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*

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

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 al[1]). One disease model that has frequently been used to analyze the function 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 ($IL\text{-}10^{-/-}$) 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 μmol/L CFDA-SE in medium at 37°C in 5% CO_2. 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 μg/mL LPS, 1 μg/mL TNF-α, and 1 μg/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 μCi/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 μg/mL)–stimulated $CD11c^+$ DCs (4×10^5/100 μL) isolated from the draining inguinal LNs by using BD OptEIA ELISA Sets (BD Biosciences, Heidelberg, Germany).

CHS

Mice were sensitized by painting 15 μL 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; Kroeplin, Schlüchtern, Germany). Afterward, mice were challenged with epicutaneous application of 0.5% TNCB solution, 10 μL 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% CO_2. 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×/1.25 oil objective and a Spot RT cooled CCD camera (Visitron, Munich, Germany). Spot software was used to gather twenty 1-μm-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, Dusseldorf, Germany). Twenty to 30 optical sections of 1.5 μm thickness were taken with an 100×/1.4 objective and recorded with a Orca-2 EM camera (Hammamatsu, 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 (SRPTEKTIFII; synthesized by Activotec, Cambridge, United Kingdom) dissolved in Hanks balanced salt solution (150 μmol/L, 300 μmol/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