **C**, Flow cytometric analysis of skin-draining LN cells (Fig E2, **B**) and peripheral blood (Fig E2, **C**) was performed. Percentages of CD4⁺FOXP3⁺ Treg cells (lymphocyte gate, upper panels) and FOXP3⁺CD25⁺ Treg cells (gated on CD4⁺ T cells, lower panels) were analyzed before treatment at days 0, 1, and 3 (phase of tolerization) and days 5 and day 7 (before sensitization) after application of DT.



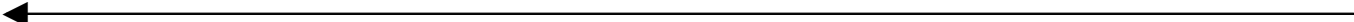


FIG E3. Percentages of CD4⁺CD25⁺FOXP3⁺ Treg cells are equivalent in tolerized versus control mice. Animals were painted with either TNCB or solvent during tolerization and subsequently sensitized and challenged. LNs were obtained after tolerization, sensitization, and challenge. Analyses were performed for CD25⁺ (**A-C**) and CD25^{high} (**D-F**) T cells. Expression of CD4 and CD25 (Fig E3, *A* and *D*) and FOXP3 (gated on CD4⁺CD25⁺ T cells; Fig E3, *B* and *E*) was analyzed by means of flow cytometry. One representative experiment (Fig E3, *A*, *B*, *D*, and *E*) and pooled data of 4 experiments (Fig E3, *C* and *F*) are demonstrated.

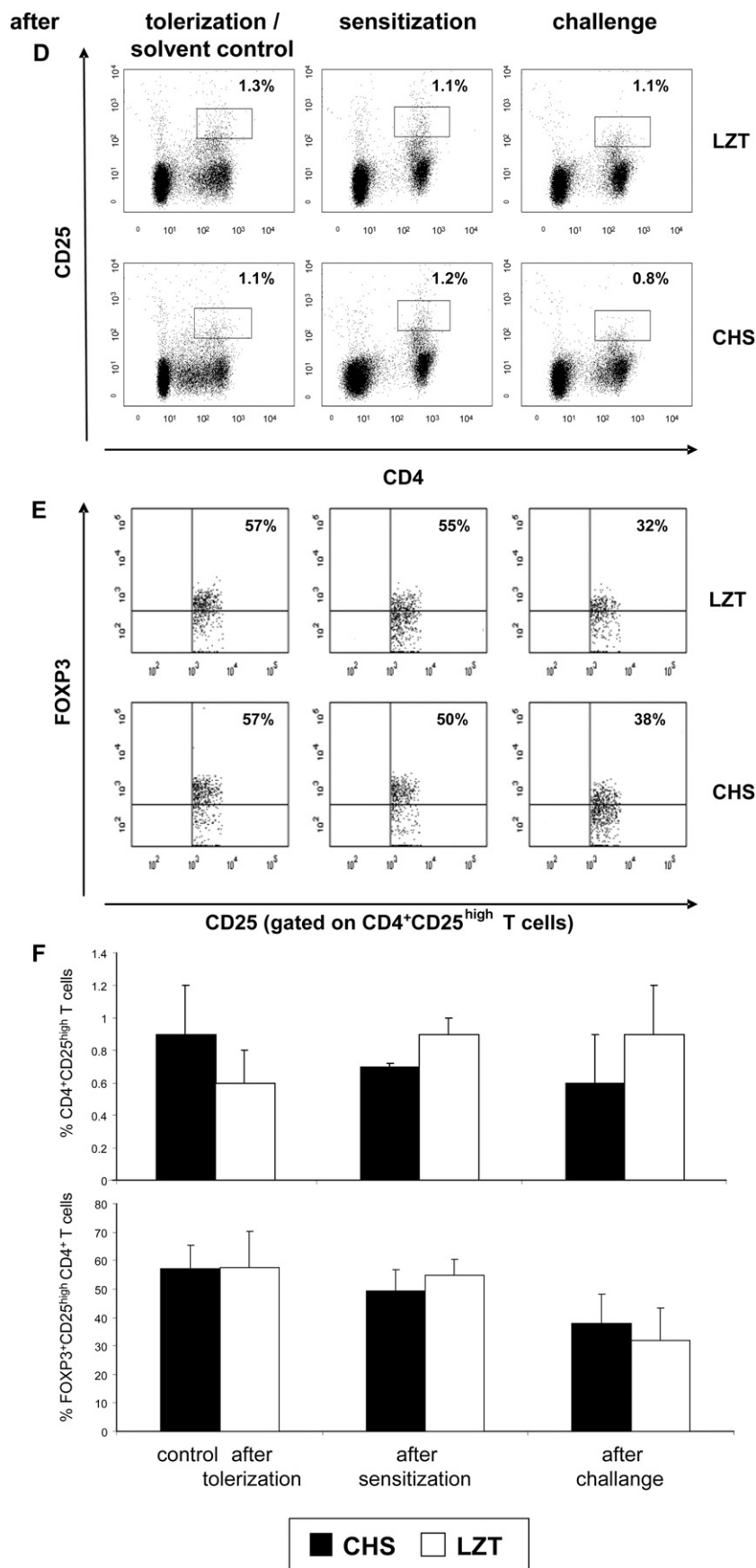


FIG E3. (Continued).

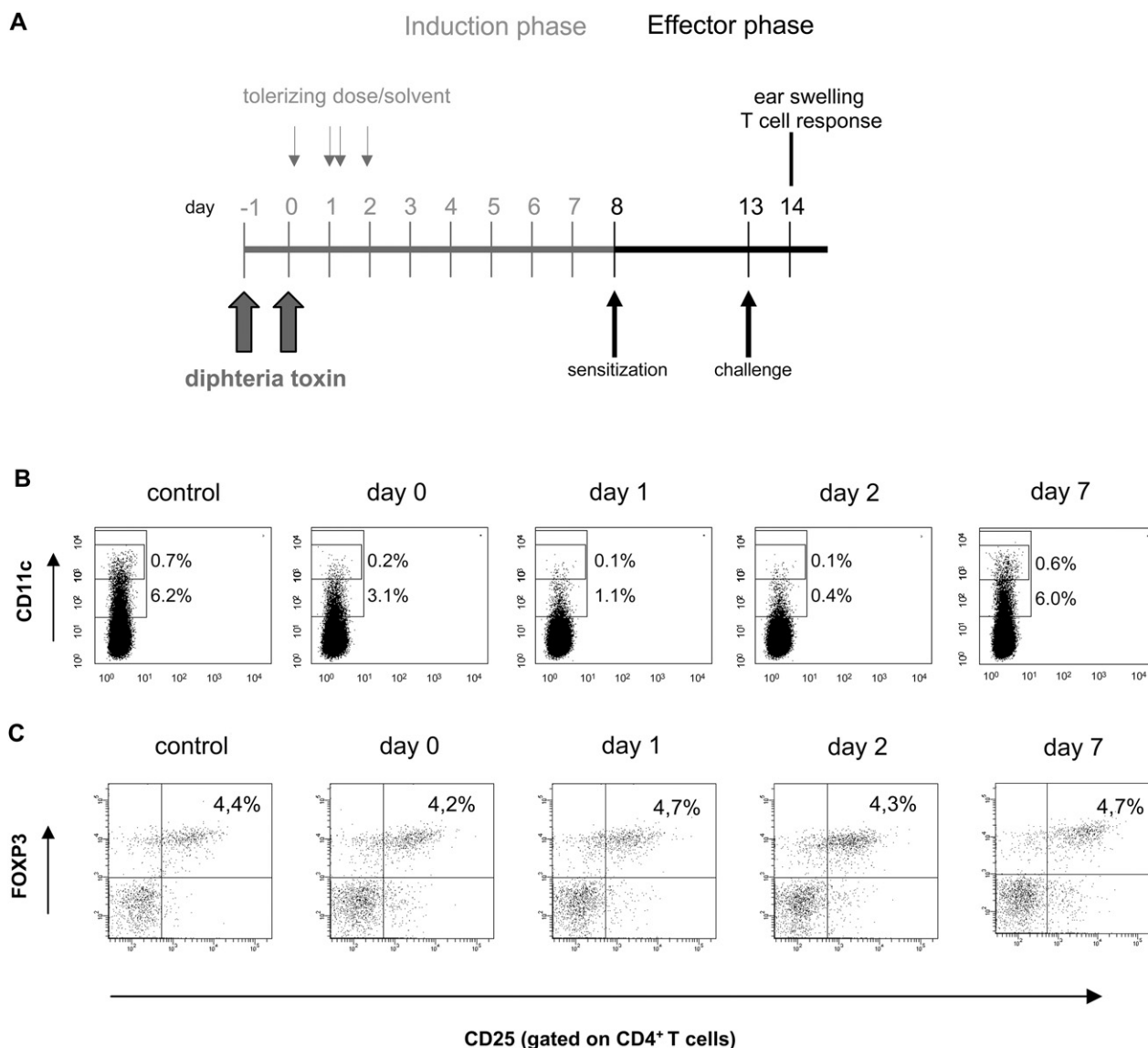


FIG E4. Evaluation of CD11c⁺ DC depletion. **A**, CD11c-DTR mice were injected with DT at days -1 and 0. Subsequently, mice were tolerized by means of 4 tolerizing doses every 12 hours. **B**, Numbers of CD11c⁺ DCs in skin-draining LNs were assessed by using flow cytometry at days 0, 1, 2, and 7 after treatment with DT. For the experiments, mice were tolerized by application of low amounts of TNCB or treated with solvent in the absence of CD11c⁺ DCs. After recovery of CD11c⁺ DCs, sensitization and challenge were performed. **C**, Percentages of CD4⁺CD25⁺FOXP3⁺ Treg cells in draining LNs after DT treatment are demonstrated.

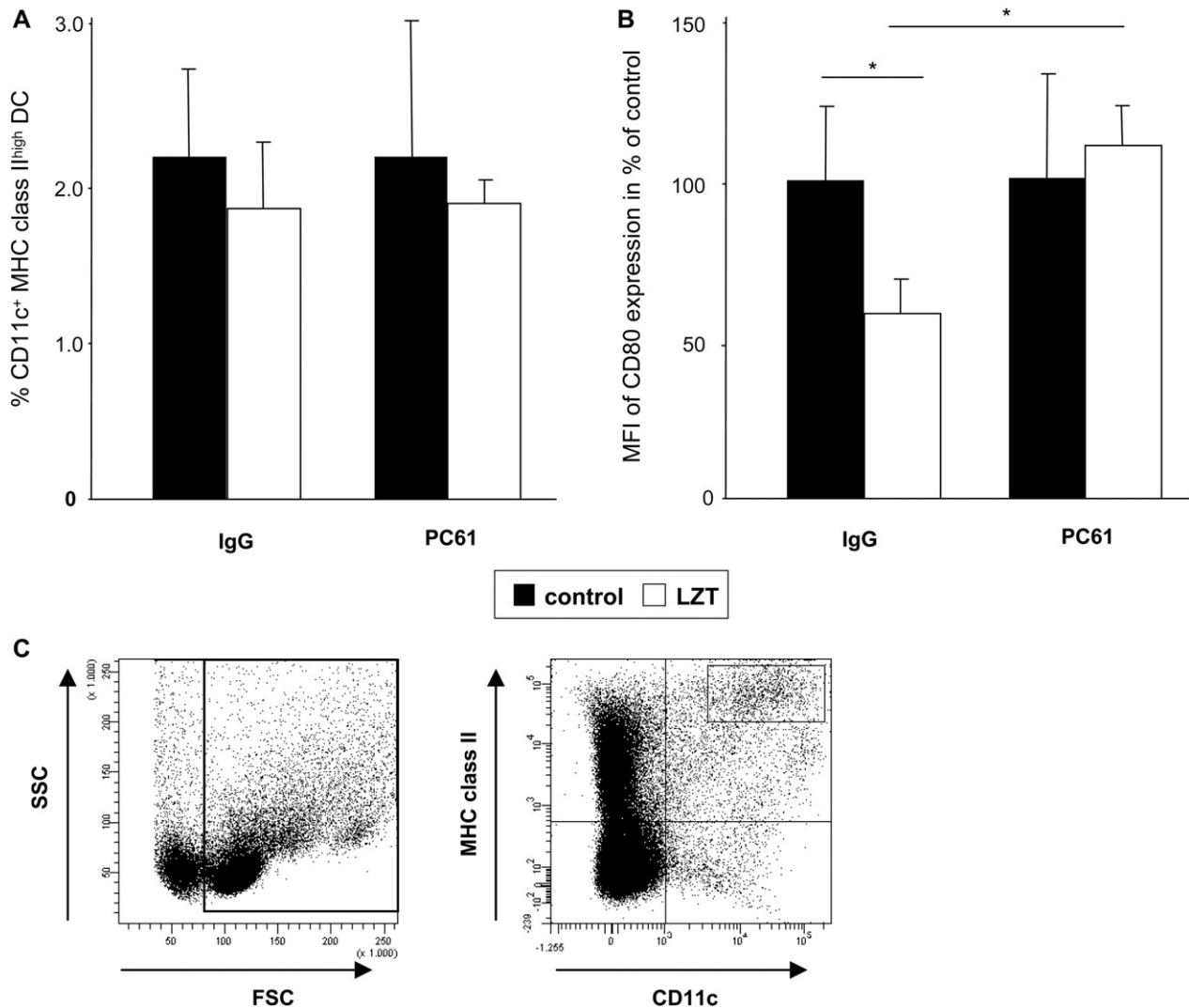


FIG E5. Increased CD80 expression on LN DCs after depletion of Treg cells. Depletion of CD4⁺CD25⁺FOXP3⁺ Treg cells was induced by means of injection of PC61 (anti-CD25 mAb). Mice were tolerized (LYT) or treated with solvent (controls). The phenotype of LN DCs was assessed by means of flow cytometry after tolerance induction. **A** and **B**, Percentages of CD11c⁺ MHC class II^{high} DCs in LN cells were demonstrated as mean $\pm$ SD (Fig E5, **A**), and mean fluorescence intensity (MFI) of CD80 expression of CD11c⁺ MHC class II^{high} DCs was depicted as percentages $\pm$ SDs of CHS controls (= 100%; Fig E5, **B**). **C**, Gating strategy for CD11c⁺ MHC class II^{high} DCs. FSC, Forward scatter; SSC, side scatter. * $P < .05$.

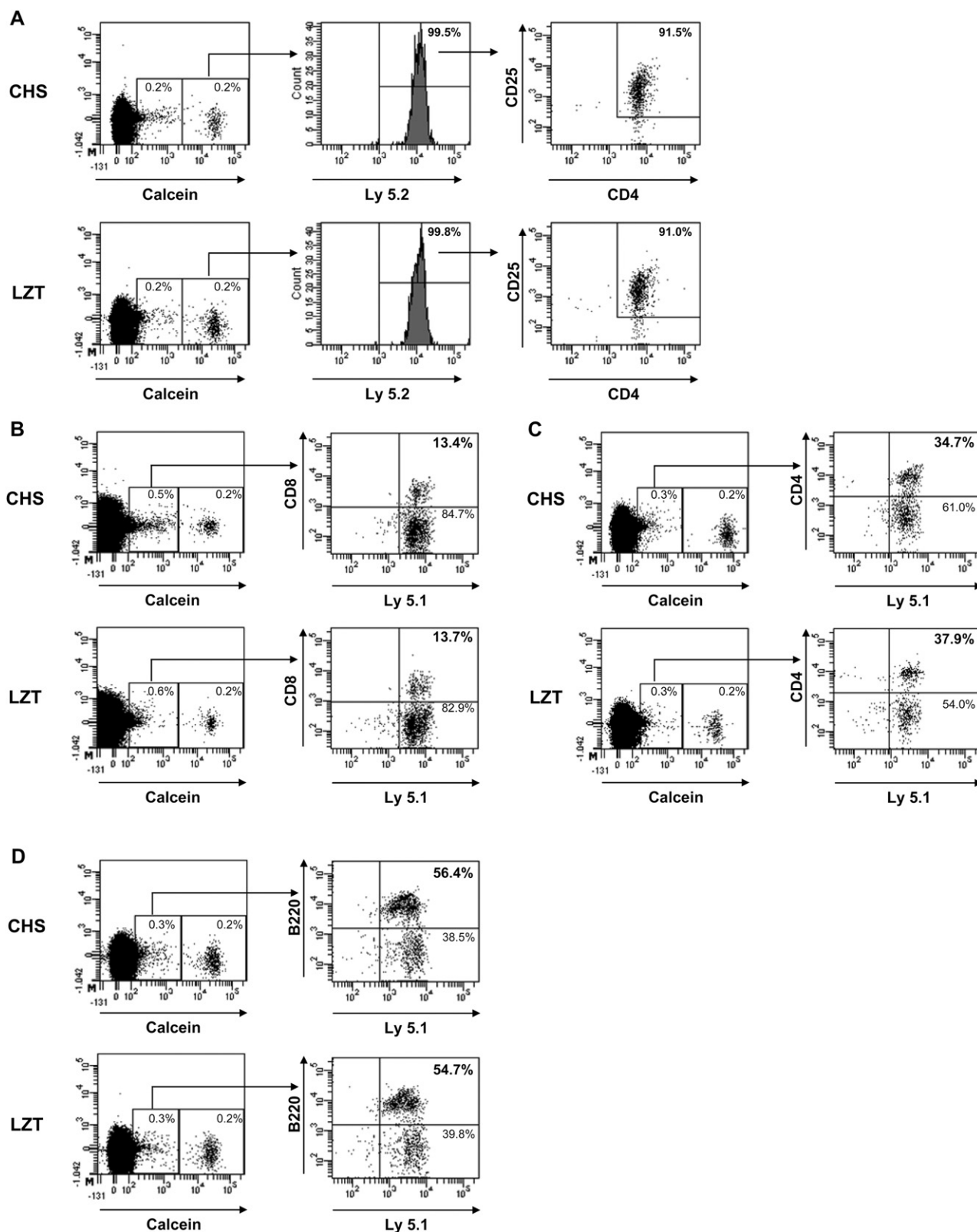


FIG E6. Identical percentages of calcein-positive CD4⁺ and CD8⁺ T cells and B cells in mice with LZT and mice with CHS after transfer of calcein-labeled CD4⁺ CD25⁺ Treg cells. CD4⁺ CD25⁺ Treg cells were obtained from skin-draining LN cells of Ly5.2 C57BL/6 WT mice after application of solvent (CHS) or tolerizing doses (LZT) of the hapten TNCB, labeled with calcein, and injected intravenously into Ly5.1 WT animals. The recipients were sensitized after 24 hours, and 72 hours after sensitization, skin-draining LNs were harvested and calcein-positive Ly5.2⁺ Treg cells (**A**) and the calcein transfer in Ly5.1 CD4⁺ T cells (**B**), CD8⁺ T cells (**C**), and B220⁺ B cells (**D**) were detected by using flow cytometry.

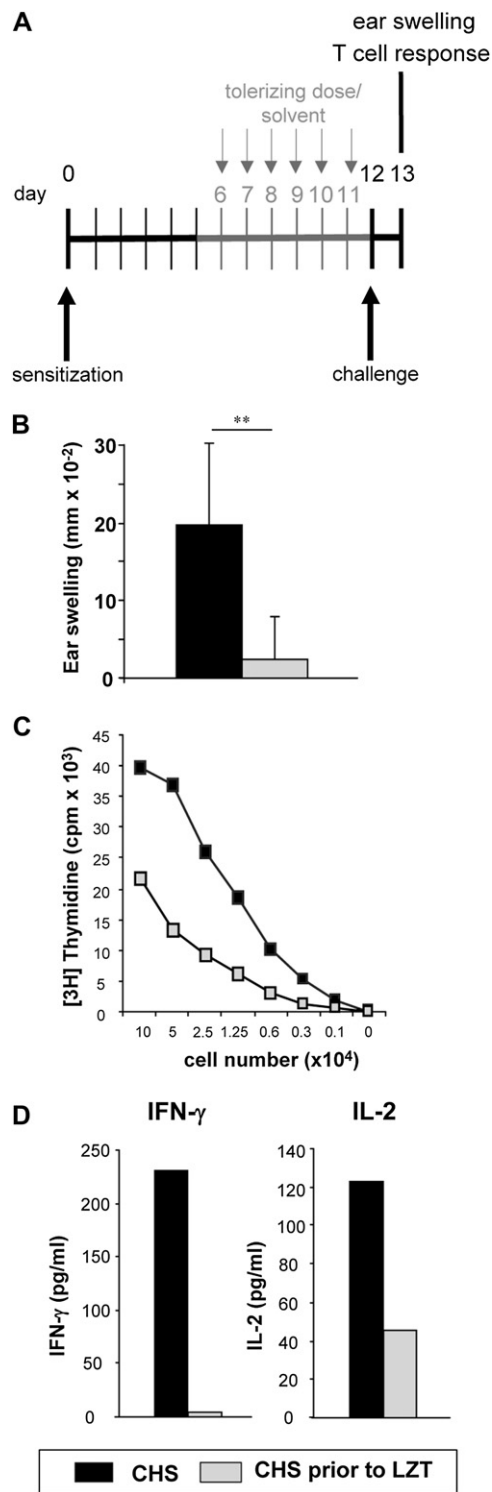


FIG E7. LZT is also efficiently induced after sensitization. **A**, Mice were sensitized and subsequently treated with solvent (CHS) or tolerizing doses (0.45 μ g) of TNCB (LZT after sensitization). **B** and **C**, After challenge, the ear-swelling response was assessed (mean of absolute values $\pm$ SDs; Fig E7, *B*) and T-cell proliferation was detected (mean values of triplicates in counts per minute; Fig E7, *C*) after restimulation. **D**, Release of IFN- γ and IL-2 by T cells is shown. Data represent one of 3 independent experiments with similar results. ***P* < .01.