

Gap junctions between regulatory T cells and dendritic cells prevent sensitization of CD8⁺ T cells

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Background: Regulatory T (Treg) cells suppress the sensitization phase of experimental contact hypersensitivity (CHS) reactions when injected before hapten application.

Objective: Our aim was to analyze the mechanisms by which Treg cells suppress the sensitization phase of CHS reactions.

Methods: Treg cells were labeled with different fluorescent dyes and injected into naive mice directly before sensitization with the hapten 2,4,6-trinitro-1-chlorobenzene. Two days after sensitization, the lymphoid organs were analyzed for the presence of Treg cells and engagement of gap junctions with other cells. Dendritic cells (DCs) and effector CD8⁺T cells were isolated from the draining lymph nodes (LNs) of the differently treated groups, analyzed by using FACS for activation markers, and assessed for the T-cell stimulatory capacity of the DCs and the priming of effector T cells.

Results: Only the LN-homing Treg cells suppressed the sensitization phase in CHS reactions by means of establishing gap junctions with DCs in the dLNs. This gap junctional intercellular communication led to downregulation of T-cell costimulatory molecules on the surface of the DCs, abrogating the priming, activation, and proliferation of hapten-specific CD8⁺T cells. Consequently, the ear-swelling response induced by challenge with the respective hapten was prevented.

Conclusion: Treg cells not only modulate ongoing CD4⁺T cell-mediated immune reactions at tissue sites but also abrogate the *de novo* induction of CD8⁺T cell-driven immune reactions by interfering with T-cell stimulatory activity of DCs through gap junctional intercellular communication. (*J Allergy Clin Immunol* 2010;125:237-46.)

Key words: Contact hypersensitivity, regulatory T cells, gap junctions, dendritic cells

CD4⁺CD25⁺ regulatory T (Treg) cells are crucial for preventing autoimmunity and exert anti-inflammatory activity *in vivo* by various means (for review, see Vignali et al¹). One disease model that has frequently been used to analyze the function

Abbreviations used

AM:	Acetoxymethyl ester
BM-DC:	Bone marrow-derived dendritic cell
cAMP:	Cyclic adenosine monophosphate
CD62L:	CD62 ligand
CFDA-SE:	5,6 Carboxyfluorescein diacetate, succinimidyl ester
CHS:	Contact hypersensitivity
DC:	Dendritic cell
dLN:	Draining lymph node
Foxp3:	Forkhead box protein 3
LN:	Lymph node
PE:	Phycoerythrin
TNCB:	2,4,6-Trinitro-1-chlorobenzene
TNBS:	2,4,6-Trinitro-benzenesulfonic acid
Treg cell:	Regulatory T cell

of Treg cells *in vivo* is the hapten-induced contact hypersensitivity (CHS) reaction.²⁻⁴ Typically, CHS reactions start with the sensitization phase, in which the skin is exposed to haptens (murine models frequently use 2,4,6-trinitro-1-chlorobenzene [TNCB], fluorescein isothiocyanate, or oxazolone). After aggregation with tissue proteins, the hapten-protein complexes are taken up by dendritic cells (DCs) and transported to regional draining lymph nodes (dLNs). Here the antigen-loaded DCs prime antigen-specific T cells, resulting in subject who are sensitized to the respective hapten.^{5,6} In this sequence of events, the priming (ie, the activation of naive CD8⁺ T cells) takes place in the dLNs, and intimate contact between DCs and naive T cells is mandatory. Although the suppressive function of Treg cells has originally been described in the context of T-cell activation,⁷ recent reports demonstrate that Treg cells also suppress the immunostimulatory functions of DCs.⁸⁻¹⁰ The means of this Treg cell-DC interaction are not clear yet; however, in general, Treg cells possess a diverse arsenal of inhibitory mechanisms, ranging from the release of inhibitory mediators (indoleamine 2,3-dioxygenase, IL-10, and adenosine) to the expression of inhibitory surface molecules (cytotoxic T lymphocyte-associated antigen 4 and glucocorticoid-induced tumor necrosis factor receptor [GITR]).¹

Recently, the establishment of gap junctions between Treg cells and T cells has been added as a novel means of Treg cell-mediated suppression of T-cell proliferation.¹¹ Gap junctions are intercellular channels formed by proteins of the connexin family, allowing the bidirectional passage of molecules of less than 1 kD. The formation of gap junctions between cells requires alignment of 2 compatible hemichannels in the membrane of 2 adjacent cells. Because both cell types, DCs and Treg cells, express the crucial proteins of the connexin family,¹²⁻¹⁴ gap junctional intercellular communication between Treg cells and DCs as a means of suppression is conceivable. This has been shown *in vitro* for

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human Treg cell-CD4⁺ T-cell interactions; however, its implication for *in vivo* diseases remains unclear thus far.

In the following, we chose the sensitization phase of the hapten-induced CHS reactions, which crucially relies on antigen-loaded DCs, to investigate the effects of Treg cells on DC function.

METHODS

Mice

BALB/c and C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany), and IL-10-deficient (IL-10^{-/-}) mice on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, Me). The mice were housed at the central animal facility of the University of Heidelberg. All experiments were performed in accordance with governmental guidelines.

Purification of T-cell subsets and labeling

The CD4⁺ T cells were isolated from lymph nodes (LNs) and spleens of naive mice by using the CD4-untouched magnetic bead separation kit from Miltenyi Biotec (Bergisch Gladbach, Germany), according to the manufacturer's protocol. Purity was greater than 97%. The CD25 MicroBead kit from Miltenyi Biotec was used to obtain CD4⁺CD25⁺ Treg cells. Briefly, CD4⁺ T cells were stained with anti-CD25 phycoerythrin (PE) mAb, followed by incubation with anti-PE Beads and separation through magnetic columns, according to the manufacturer's instructions. The purity and expression of forkhead box protein 3 (Foxp3) were always confirmed by means of flow cytometry. Ninety-eight percent or greater of the CD4⁺CD25⁺ Treg cells coexpressed Foxp3, and the average purity of CD4⁺CD25⁺Foxp3⁺ T cells was greater than 90%. The suppressive capacity of the Treg cells was always assessed by using standard suppression assays. Further separation of the CD4⁺CD25⁺Foxp3⁺ T cells into CD62 ligand (CD62L)-positive and CD62L-negative fractions was performed with CD62L Beads (Miltenyi Biotec; see the [Methods](#) section in this article's Online Repository at www.jacionline.org).

CD8⁺ T cells and CD11c⁺ and B220⁺ cells were enriched from inguinal LNs by using positive selection. Cells were labeled with CD8a, CD11c, or B220 Beads (Miltenyi Biotec) and separated in a magnetic field. Thereafter, the cells were eluted from the columns and analyzed by means of FACS. The purity of the isolated cell populations always ranged from 90% to 95%. If not stated differently, 3×10^6 cells per mouse were injected intravenously into the tail vein.

For cell labeling with PKH26 (Sigma-Aldrich, Munich, Germany), Treg cells were incubated with the dye, according to the manufacturer's protocol. For the staining of cells with 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE; Invitrogen, Germany), 2×10^7 cells were incubated with 10 μ M CFDA-SE in medium at 37°C in 5% CO₂. After 10 minutes, cells were washed and resuspended in medium.

Generation of bone marrow-derived DCs

Bone marrow-derived DCs (BM-DCs) were grown according to published protocols in RPMI medium supplemented with 10 ng/mL rmGM-CSF and 10 ng/mL rmIL-4 (Strathmann Biotec, Hamburg, Germany).¹⁵ If required, immature BM-DCs were haptenized with 10 nmol/L 2,4,6-trinitro-benzenesulfonic acid (TNBS, Sigma-Aldrich) for 10 minutes at 37°C. The reaction was stopped by adding FCS for 1 minute, followed by several washings with RPMI/10% FCS.

Functional assays

CD8⁺ T cells (1×10^5) isolated from the draining inguinal LNs of sensitized mice were cocultured in 96-well round-bottom plates with DCs isolated from the draining inguinal LNs (unstimulated, 1 μ g/mL LPS, 1 μ g/mL TNF- α , and 1 μ g/mL IFN- γ) of all differently treated groups in 1:1 ratios, or 1×10^5 CD8⁺ T cells isolated from the draining inguinal LNs of all differently treated groups were cocultured with naive or haptenized BM-DCs (1:10 ratio). After 2 days, 0.5 μ Ci/mL tritiated thymidine (Amersham, Frankfurt, Germany) was added for 18

hours, cells were harvested, and thymidine incorporation was determined with a PerkinElmer scintillation counter (PerkinElmer, Waltham, Mass).

IL-6, IL-12(p70), IL-10, and TNF were detected in the supernatants of unstimulated or LPS (1 μ g/mL)-stimulated CD11c⁺ DCs (4×10^5 /100 μ L) isolated from the draining inguinal LNs by using BD OptEIA ELISA Sets (BD Biosciences, Heidelberg, Germany).

CHS

Mice were sensitized by painting 15 μ L of 1% TNCB (Sigma-Aldrich) dissolved in acetone/olive oil (4:1) on the shaved abdomen on day 0. On day 5, ear thickness was measured with microcalipers (Oditest; Kroepelin, Schlüchtern, Germany). Afterward, mice were challenged with epicutaneous application of 0.5% TNCB solution, 10 μ L on each side of the right ear. The left ear was treated with the same amount of solvent only. After 24 hours, the ear thickness was measured again, and the difference was calculated as the change in ear response in millimeters $\times 10^{-2} \pm$ SD.

Fluorescence microscopy and immunohistochemistry of LN sections

Treg cells were labeled with Calcein Acetoxymethyl ester (AM; Invitrogen, Karlsruhe, Germany), according to the manufacturer's protocol; cocultured with DCs for 24 hours; and transferred onto alcian blue-coated slides for 1 hour at 37°C and 5% CO₂. Staining was performed with rat anti-MHC class II (M5/114.15.2; eBioscience, San Diego, Calif) and rabbit anti-connexin 43 (Z-JB1; Invitrogen), followed by matching secondary antibodies TRITC goat anti-rat IgG (Dianova, Hamburg, Germany) and Alexa Fluor 647 goat anti-rabbit IgG (Invitrogen). Specimens were visualized with a motorized microscope (DM 5500B; Leica, Solms, Germany) equipped with a 100 $\times$ /1.25 oil objective and a Spot RT cooled CCD camera (Visitron, Munich, Germany). Spot software was used to gather twenty 1- μ m-thick optical sections per specimen, and the resulting stack was deconvolved with AutoDeblur software (MediaCybernetics, Visitron). Deconvolved images were assembled with metaview software (Visitron).

Draining inguinal LNs were mounted in Tissue-Tek freezing media (Miles, Torrance, Calif) and sectioned with a cryostat (Leica). Sections were either analyzed for exogenous PKH-PE- or Calcein AM-labeled cells and/or were stained with purified hamster anti-CD11c (N418, eBioscience) and rabbit anti-connexin 43 (Z-JB1, Invitrogen) antibodies followed by the matching secondary antibodies TRITC goat anti-hamster IgG (Dianova) and Alexa Fluor 647 goat anti-rabbit IgG (Invitrogen) for immunofluorescence analysis. Sections were examined with a spinning disk confocal microscope (Ultraview; PerkinElmer Optoelectronics, Wiesbaden, Germany) attached to a Nikon inverted microscope (TM2000; Nikon, Düsseldorf, Germany). Twenty to 30 optical sections of 1.5 μ m thickness were taken with an 100 $\times$ /1.4 objective and recorded with a Orca-2 EM camera (Hamamatsu, Herrsching, Germany).

Calcein exchange experiments

For detection of Calcein AM exchange *in vivo*, 3×10^6 Calcein AM-labeled Treg cells were injected 2 hours before sensitization. Forty-eight hours after sensitization, different cell populations were isolated from the draining inguinal LNs and analyzed by means of FACS. Gap27 peptide (SRPTEKTI-FII; synthesized by Activotec, Cambridge, United Kingdom) dissolved in Hanks balanced salt solution (150 μ M/L, 300 μ M/L) was used as a gap junction inhibitor. Treg cells were incubated with Gap27 at least 60 minutes before *in vitro* culture with DCs or before intravenous injection into mice.

RESULTS

LN homing is required for Treg cells to suppress the sensitization phase of CHS

To determine the effects of differential tissue homing of Treg cells on their suppressive function in the sensitization phase of

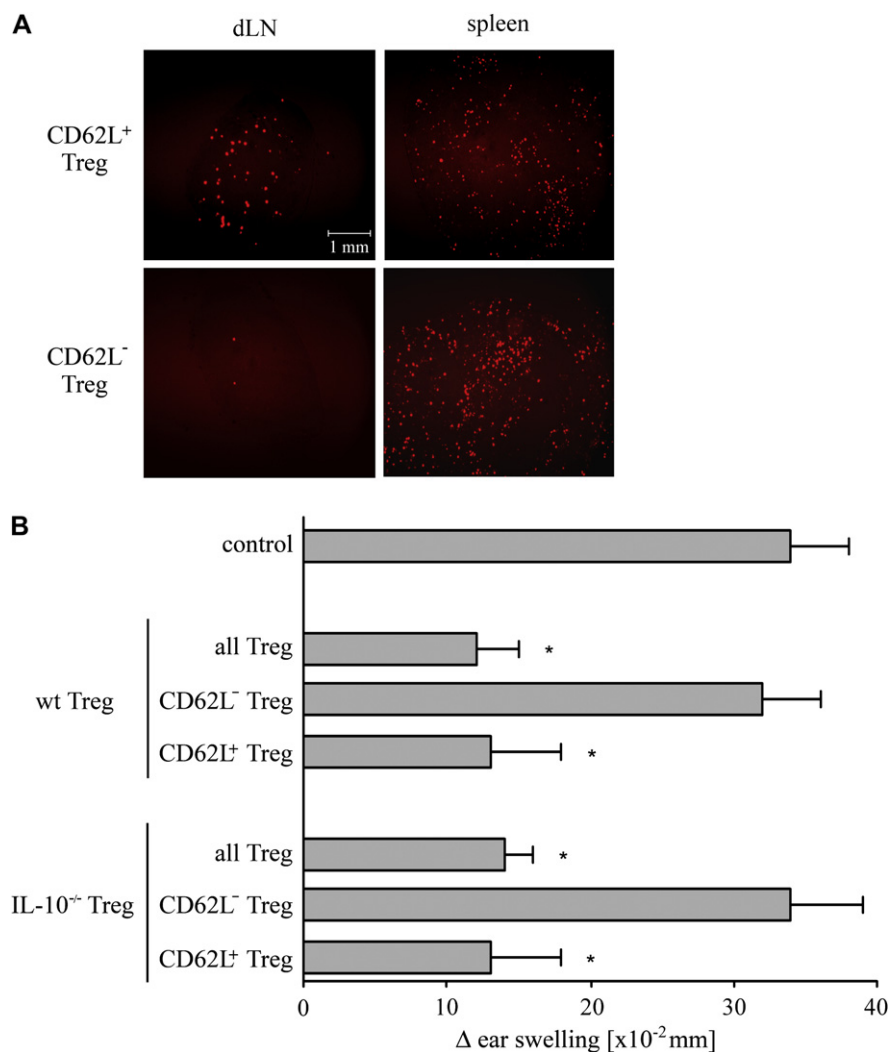


FIG 1. Treg cells suppress CHS reactions in dLNs. **A**, Migration pattern of CD62L⁺ and CD62L⁻ Treg cells. **B**, Ear swelling of C57BL/6 mice treated with all Treg cells, CD62L⁻ Treg cells, or CD62L⁺ Treg cells isolated from C57BL/6 or IL-10^{-/-} mice. Data show the mean increase in ear thickness $\pm$ SD (* $P < .001$) of 3 independent experiments comprising 5 mice each. FITC, Fluorescein isothiocyanate; wt, wild-type.

CHS, we separated CD4⁺CD25⁺Foxp3⁺CD62L⁻ and CD4⁺CD25⁺Foxp3⁺CD62L⁺ cells (see Fig E1, A and B, in this article's Online Repository at www.jacionline.org), labeled them with fluorescent dyes, and injected 3×10^6 cells of either of the 2 populations separately into naive mice 2 hours before sensitization. Analyzing the lymphoid organs 2 days after sensitization, we detected exclusively CD62L⁺ Treg cells in the LNs, whereas CD62L⁻ Treg cells did not migrate into the LNs at all (Fig 1, A). Instead, these cells were detectable in increased numbers in the blood and spleen (see Fig E1, C). Moreover, none of the Treg cells could be detected in the ears or abdominal skin (data not shown).

That homing of Treg cells to the LNs is indeed mandatory for suppressing the sensitization phase was proved by measuring the ear-swelling response 24 hours after the second application (challenge) of TNCB. Here we show that only LN-homing CD62L⁺ Treg cells were able to suppress the sensitization of mice to TNCB, as indicated by the abrogation of the ear-swelling reaction (Fig 1, B). In contrast, CD62L⁻ Treg cells did not prevent

sensitization, although both subsets of Treg cells were equally potent in suppressing T-cell proliferation *in vitro* (see Fig E1, D).

The suppressive effects of Treg cells on the CHS response was dose dependent because decreasing numbers of Treg cells (5×10^6 Treg cells down to 0.5×10^6 Treg cells per mouse) only showed partial abrogation of the ear-swelling response (see Fig E2, A, in this article's Online Repository at www.jacionline.org). Moreover, depletion of endogenous Treg cells by CD25 antibodies abrogated the suppressive effects of the injected Treg cells, indicating that the total number of Treg cells in the dLNs crucially determines the effectiveness of the suppression (see Fig E2, B and C).

Because IL-10 has immunosuppressive effects on the elicitation of CHS reactions and is produced by Treg cells in substantial amounts,^{3,16} we additionally used Treg cells derived from IL-10^{-/-} mice in our CHS setting. These CD62L⁺IL-10^{-/-} Treg cells also abrogated the sensitization phase of CHS reactions (Fig 1, B). Thus these data demonstrate that Treg cells suppress the sensitization phase of CHS reactions in the dLNs through an IL-10-independent mechanism.

Treg cells affect activation of DCs and CD8⁺ T cells in the dLNs after sensitization

Our data show that LN occupancy by Treg cells is mandatory for suppression of the sensitization phase of CHS responses. To analyze the underlying mechanism or mechanisms, we assessed the leukocyte subpopulations in the dLNs after injection of Treg cells and sensitization by means of FACS analysis. As controls, we used naive mice, sensitized mice, and only Treg cell-treated mice. Fig 2, A, shows that injection of Treg cells significantly reduced the expansion of CD8⁺ T cells in the dLNs, which is characteristically induced by sensitization (sensitization group: 28.1% CD8⁺ cells vs Treg cells injected and sensitized group: 17.5% CD8⁺ cells) compared with naive control animals (15.0%). Other subpopulations, such as CD11c⁺ DCs, CD4⁺ T cells (Fig 2, A, and see Fig E3, A, in this article's Online Repository at www.jacionline.org), B cells, or natural killer cells (data not shown) displayed no significant changes in numbers.

Because the activation status of DCs eventually determines the outcome of subsequent immune reactions, we additionally measured activation markers expressed by CD11c⁺ DCs and CD8⁺ T cells in the dLNs. Fig 2, B, and Fig E3, B, show that CD11c⁺ DCs isolated from dLNs of mice receiving Treg cells before sensitization displayed a reduced expression of the T-cell costimulatory molecules CD86 and CD80 compared with that seen in respective control animals. Likewise, the sensitization-induced activation of the CD8⁺ T cells, as indicated by upregulation of CD69 (sensitized group: 20.2% CD69⁺ cells vs naive group: 6.7% CD69⁺ cells) was abrogated by injection of Treg cells. Thus these data indicate that Treg cells block the activation of DCs in dLNs and consequently are unable to prime naive CD8⁺ T cells.

Treg cells and DCs establish gap junctions *in vitro*

Our data imply that Treg cells interact with DCs in the dLNs. We therefore started to analyze the possible means of this DC–Treg cell interaction *in vitro*. Isolated Treg cells were loaded with Calcein, followed by incubation with BM-DCs. Forty-eight hours later, sizable numbers of DCs showed a substantial fluorescence of Calcein (Fig 3, A, and see Fig E4, A, in this article's Online Repository at www.jacionline.org), which is indicative for gap junctions. Fluorescent dyes, such as CFDA-SE, that are not able to cross gap junctions were not detected in the DCs (Fig 3, A). This demonstrates that Treg cell–DC Calcein transfer was not due to unspecific uptake of the dye. Moreover, examination of the cocultures by means of fluorescence microscopy clearly indicated transfer of Calcein from the Treg cells to the DCs on a single-cell level (Fig 3, B, control). The passage of Calcein through gap junctions follows the concentration gradient between the connected cells, and therefore initially the dye accumulates close to the cell membranes, which are connected by the gap junction. However, eventually the dye distributes throughout the acceptor cell, and the whole cell displays a dim but detectable fluorescence (note the “yellow” contact zone and the dim fluorescence marked by arrows). When using Calcein-labeled conventional CD4⁺CD25[−] T cells in the cocultures with DCs, no transfer of Calcein was detectable (see Fig E4, B).

To further prove the presence of gap junctions between Treg cells and DCs, we administered the gap junction inhibitory peptide Gap27, which acts as an inhibitor for the major gap junction proteins connexin 37 and connexin 43.¹⁷ This inhibition of gap junctions prevented the transfer of Calcein from Treg cells

to DCs (Fig 3, B, Gap27) and caused a reduction of the dye exchange by 67% (300 μmol/L Gap27; Fig 3, C). However, we were not able to completely block the dye transfer (even at higher concentrations). The Gap27 peptide only blocks connexin 43/connexin 37-containing gap junctions, and therefore it is conceivable that other connexin subtype-containing gap junctions account for the residual cytosolic transfer.

On a single-cell level, the presence of gap junctions was demonstrated by means of fluorescence microscopy (Fig 3, D). Examination of the cells showed a close contact between Treg cells (green) and DCs (red) and the presence of connexin 43 (blue) at the interface of Treg cells and DCs (Fig 3, D, close up), which is suggestive for the formation of gap junctions. The specificity of the anti-connexin 43 antibody was tested by means of incubation of the antibody with a connexin 43–derived peptide that is recognized by the antibody before staining of the sections. This resulted in an abrogation of the staining, indicating that the antibody is specific for connexin 43 (data not shown). Thus *in vitro* Treg cells and DCs engage in a close contact and exchange cytoplasmic components through gap junctions.

Treg cells and DCs communicate through gap junctions *in vivo*

To analyze formation of gap junctions *in vivo*, we injected Calcein-positive Treg cells before sensitization and detected the Calcein uptake in isolated CD8⁺, B220⁺, and CD11c⁺ cells of the dLNs 48 hours later. We show that the amount of CD11c⁺ Calcein-positive cells in the LNs increased from 2.8% in naive mice (data not shown) to 14.2% in sensitized mice (Fig 4, A and B). In contrast, the CD8⁺ effector cells, as well as other antigen-presenting cells, like B220⁺ B cells, showed no significant uptake of the fluorescent dye (Fig 4, A).

In confocal images of cryosections of the dLNs (Fig 4, C), exclusively CD11c⁺ DCs (red) showed a close interaction with the Calcein-loaded Treg cells (green) combined with transfer of the green fluorescent dye. Triple staining with the connexin-specific anti-connexin 43 antibody (blue/purple) showed the presence of gap junctions at the Treg cell–DC interface, which is suggestive for the involvement of gap junctions during Treg cell–DC communication *in vivo*. In contrast, after staining the sections with anti-CD8 or anti-B220, no contacts of these cells to the Treg cells could be observed (data not shown).

Treg cells reduce the CD8⁺ T-cell stimulatory capacity of DCs through gap junctions

To prove whether the T-cell stimulatory function or functions of hapten-loaded DCs after contact with Treg cells *in vivo* is altered, we isolated DCs from dLNs of the differently treated mice (as indicated in Fig 5, A) and cocultured them with CD8⁺ T cells derived from sensitized mice. In all groups the viability of the freshly purified DCs was comparable and reached 95% (see Fig E5 in this article's Online Repository at www.jacionline.org). However, subsequent analysis of the T-cell proliferation revealed that the stimulating capacity of hapten-loaded DCs was significantly reduced after contact with Treg cells *in vivo* (Fig 5, A, DC Treg + sens group) in contrast to that seen in uninjected control animals (Fig 5, A, DC sens group). DCs obtained from untreated or naive mice did not induce any significant T-cell proliferation because the DCs lack

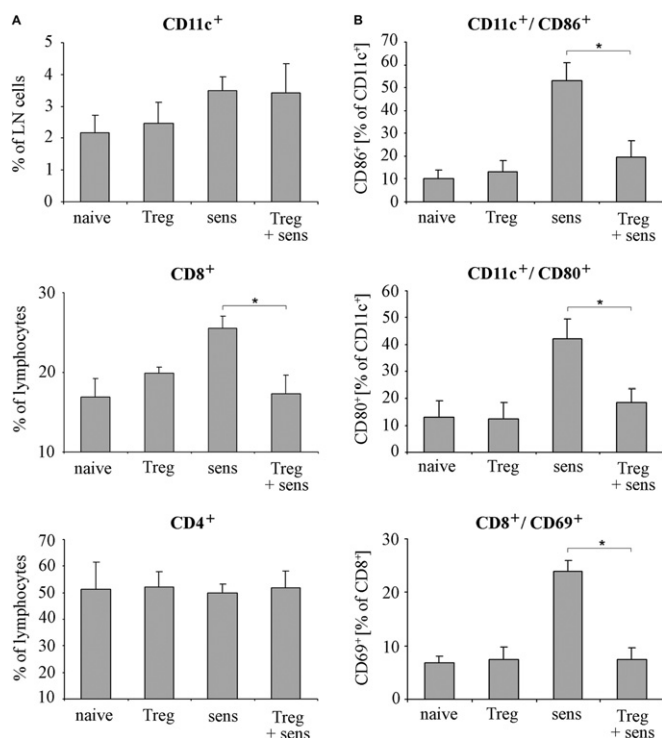


FIG 2. Treg cells inhibit the activation of DCs and CD8⁺ T cells in the dLNs. **A**, Lymphocyte populations in the dLNs of naive, sensitized, Treg cell-injected and Treg cell-injected and sensitized mice 48 hours after sensitization. **B**, Activated CD11c⁺ cells and CD8⁺ T cells in the dLNs. Data in Fig 2, A and B, show the mean $\pm$ SD of 5 independent experiments (* P < .001). FITC, Fluorescein isothiocyanate.

the MHC-antigen complexes on their surface. Of note, when DCs derived from Treg cell-injected mice were activated with LPS, TNF- α , or IFN- γ before coculture, T cell-proliferation was reestablished (see Fig E6 in this article's Online Repository at www.jacionline.org). Likewise, preincubation of the Treg cells with the gap junction inhibitory peptide Gap27 restored the T cell-activating capacity of the DCs (Fig 5, A, DC Treg + Gap27 + sens group).

The reduced priming of CD8⁺ T cells by DCs *in vivo* was further investigated by measuring the proliferation of the CD8⁺ T cells after antigen-specific restimulation. Therefore we isolated CD8⁺ T cells from mice that were treated with Treg cells and sensitized in different combinations, as indicated in Fig 5, B, and restimulated them with naive or TNBS-haptenized DCs. Our data show (Fig 5, B) that CD8⁺ T cells isolated from TNBS-sensitized mice proliferated strongly in response to antigenic stimulation by DCs (Fig 5, B, group CD8⁺sens), indicating successful priming *in vivo*. In contrast, CD8⁺ T cells from mice treated with Treg cells showed a significantly reduced proliferation after antigen-specific restimulation (Fig 5, B, CD8⁺ Treg + sens group). Remarkably, this abrogation of T-cell proliferation was neutralized when gap junctions between Treg cells and DCs were blocked by the Gap27 peptide (Fig 5, B, CD8⁺ Treg + Gap27 + sens group). However, as expected, no proliferation was detected after restimulation of CD8⁺ T cells with DCs that lack the antigen TNBS (naive DCs; Fig 5, B, left group).

To determine whether cytokine production of DCs is also affected by Treg cells *in vivo*, we isolated DCs from differently

treated mice (Fig 5, C; naive, sens, and Treg + sens groups) and tested the cell-culture supernatants after 24 hours for IL-10, IL-6, IL-12, and TNF- α , respectively. In Fig 5, C, we show that injection of Treg cells reduced the production of IL-6, whereas IL-10 levels were increased compared with those seen in control animals. Thus these data corroborate a previous report by Veldhoen et al¹⁰ and indicate that Treg cells are able to shift DCs toward a tolerogenic function (less IL-6 and more IL-10). However, neither IL-12 nor TNF- α expression was affected significantly by Treg cells (data not shown).

To test for involvement of gap junctions during the sensitization phase, we injected Gap27-treated or control Treg cells into mice before sensitization. Five days later, a CHS reaction was elicited at one ear, and ear swelling was measured after 24 hours. Here we show (Fig 5, D) that the suppression of the sensitization by Treg cells is abrogated by pretreatment with Gap27. Thus these data indicate that Treg cells inhibit the antigen-specific priming of CD8⁺ effector T cells by engaging in gap junctions with DCs in the dLNs.

DISCUSSION

Our data indicate that Treg cells interact with DCs in the dLNs by establishing gap junctions, leading to downregulation of the T cell-activating capacities of the DCs.

The effects of Treg cells on differentiation of DCs has been investigated recently, showing that Treg cells impair maturation of the DCs⁹ and cause upregulation of T-cell inhibitory molecules,^{8,18} as well as secretion of immunosuppressive molecules, such as indoleamine 2,3-dioxygenase and IL-10.^{10,19} According to Onishi et al,²⁰ these effects are dependent on the formation of aggregates between DCs and Treg cells, and thus the Treg cells might shield the DCs from conventional T cells. Consequently, activation of effector T cells by DCs is abrogated. Although most of these results were obtained *in vitro* by using cocultivation of DCs and transgenic CD4⁺ T cells, recent results with *in vivo* microscopy support these concepts.²¹ In line with these observations, our results demonstrate that the DC-suppressing activity of Treg cells operates also *in vivo* in a CD8⁺ T cell-driven CHS model by means of gap junctional intercellular communication.

The suppressive effects of Treg cells are dependent on their homing to the dLNs because CD62L⁻ Treg cells, which do not enter the dLNs, also do not suppress hapten sensitization. This is in line with observations showing that β_7 -integrin-deficient Treg cells, which home to the LNs but not to the gut, are nevertheless able to suppress experimental colitis.²² Moreover, in a transplantation model²³ tolerance-inducing Treg cells accumulated in the draft dLNs and not in the graft itself, and CCR7-deficient mice display augmented immune responses that are due to the defective LN positioning of Treg cells.²⁴ Therefore these data indicate that LN-positioning of Treg cells determines their function in different disease models.

It is conceivable that Treg cells can suppress different subsets of LN-residing leukocytes, either through the release of soluble factors, such as IL-10, TGF- β , and adenosine, or through the expression of inhibitory surface molecules.¹ IL-10 in particular has been shown to be of importance for suppressive activity of Treg cells *in vivo*,^{25,26} but our data, together with recent findings, imply that IL-10 is dispensable for the effects exerted on DCs.⁹ This might be explained by the fact that exclusively immature DCs are sensitive to IL-10, whereas mature DCs do not react to

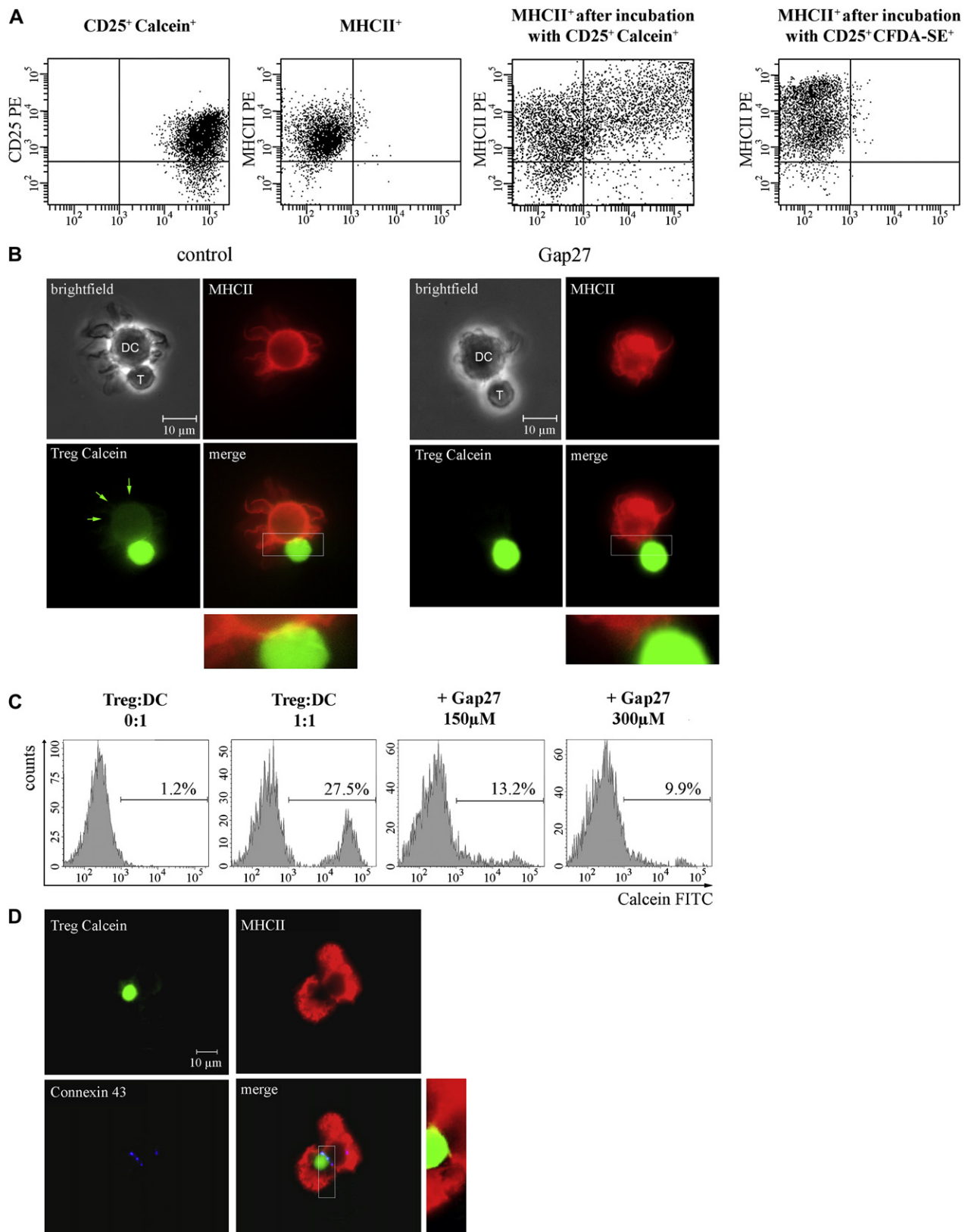


FIG 3. Treg cells interact with DCs *in vitro* through gap junctions. **A**, Calcein and CFDA-SE uptake of BM-DCs (MHC II⁺) from Calcein-positive or CFDA-SE⁺ Treg cells. **B**, Fluorescence microscopy of Treg/BM-DC cocultures (*control*) and Treg-Gap27/BM-DC cocultures (*Gap27*). **C**, Influence of Gap27 on Calcein uptake of MHC class II-positive cells. **D**, Gap junctional intercellular communication, stained with connexin 43, between Treg cells and DCs. FITC, Fluorescein isothiocyanate.

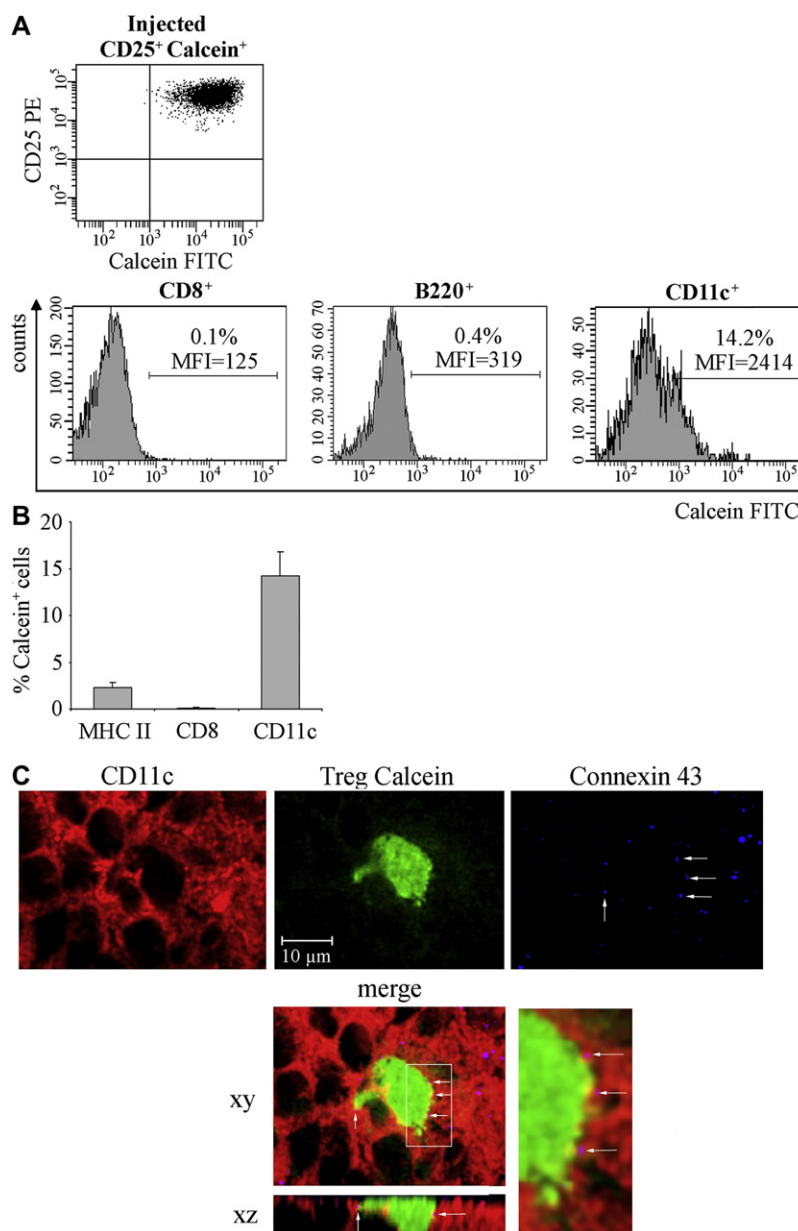


FIG 4. Intercellular communication between Treg cells and CD11c⁺ DCs in the dLNs *in vivo*. **A**, Calcein uptake of isolated CD8⁺, B220⁺, and CD11c⁺ cells from Calcein-positive Treg cells. *MFI*, Mean fluorescence intensity. **B**, Mean percentages of Calcein-positive cells of 3 independent experiments $\pm$ SD. **C**, Confocal microscopy of Calcein transfer through gap junctions (blue) from Calcein-positive Treg cells (green) to CD11c⁺ DCs (red) in the dLNs *in vivo*. *FITC*, Fluorescein isothiocyanate.

IL-10.²⁷ Thus the skin-immigrated DCs, which are the targets of the Treg cells, might have lost their ability to react to IL-10.

Instead of IL-10 release, we demonstrate gap junctional intercellular communication as a means of interaction between Treg cells and DCs. The relevance of gap junctions for DCs has thus far been attributed to proinflammatory functions because DCs use gap junctional intercellular communication to carry activation over to other DCs and use gap junctions to facilitate antigen transfer between DCs.^{12,13,28} However, these results only refer to DC–DC gap junctions, whereas we observe for the first time suppressive effects exerted by Treg cell–DC gap junctions.

Gap junction formation between different cell types was shown in germinal centers, where antigenic challenge induced

upregulation of connexin 43 mRNA and protein in follicular DCs and B cells.²⁹ Moreover, blockage of gap junctions in T/B-cell mixed leukocyte reactions led to reduced secretion of immunoglobulins.³⁰

Gap junctions allow small molecules (<1 kD) to travel between cells. Although originally described as unselective pores, it became clear that the composition of the gap junctions comprising different connexins is at least partially determining the specificity of the gap junction. Certain combinations of connexins favor the passage of cations, whereas others preferentially let anions pass between cells.³¹ Moreover, the opening of the gap junctions is a regulated process that can be triggered by voltage differences between cells or by phosphorylation events.³²

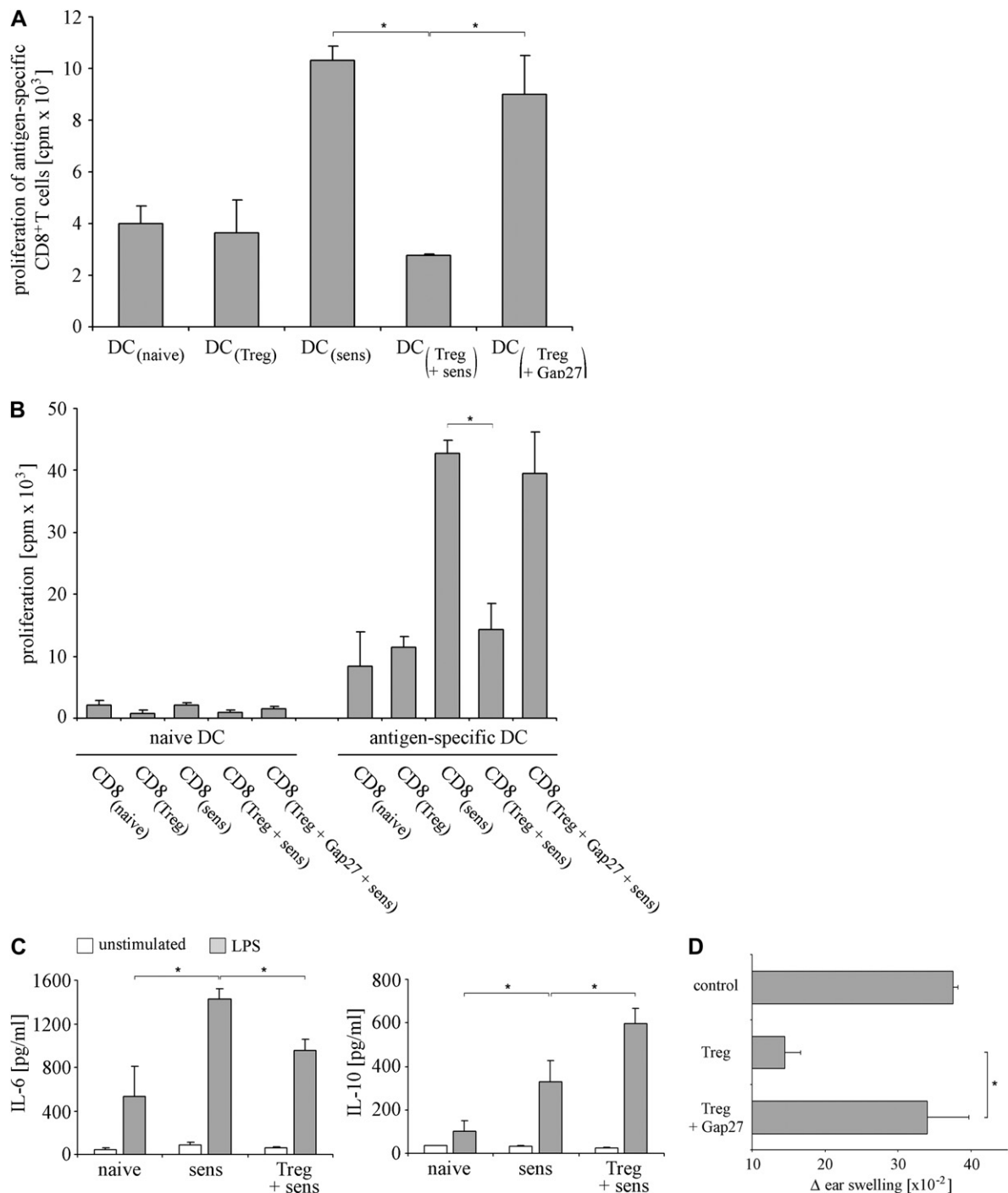


FIG 5. Treg cells reduce the stimulatory capacity of DCs. **A**, Antigen-presenting capacity of CD11c⁺ DCs. **B**, Antigen-specific response of CD8⁺ T cells. Data show the mean counts per minute ± SD of 4 (Fig 5, A) and 3 (Fig 5, B) experiments (**P* < .001). **C**, Cytokine production of CD11c⁺ DCs. **D**, Mean increase in ear thickness ± SD (**P* < .001) of 3 independent experiments comprising 5 mice each.

Initially, the passage of K⁺, Na⁺, and Ca²⁺ ions through gap junctions has been confirmed, and transport of these ions through gap junctions is of importance in electrical conductivity in heart, muscle, and neuronal cells. Beyond ions, second messengers, such as inositol trisphosphate, glutamate, glutathione, and glucose, use gap junctions as well, and depending on the type of acceptor cell, these substances induce intracellular signaling mainly

by increasing Ca²⁺ levels.³³ Treg cell-derived cyclic adenosine monophosphate (cAMP) has recently been described to suppress T-cell proliferation,¹¹ and it has also been attributed to have a role in DC maturation. For instance, it has been shown to impair the differentiation of mature DCs and to suppress antigen uptake, T-cell stimulatory capacity, and IL-12 production of DCs by means of intracellular interaction with interferon regulatory

factor 8.³⁴⁻³⁶ Therefore increased levels of cAMP in DCs after contact with Treg cells, which can either be induced by means of direct transfer through gap junctions or transfer of cAMP level-increasing second messengers, might provide a means to render DCs nonimmunogenic and to block sensitization.

Other nucleotide-based intermediates, such as ATP, adenosine, and nicotinamide adenine nucleotides, are also frequently involved in gap junctional intercellular communication.³⁷⁻³⁹ These messengers regulate the adhesion and transendothelial diapedesis of leukocytes during inflammatory reactions.^{40,41} In particular, ATP derivatives might be important because it has recently been shown that Treg cells augment adenosine levels *in vivo* and *in vitro*, leading to suppression of effector T-cell proliferation.^{42,43} These intermediates have been shown to travel through gap junctions, and therefore it is conceivable that signaling through nucleotide intermediates by means of gap junctional intercellular communication is a major communication pathway of Treg cells.

Our results showing active downregulation of CD86 expression by DCs *in vivo* are corroborated by Schmidt et al.⁴⁴ Here the authors demonstrate reduction of CD86 expression by DCs after coculture with Treg cells *in vitro*, but the exact means are not clear yet. Generally, surface molecules can actively be removed from cell membranes by means of shedding, which has been shown for adhesion molecules, such as CD62L,⁴⁵ or by means of internalization and subsequent degradation in lysosomes (as shown for the T-cell receptor).⁴⁶ Both mechanisms are not confirmed for CD80/CD86 regulation yet, but the intracellular tail of CD80 contains several domains that are critical for its localization in the immunologic synapse and interact with protein kinase C θ .^{47,48} Therefore it is conceivable that transfer of second messengers through gap junctions (eg, cAMP or calcium ions) leads to downregulation of CD80/CD86 surface expression through interference with their intracellular domains.

The formation of gap junctions between Treg cells and DCs, as shown for the first time in our report, broadens the suppressive capacity of Treg cells beyond the mere cell-cell interaction with CD4⁺ effector T cells. By suppressing DCs, the most potent T-cell activators *in vivo*, Treg cells not only suppress ongoing immune responses but are also able to abrogate the *de novo* generation of CD8-driven immune responses.

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Clinical implications: Modification of the gap junctional intercellular communication of Treg cells might augment the Treg cell-mediated immune suppression of allergic and autoimmune diseases.

REFERENCES

1. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* 2008;8:523-32.
2. 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.
3. Ring S, Schafer SC, Mahnke K, Lehr HA, Enk AH. CD4⁺ CD25⁺ regulatory T cells suppress contact hypersensitivity reactions by blocking influx of effector T cells into inflamed tissue. *Eur J Immunol* 2006;36:2981-92.
4. Ring S, Thome M, Pretsch L, Enk AH, Mahnke K. Expanded murine regulatory T cells: analysis of phenotype and function in contact hypersensitivity reactions. *J Immunol Methods* 2007;326:10-21.
5. Allan RS, Waithman J, Bedoui S, Jones CM, Villadangos JA, Zhan Y, et al. Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. *Immunity* 2006;25:153-62.
6. Bursch LS, Wang L, Igyarto B, Kissenfennig A, Malissen B, Kaplan DH, et al. Identification of a novel population of Langerin⁺ dendritic cells. *J Exp Med* 2007;204:3147-56.
7. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α -chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995;155:1151-64.
8. Mahnke K, Ring S, Johnson TS, Schallenberg S, Schonfeld K, Storn V, et al. Induction of immunosuppressive functions of dendritic cells *in vivo* by CD4⁺ CD25⁺ regulatory T cells: role of B7-H3 expression and antigen presentation. *Eur J Immunol* 2007;37:2117-26.
9. 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.
10. Veldhoen M, Moncrieffe H, Hocking RJ, Atkins CJ, Stockinger B. Modulation of dendritic cell function by naive and regulatory CD4⁺ T cells. *J Immunol* 2006;176:6202-10.
11. 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.
12. Matsue H, Yao J, Matsue K, Nagasaka A, Sugiyama H, Aoki R, et al. Gap junction-mediated intercellular communication between dendritic cells (DCs) is required for effective activation of DCs. *J Immunol* 2006;176:181-90.
13. Mendoza-Naranjo A, Saez PJ, Johansson CC, Ramirez M, Mandakovic D, Pereda C, et al. Functional gap junctions facilitate melanoma antigen transfer and cross-presentation between human dendritic cells. *J Immunol* 2007;178:6949-57.
14. Oviedo-Orta E, Hoy T, Evans WH. Intercellular communication in the immune system: differential expression of connexin 40 and 43, and perturbation of gap junction channel functions in peripheral blood and tonsil human lymphocyte subpopulations. *Immunology* 2000;99:578-90.
15. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992;176:1693-702.
16. Yoshiki R, Kabashima K, Sugita K, Atarashi K, Shimauchi T, Tokura Y. IL-10-producing Langerhans cells and regulatory T cells are responsible for depressed contact hypersensitivity in grafted skin. *J Invest Dermatol* 2009;129:705-13.
17. Evans WH, Leybaert L. Mimetic peptides as blockers of connexin channel-facilitated intercellular communication. *Cell Commun Adhes* 2007;14:265-73.
18. Kryczek I, Wei S, Zou L, Zhu G, Mottram P, Xu H, et al. Cutting edge: induction of B7-H4 on APCs through IL-10: novel suppressive mode for regulatory T cells. *J Immunol* 2006;177:40-4.
19. Fallarino F, Grohmann U, Hwang KW, Orabona C, Vacca C, Bianchi R, et al. Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol* 2003;4:1206-12.
20. Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S. Foxp3⁺ natural regulatory T cells preferentially form aggregates on dendritic cells *in vitro* and actively inhibit their maturation. *Proc Natl Acad Sci U S A* 2008;105:10113-8.
21. Tang Q, Adams JY, Tooley AJ, Bi M, Fife BT, Serra P, et al. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat Immunol* 2006;7:83-92.
22. Denning TL, Kim G, Kronenberg M. Cutting edge: CD4⁺ CD25⁺ regulatory T cells impaired for intestinal homing can prevent colitis. *J Immunol* 2005;174:7487-91.
23. Ochando JC, Yopp AC, Yang Y, Garin A, Li Y, Boros P, et al. Lymph node occupancy is required for the peripheral development of alloantigen-specific Foxp3⁺ regulatory T cells. *J Immunol* 2005;174:6993-7005.
24. 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.
25. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin-10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 1999;190:995-1002.
26. Zhang X, Koldzic DN, Izikson L, Reddy J, Nazareno RF, Sakaguchi S, et al. IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25⁺ CD4⁺ regulatory T cells. *Int Immunol* 2004;16:249-56.
27. Steinbrink K, Wölfl M, Jonuleit H, Knop J, Enk AH. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* 1997;159:4772-82.
28. Neijssen J, Herberths C, Drijfhout JW, Reits E, Janssen L, Neeffjes J. Cross-presentation by intercellular peptide transfer through gap junctions. *Nature* 2005;434:83-8.
29. Krenacs T, van DM, Lindhout E, Rosendaal M. Direct cell/cell communication in the lymphoid germinal center: connexin43 gap junctions functionally couple follicular dendritic cells to each other and to B lymphocytes. *Eur J Immunol* 1997;27:1489-97.

30. Oviedo-Orta E, Gasque P, Evans WH. Immunoglobulin and cytokine expression in mixed lymphocyte cultures is reduced by disruption of gap junction intercellular communication. *FASEB J* 2001;15:768-74.
31. Scemes E, Suadicani SO, Dahl G, Spray DC. Connexin and pannexin mediated cell-cell communication. *Neuron Glia Biol* 2007;3:199-208.
32. Qu Y, Dahl G. Function of the voltage gate of gap junction channels: selective exclusion of molecules. *Proc Natl Acad Sci U S A* 2002;99:697-702.
33. Harris AL. Connexin channel permeability to cytoplasmic molecules. *Prog Biophys Mol Biol* 2007;94:120-43.
34. la SA, He J, Laricchia-Robbio L, Gorini S, Iwasaki A, Braun M, et al. Cholera toxin inhibits IL-12 production and CD8alpha+ dendritic cell differentiation by cAMP-mediated inhibition of IRF8 function. *J Exp Med* 2009;206:1227-35.
35. Giordano D, Magaletti DM, Clark EA, Beavo JA. Cyclic nucleotides promote monocyte differentiation toward a DC-SIGN+ (CD209) intermediate cell and impair differentiation into dendritic cells. *J Immunol* 2003;171:6421-30.
36. Li K, Anderson KJ, Peng Q, Noble A, Lu B, Kelly AP, et al. Cyclic AMP plays a critical role in C3a-receptor-mediated regulation of dendritic cells in antigen uptake and T-cell stimulation. *Blood* 2008;112:5084-94.
37. Belliveau DJ, Bani-Yaghoob M, McGirr B, Naus CC, Rushlow WJ. Enhanced neurite outgrowth in PC12 cells mediated by connexin hemichannels and ATP. *J Biol Chem* 2006;281:20920-31.
38. Bruzzone S, Guida L, Zocchi E, Franco L, De FA. Connexin 43 hemi channels mediate Ca2+-regulated transmembrane NAD+ fluxes in intact cells. *FASEB J* 2001;15:10-2.
39. Kanaporis G, Mese G, Valiuniene L, White TW, Brink PR, Valiunas V. Gap junction channels exhibit connexin-specific permeability to cyclic nucleotides. *J Gen Physiol* 2008;131:293-305.
40. Eltzschig HK, Eckle T, Mager A, Kuper N, Karcher C, Weissmuller T, et al. ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function. *Circ Res* 2006;99:1100-8.
41. Wong CW, Christen T, Roth I, Chadichristos CE, Derouette JP, Foglia BF, et al. Connexin37 protects against atherosclerosis by regulating monocyte adhesion. *Nat Med* 2006;12:950-4.
42. Borsellino G, Kleinewietfeld M, Di MD, Sternjak A, Diamantini A, Giometto R, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* 2007;110:1225-32.
43. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 2007;204:1257-65.
44. Schmidt EM, Wang CJ, Ryan GA, Clough LE, Qureshi OS, Goodall M, et al. CtlA-4 controls regulatory T cell peripheral homeostasis and is required for suppression of pancreatic islet autoimmunity. *J Immunol* 2009;182:274-82.
45. Scheuplein F, Schwarz N, Adriouch S, Krebs C, Bannas P, Rissiek B, et al. NAD+ and ATP released from injured cells induce P2X7-dependent shedding of CD62L and externalization of phosphatidylserine by murine T cells. *J Immunol* 2009;182:2898-908.
46. Liu H, Rhodes M, Wiest DL, Vignali DA. On the dynamics of TCR: CD3 complex cell surface expression and downmodulation. *Immunity* 2000;13:665-75.
47. Tseng SY, Liu M, Dustin ML. CD80 cytoplasmic domain controls localization of CD28, CTLA-4, and protein kinase Ctheta in the immunological synapse. *J Immunol* 2005;175:7829-36.
48. Doty RT, Clark EA. Subcellular localization of CD80 receptors is dependent on an intact cytoplasmic tail and is required for CD28-dependent T cell costimulation. *J Immunol* 1996;157:3270-9.

METHODS

Isolation of CD62L⁺ and CD62L⁻ Treg cells

Treg cells were isolated from LNs and spleens of naive mice by using the CD4 untouched magnetic bead separation kit from Miltenyi Biotec followed by staining of the CD4⁺ T cells with anti-CD25 PE mAb and anti-PE Beads. Separation was performed through magnetic columns (average purity of CD4⁺CD25⁺ cells, >90%). Separation of the CD4⁺CD25⁺ T cells into CD62L⁺ and CD62L⁻ fractions was performed with CD62L Beads (Miltenyi Biotec). The purity of the isolated cell fractions and the expression of Foxp3 were always confirmed with flow cytometry. Cells were labeled with PKH26 (Sigma-Aldrich), according to the manufacturer's protocol.

Migration pattern of injected Treg cells

Mice were injected with 3×10^6 PKH-PE-labeled CD62⁺ Treg cells or CD62L⁻ Treg cells 2 hours before sensitization with 15 μ L of 1% TNCB. Two days later, LNs, spleen, and blood were isolated and analyzed by means of flow cytometry for the amount of injected PKH-PE⁺ Treg cells.

Functional assay

CD4⁺ T cells (1×10^5) isolated from LNs and spleens of naive mice were cocultured with different amount of CD62L⁺ or CD62L⁻ Treg cells and stimulated with anti-CD3 and anti-CD28 (0.5 μ g/mL each). Proliferation was measured by using tritiated thymidine (Amersham) incorporation with a PerkinElmer scintillation counter.

CHS

Mice were sensitized by painting 15 μ L of 1% TNCB (Sigma-Aldrich) dissolved in acetone/olive oil (4:1) on the shaved abdomen on day 0. On day 5, mice were challenged with epicutaneous application of 10 μ L of 0.5% TNCB on each side of the right ears. Left ears were treated with solvent only. After 24 hours, ear thickness was measured. Treg cells (0.5×10^6 to 5×10^6) were injected intravenously 2 hours before sensitization. CD25-depleting antibody (PC61, 300 μ g per mouse) was injected intraperitoneally 4 days before Treg cell injection.

Calcein exchange

Conventional CD4⁺CD25⁻ T cells were labeled with Calcein AM (Invitrogen), according to the manufacturer's protocol, and cocultured with MHC class II-positive BM-DCs for 24 hours. Transfer of Calcein was detected by means of flow cytometry. Gap27 peptide (SRPTEKTIFII; synthesized by Activotec) dissolved in Hanks balanced salt solution (300 μ mol/L) was used as a gap junction inhibitor. CD4⁺CD25⁻ T cells were incubated with Gap27 at least 60 minutes before *in vitro* culture with BM-DCs.

Viability of CD11c⁺ DCs

CD11c⁺ DCs, freshly isolated from the draining inguinal LNs of all differently treated groups or after 24 hours in culture without any further stimulation, were analyzed by means of flow cytometry for fluorescence of propidium iodide and Annexin V (both from BD Biosciences, Germany).

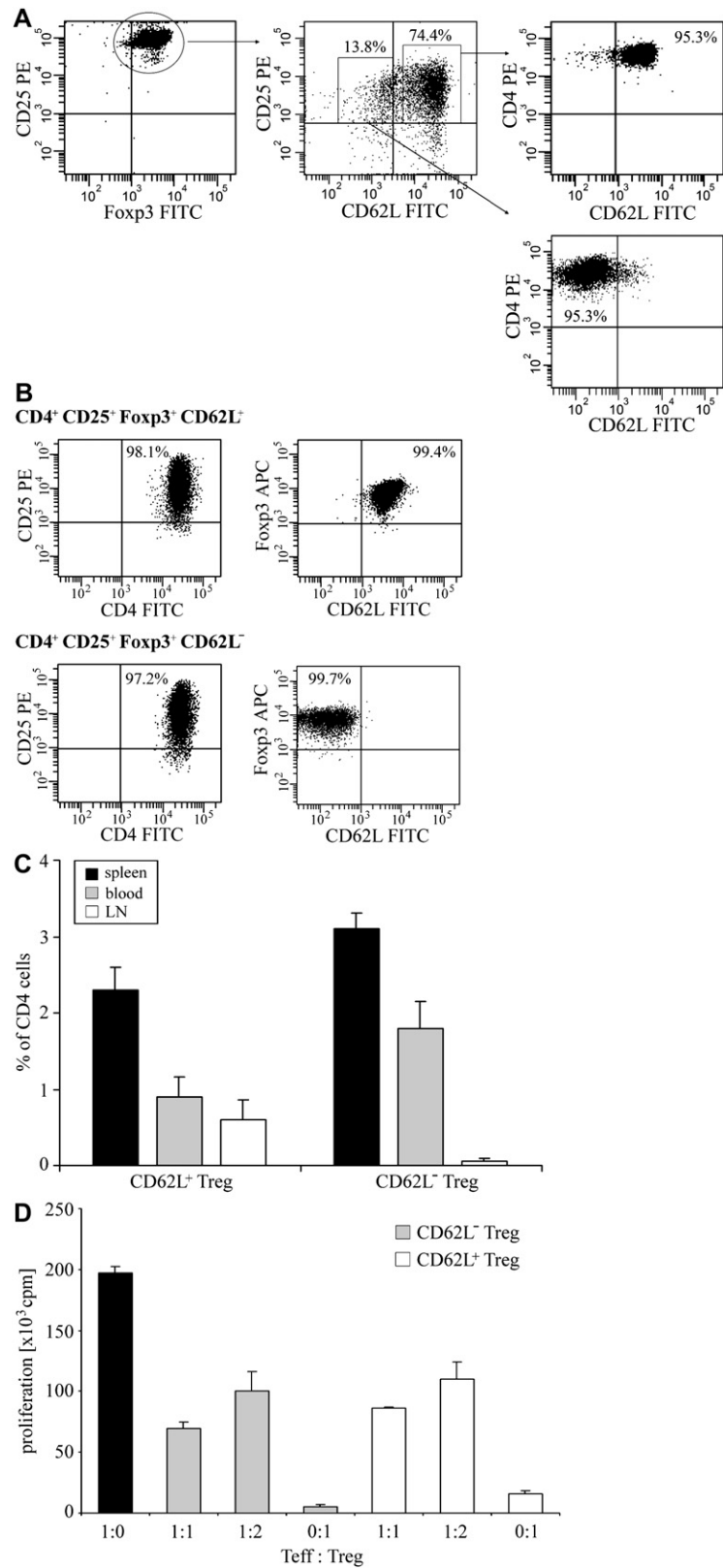


FIG E1. *In vitro* suppression of Treg cells independent of their *in vivo* homing pattern. **A**, Isolated CD62L⁺ and CD62L⁻ subpopulations of Treg cells. *FITC*, Fluorescein isothiocyanate. **B**, Foxp3 expression on CD62L⁻ and CD62L⁺ Treg cells. **C**, Migration pattern of injected CD62L⁺ and CD62L⁻ Treg cells. The frequency of the injected PKH-PE⁺ Treg cells in the organs is shown as a percentage of CD4⁺ T cells. **D**, Suppressive function of CD62L⁺ and CD62L⁻ Treg cells on the proliferation of conventional CD4⁺CD25⁻ T cells. Data show the mean proliferation $\pm$ SD from triplicates of one characteristic experiment of 5 total experiments.

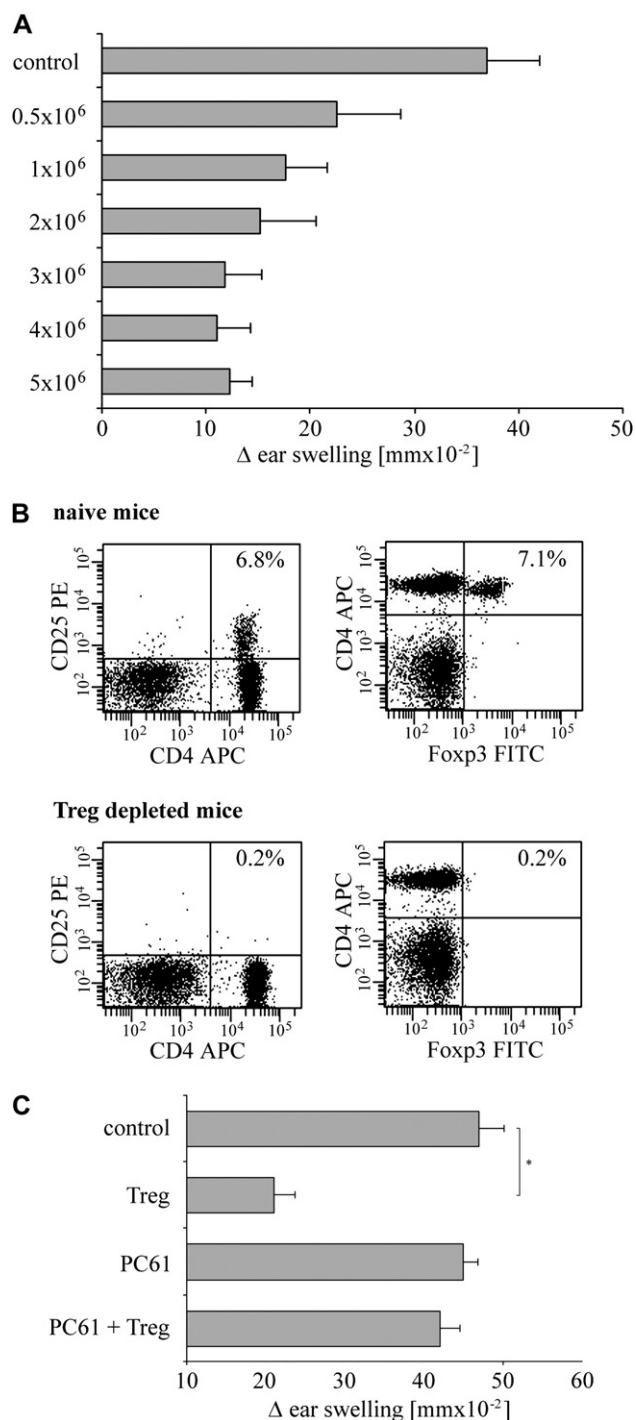


FIG E2. Total number of Treg cells in the dLNs determines the effectiveness of suppression. **A**, Ear-swelling reaction of mice treated with graded doses of Treg cells (0.5×10^6 to 5×10^6). **B**, CD25 and Foxp3 expression in LNs from naive or Treg cell-depleted mice. *APC*, Allophycocyanin; *FITC*, fluorescein isothiocyanate. **C**, Ear-swelling reaction of mice treated with Treg cells, with the Treg cell-depleting antibody PC61, with PC61 and Treg cells, or left untreated (*control*). Data in Fig 2, A and C, show the mean increase in ear thickness $\pm$ SD ($*P < .001$) of 3 independent experiments comprising 3 mice each.

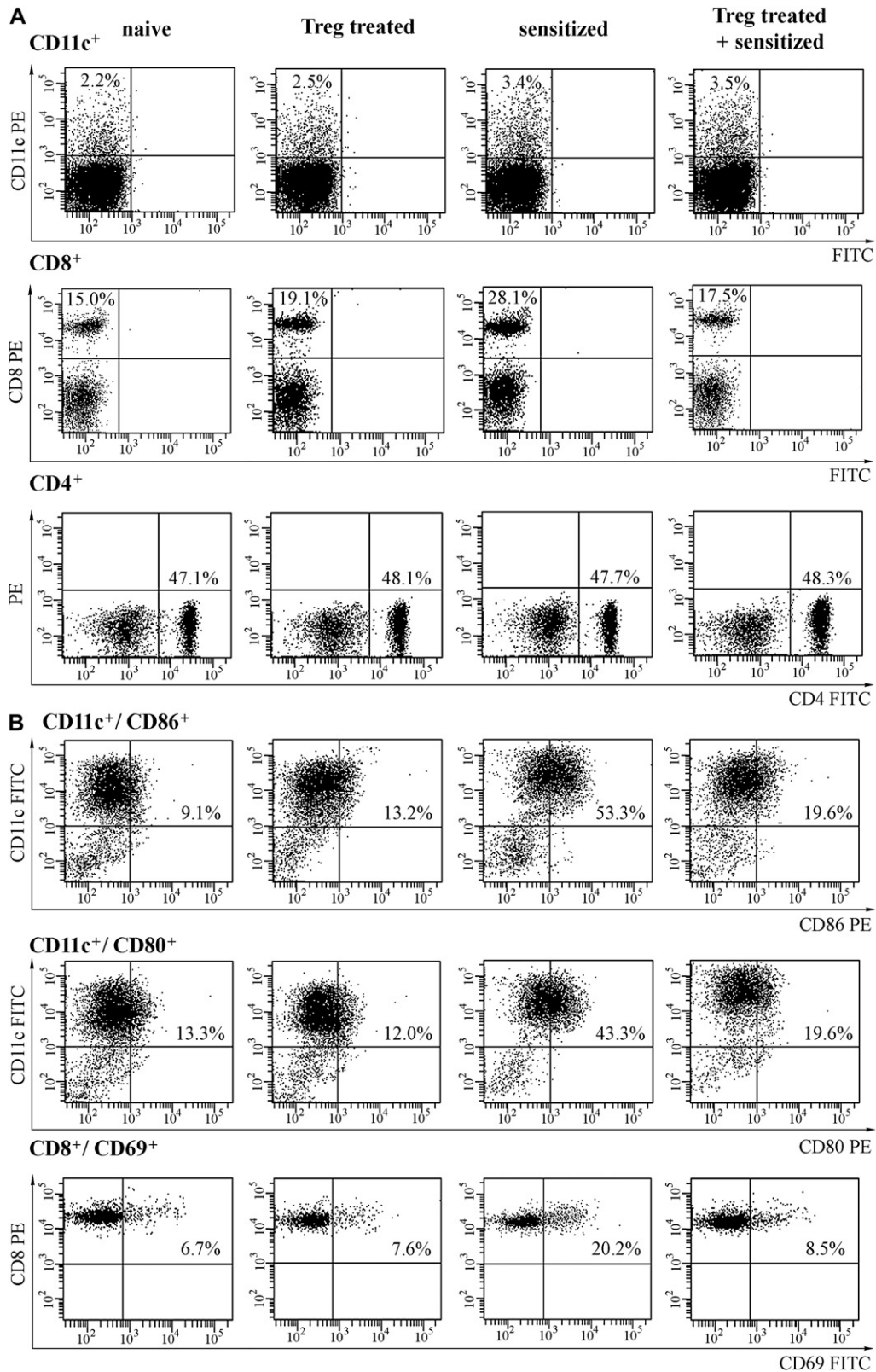


FIG E3. Treg cells inhibit the activation of DCs and CD8⁺ T cells in the dLNs. **A**, Lymphocyte populations in the dLNs of naive, sensitized, Treg cell-injected and Treg cell-injected and sensitized mice 48 hours after sensitization. **B**, Activated CD11c⁺ cells and CD8⁺ T cells in the dLNs. FITC, Fluorescein isothiocyanate.

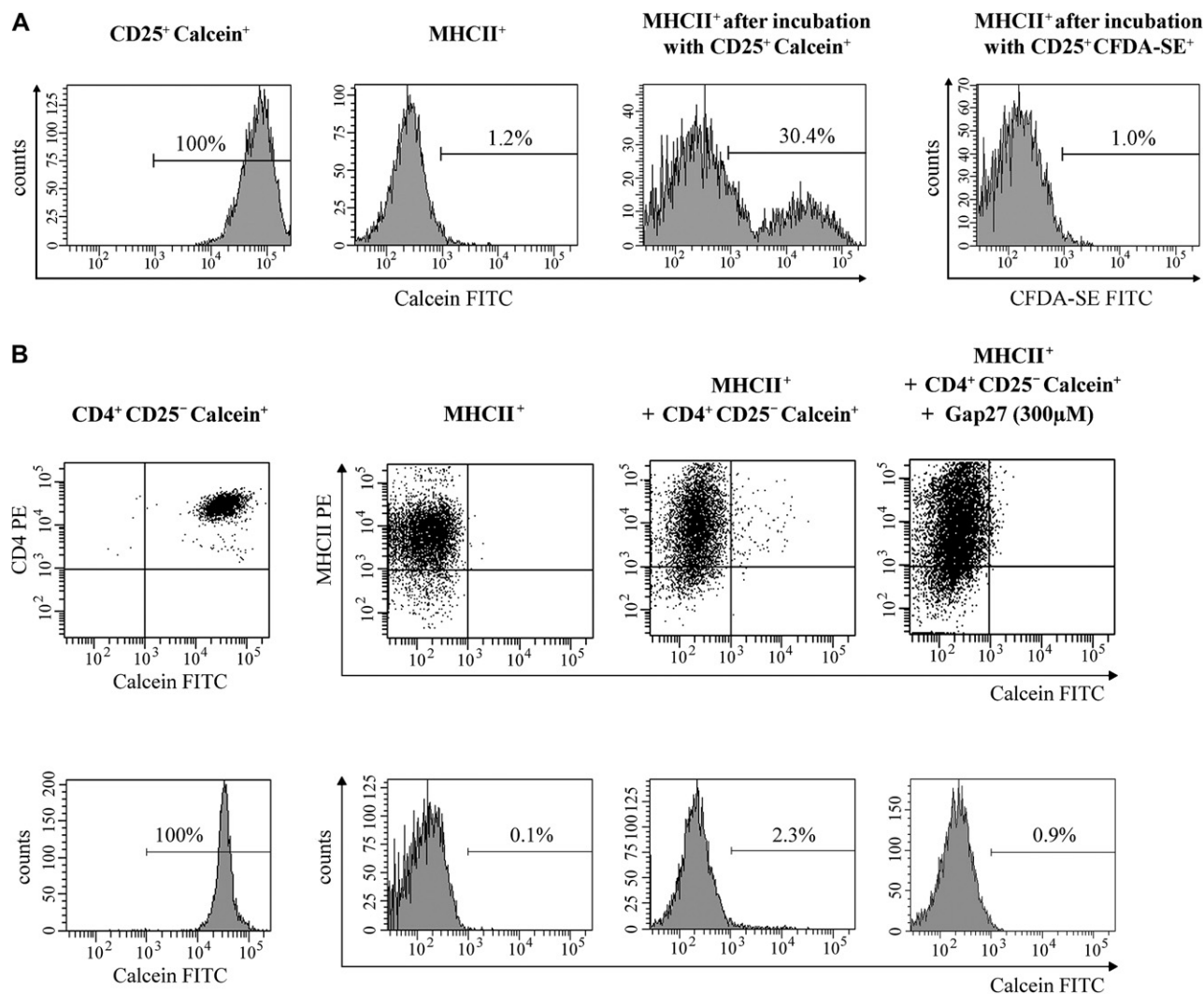


FIG E4. Treg cells interact with DCs *in vitro* through gap junctions. **A**, Calcein and CFDA-SE uptake of BM-DCs (MHC II⁺) from Calcein-positive or CFDA-SE⁺ Treg cells. **B**, CD4⁺CD25⁻ T cells do not interact with DCs *in vitro*. Calcein uptake of BM-DCs (MHC II⁺) after coculture with CD4⁺CD25⁻ Calcein-positive T cells (ratio 1:1) measured by means of flow cytometry. FITC, Fluorescein isothiocyanate.

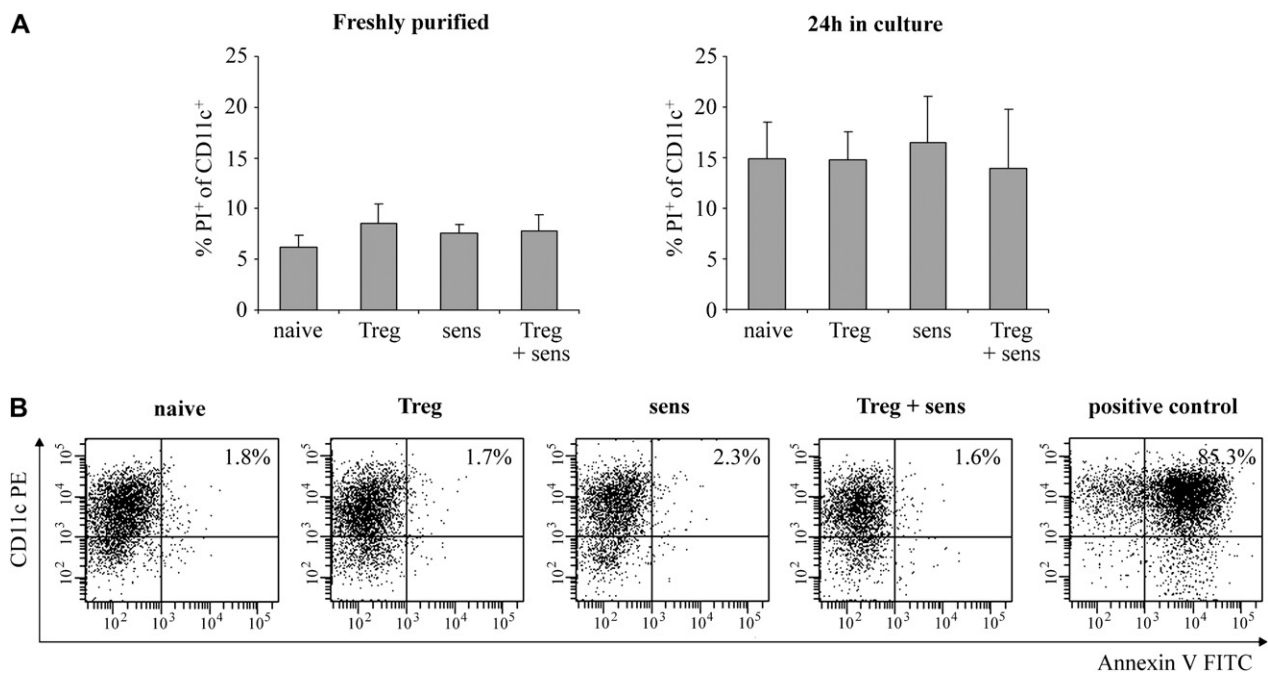


FIG E5. Viability of isolated CD11c⁺ DCs. **A**, Propidium iodide–positive CD11c⁺ cells in the purified DC populations directly after isolation (*left*) and after 24 hours in culture (*right*). **B**, Isolated CD11c⁺ cells from the 4 experimental groups were stained with CD11c and Annexin V. In positive controls apoptosis was induced by incubating the cells at 42°C for 45 minutes.

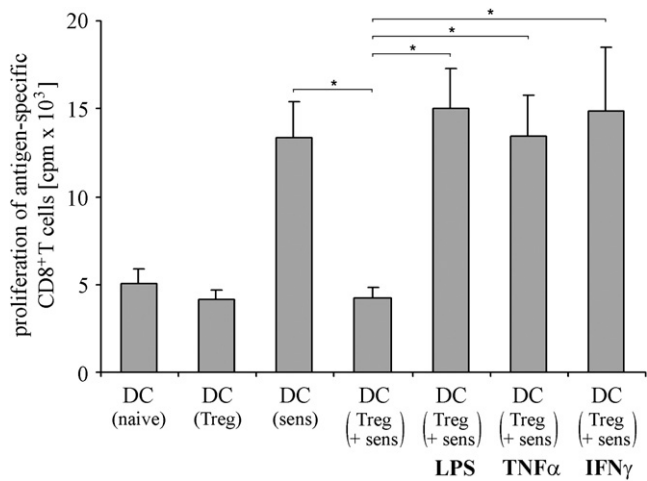


FIG E6. Re-establishment of the reduced stimulatory capacity, mediated by Treg cells, of DCs. Antigen-presenting capacity of freshly isolated or prestimulated (LPS, TNF- α , and IFN- γ) CD11c⁺ DCs isolated from untreated (*naive*), Treg cell-treated (*Treg*), sensitized (*sens*), and Treg cell-treated and sensitized mice (*Treg + sens*).