

Epicutaneous Immunization with Protein Antigen in the Presence of TLR4 Ligand Induces TCR $\alpha\beta$ ⁺CD4⁺ T Contrasuppressor Cells That Reverse Skin-Induced Suppression of Th1-Mediated Contact Sensitivity¹

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Our previous work showed that epicutaneous (EC) immunization of mice with different protein Ags applied on the skin in the form of a patch induces a state of subsequent Ag-nonspecific unresponsiveness due to suppressor CD4⁺8⁺ T cells (Ts) that inhibit Th1-mediated contact sensitivity (CS) reactions via released TGF- β . In the present work we show that EC immunization with Ag together with the TLR4 ligand LPS induced cells that could prevent suppression by the Ag-nonspecific Ts. These up-regulatory cells, called contrasuppressor T cells (Tcs), belong to a population of Ag-specific TCR $\alpha\beta$ CD4⁺ lymphocytes and are different from Th1 CD4⁺ cells that mediate the CS reaction. Experiments using knockout mice showed that EC induced contrasuppression is MyD88, INF- γ , and IL-12 dependent, whereas IL-6 is not involved in this phenomenon. Additional experiments with anti-INF- γ mAb showed that INF- γ is required for induction of Tcs cells but does not play a crucial role in the effector phase of contrasuppression. Additionally, treatment of CS effector cells with rIL-12 makes them resistant to EC induced suppression without affecting Ts cells, whereas IL-12 neutralization in vitro abrogates contrasuppression. These data show that IL-12 is indeed involved in the effector phase of EC induced contrasuppression and that this cytokine does not act directly on Ts cells. The mechanism of action of Tcs protects Th1 effector cells mediating CS from the nonspecific Ts, leaving suppression to other Ags intact. Ts and Tcs cells do not influence each other and can be induced simultaneously in the same animal. *The Journal of Immunology*, 2009, 182: 837–850.

Surfaces of both the skin and mucosa are constantly exposed to many Ags that play a crucial role in body protection from different pathogens present in the external world. Although development of the immune response to pathogens is of vital importance, responses to innocuous Ags are not required and sometimes can be harmful and lead to allergies. It is well known that immunization with Ag via the digestive tract or nasal mucosa leads to a state of profound immunosuppression (1–3) that seems to play an important role in avoiding the development of immune responses to nonpathogenic environmental Ag.

Our previous work showed that similarly to mucosa, epicutaneous (EC)³ immunization of mice with different protein Ag applied

on the skin in the form of a patch or cream emulsion induces a state of subsequent Ag-nonspecific unresponsiveness due to suppressor T cells (Ts) that inhibit sensitization and elicitation of effector T cell responses (4, 5). Suppression was transferable in vivo by $\alpha\beta$ TCR CD4⁺CD8⁺ double positive lymphocytes harvested from lymphoid organs of skin-patched animals and was mediated via TGF- β (6).

We found similar results in an animal model of multiple sclerosis where EC immunization with myelin basic protein reduced disease severity and decreased disease incidence (7, 8). Our further work using allogeneic skin grafts showed that EC immunization with a protein Ag delays graft rejection (9).

In recent studies we found that EC induced Ag-nonspecific suppression could be reversed by crude bacterial material as well as purified TLR-2, TLR-3, TLR-4, and TLR-9 ligands. The effect of the TLR4 ligand LPS was not observed in the *Tlr-4* mutant C3H/HeJ mouse, indicating that this effect was dependent upon intact TLR-4 signaling (10).

In the current work we show that EC immunization with the TLR4 ligand LPS induces regulatory cells that can reverse suppression. These regulatory cells, called contrasuppressor T cells (Tcs), belong to a population of TCR $\alpha\beta$ ⁺CD4⁺ lymphocytes and are Ag specific. Induction of these Tcs cells is MyD88 dependent and the reversal of suppression is dependent on INF- γ and IL-12, whereas IL-6 is not involved. Furthermore, we found that INF- γ is required for induction of Tcs cells but does not seem to play a crucial role in the effector phase of EC induced contrasuppression. Finally, we show that IL-12 is involved in EC induced contrasuppression and that this cytokine protects contact sensitivity (CS) effector cells from Ts cells and does not act directly on Ts cells.

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³ Abbreviations used in this paper: EC, epicutaneous; CS, contact sensitivity; LN, lymph node; OX, oxazolone; RC, rabbit complement; TNP, trinitrophenyl; TNP-Cl, TNP chloride; Tcs, T contrasuppressor cell; Ts, T suppressor cell.

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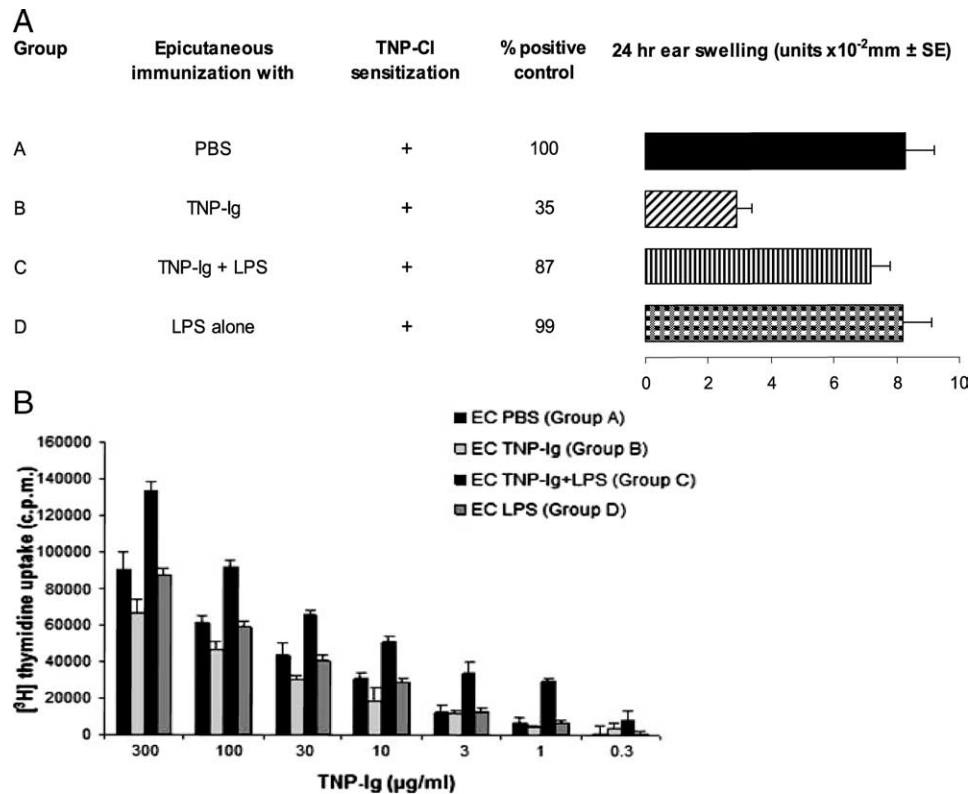


FIGURE 1. EC immunization with TNP-Ig and LPS reverses skin-induced tolerance. **A**, EC application of TNP-Ig with LPS reverses skin-induced tolerance in vivo. CBA/J mice were EC immunized via patches with TNP-Ig alone (group B) or TNP-Ig plus LPS (group C). In control groups, mice were EC exposed to PBS (group A) or LPS alone (group D). On day 7 patches were removed and mice were actively immunized with 5% TNP-Cl and then tested for CS. Results were expressed in units of $10^{-2} \text{ mm} \pm \text{SE}$. Each experimental group consisted of five or six mice. Statistical significance: $p < 0.001$ for group B vs A; and $p < 0.01$ for group C vs B. **B**, EC immunization with TNP-Ig and LPS reverses skin-induced suppression in vitro. CBA/J mice were EC exposed to PBS (group A), TNP-Ig (group B), TNP-Ig plus LPS (group C), or LPS alone (group D). On day 7, patches were removed and mice from all groups were sensitized with 5% TNP-Cl. Four days later, auxiliary and inguinal LN were collected and single cell suspensions were prepared and tested in a proliferation assay. LN cells (3×10^5 per well) were cultured in U-bottom, 96-well microplates in tetraplicate in RPMI 1640 plus FCS, L-glutamine, HEPES, and 2-ME. Three-fold dilutions of TNP-Ig were added to the wells (consecutively 300, 100, 30, 10, 3, 1, and 0.3 $\mu\text{g/ml}$). After the addition of 0.5 $\mu\text{Ci/well}$ [^3H]thymidine, the cells were incubated for 18 h and then [^3H]thymidine incorporation was measured in a beta scintillation counter. Results are expressed as cpm $\pm \text{SE}$. Statistical significance: $p < 0.01$ for 300 $\mu\text{g/ml}$, groups B vs group A; $p < 0.02$ for both 100 and 30 $\mu\text{g/ml}$, group B vs group A; and $p < 0.001$ for 300-1 $\mu\text{g/ml}$, group C vs group B.

Materials and Methods

Mice

Male CBA/J and BALB/c mice 6–8 wk old were from the breeding unit of the Department of Human Developmental Biology, Jagiellonian University, College of Medicine (Kraków, Poland). MyD88^{-/-} mice on BALB/c (H-2^d) background were a gift from Dr. D. R. Goldstein of the Section of Cardiovascular Medicine, Yale University School of Medicine (New Haven, CT). Additionally, IL-12^{-/-} mice on BALB/c background were from Taconic and C57BL/6J, IL-6^{-/-} (B6.129S-2-IL6^{tm1kopt/J}) mice on C57BL/6J background and IFN- γ ^{-/-} mice on BALB/c background were purchased from The Jackson Laboratory. Mice were fed autoclaved food and water. All experiments were conducted according to guidelines of both the Jagiellonian University College of Medicine and Yale University School of Medicine.

Reagents

Trinitrophenyl chloride (TNP-Cl) was from Chemica Alta and 4-ethoxymethylene-2-phenyloxazolone, oxazolone (OX), and rIL-12 were purchased from Sigma-Aldrich. Protein A was from Pharmacia Fine Chemicals and Sepharose 4 Fast Flow was obtained from Pharmacia LKB Biotechnology.

Low-Tox rabbit complement (RC) and LPS from *Escherichia coli* 026:B6 were obtained from Pel-Freez Biologicals and Sigma-Aldrich, respectively. Mouse Ig was prepared from CBA/J mouse sera and conjugated with TNP hapten (11, 12). A single preparation with a level of substitution of 40 TNP molecules per Ig molecule (TNP₄₀-Ig) was used throughout. Mouse Ig were conjugated with OX (OX₂₀-Ig), as described by Askenase and Asherson (13).

Additionally, HRP-streptavidin (Vector Laboratories), granulated milk (Marvel; Chivers) and *o*-phenylenediamine, and hydrogen peroxide (Sigma-Aldrich) were used.

mAbs and hybridomas

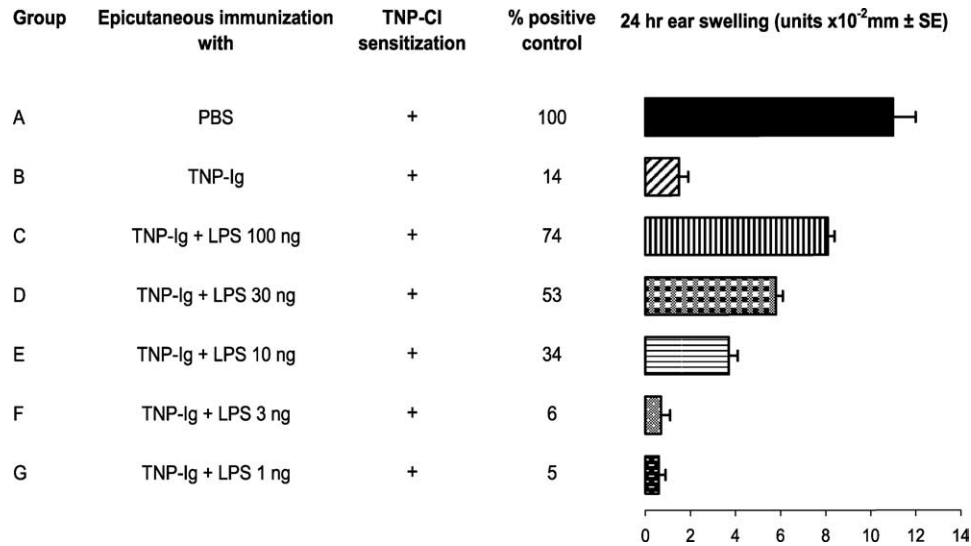
The following hybridomas were cultured: anti-TCR β clone (H57-597) from Dr. R. Kubo, Cytel Inc.; anti-TCR δ (clone UC7-13D5) from Dr. J. Bluestone, University of California, San Francisco, CA; and anti-CD4 (clone TIB 207) and anti-CD8 (clone TIB 105.3) from Dr. C.A. Janeway, Jr., Yale University, New Haven, CT. The culture supernatants were then purified on protein A as described previously (14).

Neutralizing anti-IFN- γ and anti-IL-12 mAb were purchased from BD Pharmingen. The following mouse recombinant cytokines were used as standards: TNF- α (Sigma-Aldrich), IL-6 (PeproTech), IL-12 (Genzyme), and TGF- β (BD Pharmingen). The following Abs were used in ELISA test: mAb rat anti-mouse IL-6 (MP5-20F3), biotinylated mAb rat anti-mouse IL-6 (MP532C11), mAb rat anti-mouse IL-12 (C15.6), biotinylated mAb rat anti-mouse IL-12 (C17.8 p40/p70), mAb rat anti-mouse TNF- α (G281-2626), biotinylated mAb rat anti-mouse TNF- α (MP6-XT3), mAb rat anti-mouse TGF- β 1, and biotinylated anti-mouse, anti-human, and anti-pig TGF- β 1 Abs purchased from BD Pharmingen. Anti-mouse CD11c MicroBeads were purchased from Miltenyi Biotec.

Epicutaneous immunization with TNP-Ig and TLR4 ligand LPS

EC immunization was performed by applying to the shaved skin of the mouse dorsum a 1-cm² gauze patch soaked with a solution containing 100 μg of TNP-Ig and 100 ng of the TLR4 ligand LPS in a volume of 100 μl of PBS on day 0. The patch was secured by adhesive tape wrapped around

FIGURE 2. Skin-induced suppression is reversed by TLR4 ligand LPS in dose-dependent manner. CBA/J mice were EC immunized with TNP-Ig alone (group B) or TNP-Ig with graded doses of LPS (groups C–G), or treated with PBS before sensitization with TNP-Cl. Then mice were tested for CS. Results were expressed in units of 10^{-2} mm $\pm$ SE. Each experimental group consisted of five or six mice. Statistical significance: $p < 0.001$ for group B vs A; $p < 0.001$ for groups C and D vs B; $p < 0.02$ for group E vs B; and $p = \text{NS}$ for groups F and G vs B.



the midsection. In some groups, mice were EC immunized with TNP-Ig or LPS alone. In our positive controls, mice were patched with PBS. The patch was left in place from day 0 until day 4, when it was replaced by a fresh patch. On day 7, patches were removed and mice were actively sensitized with TNP-Cl.

An identical procedure was used for EC immunization with OX-Ig.

Active sensitization and measurement of CS in vivo

Mice were actively sensitized by topical application of 0.15 ml of 5% TNP-Cl or 3% OX in acetone-ethanol mixture (1:3) to the shaved abdomen and hind feet. Control mice were shaved and painted with the acetone-ethanol mixture alone as a sham sensitization. Four days later, mice were challenged on both sides of the ears either with 10 μ l of 0.4% TNP-Cl or 10 μ l of 0.4% OX in olive oil-acetone mixture (1:1).

The subsequent increase in ear thickness was measured 24 h later with an engineer's micrometer (Mitutoyo) and expressed in units of 10^{-2} mm $\pm$ SE (15). Background increase in ear thickness (± 2 U at 24 h) of littermate nonsensitized animals that were similarly challenged was subtracted from each experimental group to yield the net ear swelling expressed in units of 10^{-2} mm $\pm$ SE. Each experimental and control group consisted of 5–6 mice.

Adoptive cell transfer of CS and cell mixing assay to evaluate contrasuppression ("transfer out" protocol)

Donors of CS-immune effector cells were contact sensitized with 5% TNP-Cl. Auxiliary and inguinal lymph nodes (LN) and spleens were harvested on day 4 and 7×10^7 immune cells were incubated for 30 min at 37°C in RPMI 1640 medium alone, washed, and then injected i.v. into normal syngeneic recipients (positive transfer). For the cell mixing assay, 7×10^7 of the CS effector immune cells from TNP-Cl contact-sensitized donors were incubated for 30 min at 37°C with 5×10^7 auxiliary and inguinal LN cells (Ts cells) from mice tolerized by EC immunization with TNP-Ig and harvested on day 7 (6). After incubation, the cell mixture was transferred i.v. into naive recipients (suppression control). To test whether EC immunization with TNP-Ig together with LPS induces regulatory cells that could reverse skin-induced suppression, 7×10^7 of the CS effector immune cells from TNP-Cl contact-sensitized donors were incubated for 30 min at 37°C with 5×10^7 auxiliary and inguinal LN cells (Tcs cells) from mice EC immunized with TNP-Ig plus LPS and harvested on day 7. Then, resultant cells were washed and incubated for 30 min at 37°C with 5×10^7 lymphoid cells from mice tolerized by EC immunization with TNP-Ig alone and harvested on day 7. After the last incubation the cell mixture was

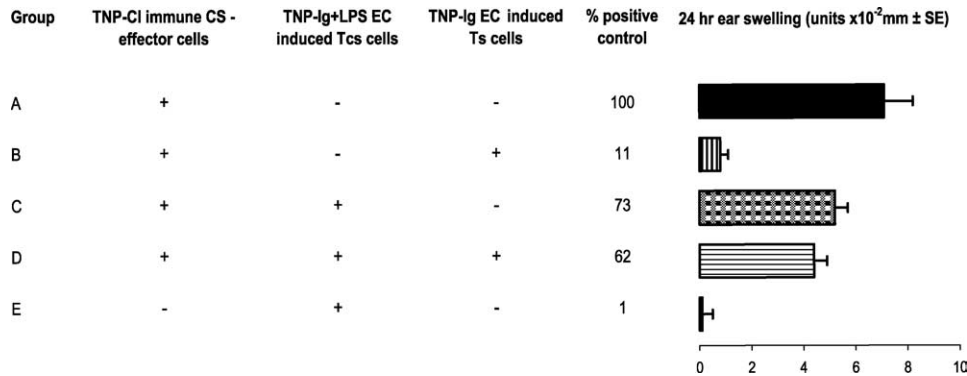


FIGURE 3. EC immunization with Ag and LPS induces Tcs cells. To test whether EC immunization with TNP-Ig together with LPS induces regulatory cells (Tcs) that could reverse skin induced suppression, 7×10^7 CS effector immune cells from TNP-Cl contact-sensitized donors were incubated for 30 min at 37°C with 5×10^7 lymphoid cells from mice EC immunized with TNP-Ig plus LPS and harvested on day 7. The resultant cells were then washed and incubated for 30 min at 37°C with 5×10^7 lymphoid cells (Ts) from mice tolerized by EC immunization with TNP-Ig and harvested on day 7. After the last incubation, the cell mixture was transferred i.v. into naive recipients (contrasuppression control) (group D). Positive control recipients received immune cells only (group A), whereas for suppression controls immune cells were cotransferred together with EC induced Ts cells (group B). To check whether Tcs affect either the function of CS effector cells or could transfer CS reaction by itself, immune cells with Tcs (group C) or Tcs alone (group E), were transferred into naive recipients. Then, all recipient mice were challenged with 0.4% TNP-Cl and tested for CS. Statistical significance: $p < 0.001$ for group B vs A; and $p < 0.001$ for group D vs B.

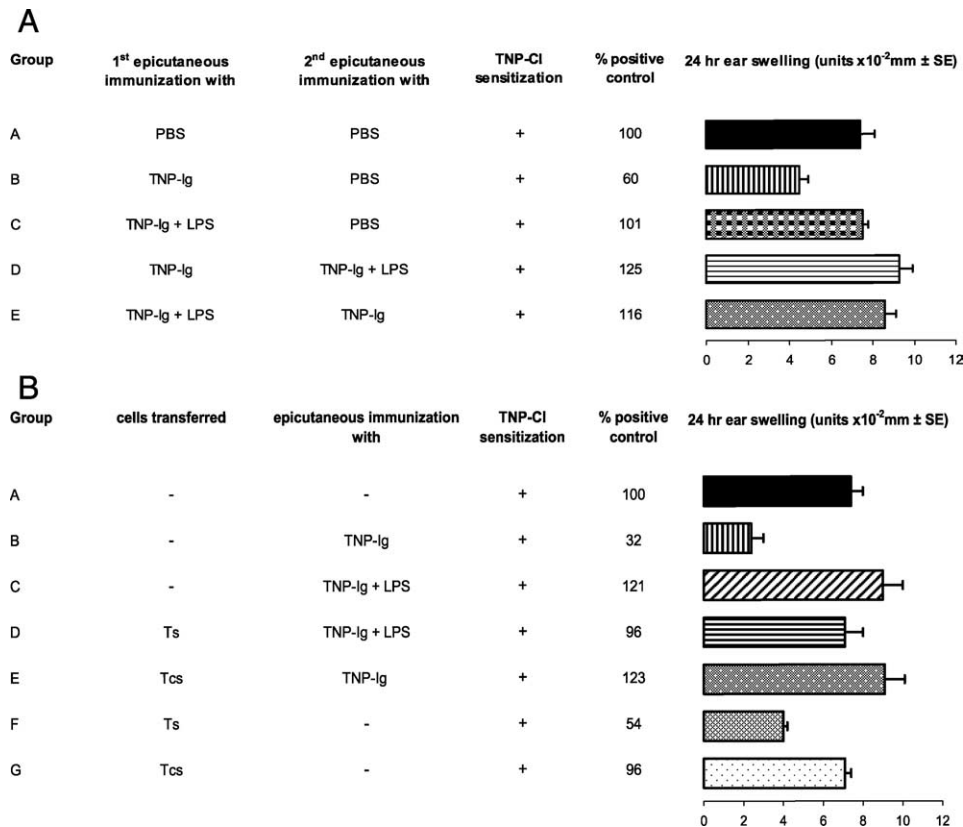


FIGURE 4. Prior exposure to Ag with LPS does not allow for induction of suppression via EC immunization with Ag alone. **A**, EC immunization with TNP-Ig plus LPS before exposure to TNP-Ig alone blocks suppression. CBA/J mice were EC treated with PBS (group A), TNP-Ig (group B), or TNP-Ig plus LPS (group C) only. In group D, mice were first EC treated with TNP-Ig alone and then with TNP-Ig plus LPS. In contrast, mice in group E were first EC treated with TNP-Ig plus LPS and subsequently with TNP-Ig alone. After patch removal, mice in all experimental groups were sensitized with TNP-Cl and tested for CS. Results were expressed in units of 10^{-2} mm $\pm$ SE. Each experimental group consisted of five or six mice. Statistical significance: $p < 0.02$ for group B vs A; and $p < 0.001$ for groups C, D, and E vs B. **B**, Transfer of Ts cells before EC immunization with TNP-Ig plus LPS does not disturb the induction of contrasuppression. CBA/J mice were EC exposed to PBS (group A), TNP-Ig (group B), or TNP-Ig plus LPS (group C). In group D, mice received EC induced Ts cells before patching with TNP-Ig plus LPS, whereas animals in group E were transferred with EC induced Tcs before EC immunization with TNP-Ig alone. In control groups, animals received Ts (group F) or Tcs (group G) but were not patched with Ag. After patch removal, mice in all experimental groups were sensitized with TNP-Cl and tested for CS. Results were expressed in units of 10^{-2} mm $\pm$ SE. Each experimental group consisted of five or six mice. Statistical significance: $p < 0.001$ for group B vs A; $p < 0.001$ for groups C and E vs B; and $p < 0.001$ for group D vs B.

transferred i.v. into naive recipients (contrasuppression control). To determine the involvement of APCs in EC induced contrasuppression, before incubation with T effector and Ts cells and subsequent adoptive transfer, Tcs cells were depleted of APC by adherence to plastic (16) followed by MACS isolation with anti-CD11c beads (17). The resulting Tcs cell population was completely free of CD11c cells as determined by FACS. After APC depletion, Tcs cells were incubated with T effector and Ts cells before adoptive transfer into naive recipients.

Mice were subsequently ear challenged with Ag within 30 min after cell transfer and tested for CS at 24 h, as described above.

Phenotype of contrasuppressor cells

To determine the phenotype of patch immunization-induced contrasuppressor cells in vivo, LN cells isolated from mice EC immunized with TNP-Ig plus LPS were incubated in PBS on ice with purified anti-TCR β , anti-TCR δ , anti-CD4, or anti-CD8 mAbs ($1 \mu\text{g Ab}/10^6$ cells) or with PBS alone for 45 min. Then, the cells were washed and incubated with a predetermined dilution of RC for 60 min at 37°C and then washed and resuspended in PBS. After that, on day 4 TNP-Cl immune CS effector cells (7×10^7) were incubated for 30 min at 37°C with medium alone (positive control) or with 5×10^7 EC induced contrasuppressor cells treated with RC alone (contrasuppression control) or with 5×10^7 cell aliquots of contrasuppressor cells treated with each appropriate mAb and RC and then washed and incubated for 30 min at 37°C with 5×10^7 EC induced suppressor cells and finally transferred i.v. into naive recipients that were challenged with TNP-Cl, and their CS reactions measured 24 h later. Positive control and suppression control were prepared as described in previous paragraph.

Transfer of regulatory cells into EC tolerized or contrasuppressed mice ("transfer in" protocol)

To test whether already established suppression or contrasuppression could be reversed by adoptive transfer of Tcs or Ts cells, respectively, we developed a "transfer in" protocol. Recipient mice were patched with PBS or TNP-Ig alone or with TNP-Ig plus LPS for 1 wk. On day 7, half of the EC recipients treated with TNP-Ig (suppressed recipients) and half of the EC immunized recipient mice treated with TNP-Ig plus LPS (contrasuppressed recipients) received i.v. 5×10^7 Tcs or Ts cells, respectively. Immediately after cell transfer, mice were sensitized with 5% TNP-Cl. Also, mice in control groups (patched with PBS or TNP-Ig or TNP-Ig plus LPS without receiving cell transfers) were sensitized with TNP-Cl. Four days later, mice were challenged and tested for CS.

Reversal of skin-induced suppression in vitro

CBA/J mice were EC exposed to TNP-Ig, TNP-Ig plus LPS, or LPS alone, as described above. Then, on day 7 mice were actively contact immunized by topical application of 5% TNP-Cl. As positive controls we used mice that were EC treated with PBS alone, before active TNP-Cl sensitization. Four days after TNP-Cl immunization, single cell suspensions of auxiliary and inguinal lymph node cells were prepared under aseptic conditions and 3×10^5 lymph node cells were incubated in U-bottom 96-well micro plates (Falcon) in tetraplicate with 3-fold decreasing dilutions of TNP-Ig starting with $300 \mu\text{g}/\text{ml}$ in $200 \mu\text{l}$ of RPMI 1640 containing 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamine, 25 mM HEPES, 5×10^{-5} M 2-ME, and 10% FCS for 48 h. Then, 0.5 $\mu\text{Ci}/\text{well}$ [^3H]thymidine was

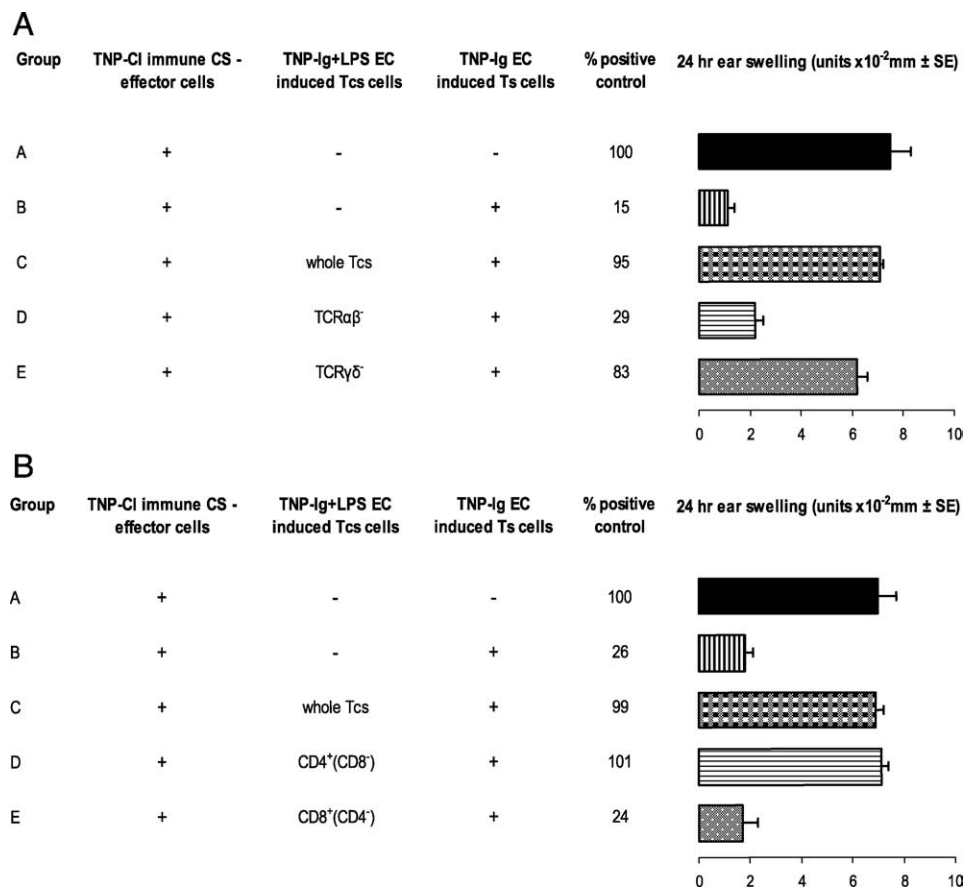


FIGURE 5. EC immunization with TNP-Ig plus LPS induces TCR $\alpha\beta$ ⁺ CD4⁺ Tcs cells. **A**, EC exposure to Ag together with LPS induces TCR $\alpha\beta$ ⁺ cells that protect from suppression. To determine the phenotype of skin-induced contrasuppressor cells *in vivo*, LN cells isolated from mice EC immunized with TNP-Ig plus LPS were incubated with purified anti-TCR β (group D) or anti-TCR δ (group E) mAb and RC. Then, on day 4, TNP-CI immune CS effector cells (7×10^7) were incubated for 30 min at 37°C with medium alone (positive control) (group A), 5×10^7 EC induced contrasuppressor cells treated with RC alone (contrasuppression control) (group C), or 5×10^7 cell aliquots of contrasuppressor cells treated with each appropriate mAb and RC and then washed and incubated for 30 min at 37°C with 5×10^7 EC induced suppressor cells and finally transferred *i.v.* into naive recipients that were challenged with TNP-CI and their CS reactions were measured 24 h later. In the suppressor control group, immune cells were cotransferred together with EC induced Ts cells (group B). Statistical significance: $p < 0.001$ for group B vs A; $p < 0.001$ for groups C and E vs B; and $p = \text{NS}$ for group D vs B. **B**, Skin-induced Tcs cells belong to the population of CD4⁺ T lymphocytes. LN cells isolated from mice EC immunized with TNP-Ig plus LPS were incubated with purified anti-CD8 (group D) or anti-CD4 (group E) mAb and RC. Then, on day 4 TNP-CI immune CS effector cells (7×10^7) were incubated for 30 min at 37°C with medium alone (positive control) (group A), 5×10^7 EC induced contrasuppressor cells treated with RC alone (contrasuppression control) (group C), or 5×10^7 cell aliquots of contrasuppressor cells treated with each appropriate mAb and RC and then washed and incubated for 30 min at 37°C with 5×10^7 EC induced suppressor cells and finally transferred *i.v.* into naive recipients that were challenged with TNP-CI and their CS reactions measured 24 h later. In the suppressor control group, immune cells were cotransferred together with EC induced Ts cells (group B). Statistical significance: $p < 0.001$ for group B vs A; $p < 0.001$ for groups C and D vs B; and $p = \text{NS}$ for group E vs B.

added and cells were incubated for an additional 18 h. Then, cells were harvested and [³H]thymidine incorporation was determined by beta scintillation counting (6). Results are presented as mean cpm $\pm$ SE. Background scintillation (cells cultured without any Ag) was subtracted from all tested groups.

Cytokines (TNF- α , IL-6, IL-12, IFN- γ , and TGF- β) immunoassays

CBA/J mice were EC exposed to TNP-Ig, TNP-Ig plus LPS, or LPS alone as described in *Material and Methods*. Then, on day 7 auxiliary and inguinal LN cells were collected and processed under aseptic conditions.

LN cells (3×10^6) from mice EC treated with PBS (control group), TNP-Ig, TNP-Ig plus LPS, or LPS alone were cultured in 1 ml RPMI 1640 medium supplemented with 5% FCS in the presence of 100 $\mu\text{g}/\text{ml}$ TNP-₄₀-Ig. Cells were distributed in triplicate wells in flat 24-well Falcon plates. After 48 h of culture, supernatants were collected and then tested for cytokine concentration (18).

Concentrations of TNF- α , IL-6, and IL-12 in culture supernatants were measured by capture ELISA test using Corning Easy Wash plates (Corning). Recombinant murine cytokines were used as standards. To each well a suitably diluted Ab was added (50 $\mu\text{l}/\text{well}$). After overnight incubation,

wells were washed twice with PBS containing 0.1% Tween 20 and then blocked with 200 μl of 3% granulated milk in PBS. Plates were thoroughly washed and, after the addition to each well of 100 μl of approximately diluted standards or tested supernatants, were incubated overnight at 4°C. After several washes, 100 μl of biotinylated Ab was added to each well and plates were incubated for 1 h at 37°C and again thoroughly washed. Then, after the addition of 100 μl of streptavidin-HRP and 100 μl of *o*-phenylenediamine plus H₂O₂ to each well, plates were incubated for 30 min at room temperature and the reaction was stopped with 3 M H₂SO₄. The OD of each well was measured at 492 nm in a 96-well plate reader and the concentration of cytokines in samples was read from a standard curve. Sensitivity of ELISA test was as follows: IL-6, 15 pg/ml; IL-12 p40, 30 pg/ml; TNF- α , 10 pg/ml.

Samples were tested for IFN- γ concentration with the use of BD OptEIA Set (BD Biosciences).

For estimating TGF- β concentrations, supernatants were at first acidified with 1N HCl diluted 1/25 and then neutralized with 1N NaOH to pH 7.0 in the same proportion. Cytokine concentrations in culture supernatants were measured by sandwich ELISA using monoclonal rat anti-mouse TGF- β Abs as capture Ab and biotinylated monoclonal rat anti-mouse, anti-human, and anti-pig TGF- β Abs as the secondary Ab. The reaction

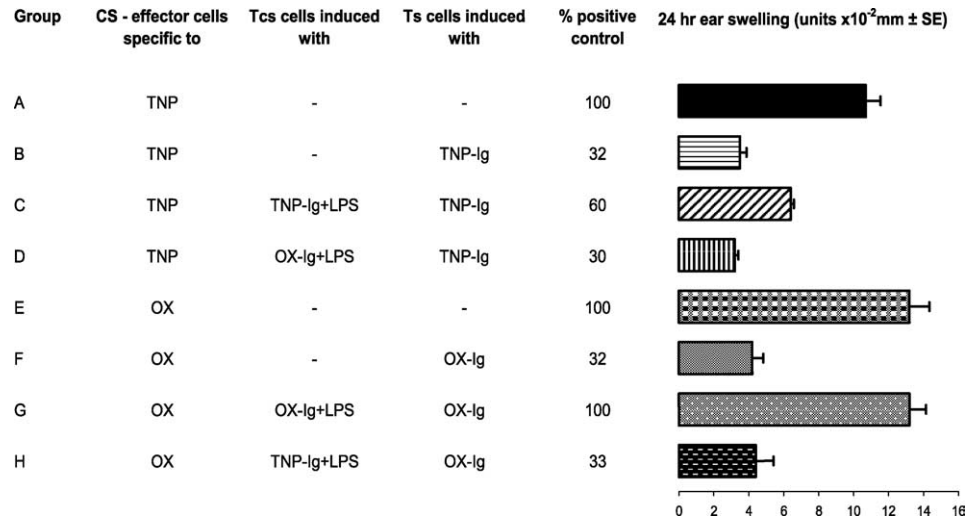


FIGURE 6. Tcs cells induced via EC immunization are Ag specific. On day 4 TNP-Cl immune CS effector cells (7×10^7) were incubated for 30 min at 37°C with medium alone (positive control) (group A) or 5×10^7 contrasuppressor cells induced via EC immunization with either TNP-Ig plus LPS (group C) or OX-Ig plus LPS (group D). Then, cell mixtures (groups C and D) were incubated for 30 min at 37°C with 5×10^7 suppressor cells induced via TNP-Ig patching and finally transferred i.v. into naive recipients that were challenged with TNP-Cl and their CS reactions measured 24 h later. In the suppressor control group, immune cells were cotransferred together with EC induced Ts cells (group B). In the other part of the experiment, on day 4 OX immune CS effector cells (7×10^7) were incubated for 30 min at 37°C with medium alone (positive control) (group E) or 5×10^7 contrasuppressor cells induced via EC immunization with either OX-Ig plus LPS (group G) or TNP-Ig plus LPS (group H). Then, cell mixtures (groups G and H) were incubated for 30 min at 37°C with 5×10^7 suppressor cells induced via OX-Ig patching and finally transferred i.v. into naive recipients that were challenged with OX, and CS the reaction was measured 24 h later. In the suppressor control group, immune cells were cotransferred together with EC induced Ts cells (group F). Statistical significance: $p < 0.001$ for group B vs A; $p < 0.001$ for group C vs B; $p = \text{NS}$ for group D vs B; $p < 0.001$ for group F vs E; $p < 0.001$ for group G vs F; and $p = \text{NS}$ for group H vs F.

was developed with HRP-streptavidin followed by *o*-phenylenediamine and H_2O_2 as substrate and was stopped with 3M H_2SO_4 . The OD of each well was measured in a 96-well plate reader at 492 nm. All determinations were done in triplicate. Standard curve was generated with recombinant mouse TGF- β . The lower concentration limit was 30 pg/ml TGF- β .

Statistics

The paired two-tailed Student's *t* test was used with $p < 0.05$ taken as the level of significance.

Results

Skin induced suppression is reversed by TLR4 ligand LPS in a dose-dependent manner

Our previous work showed that EC immunization with protein Ag, e.g., TNP-Ig, induces Ag-nonspecific suppression of CS in H-2^k CBA/J mice. In the current work, when using CBA/J mice we show that EC exposure to TNP-Ig plus LPS reversed skin-induced suppression both in vivo (Fig. 1A, group C vs group B) and in vitro (Fig. 1B, group C vs group B). A similar effect to that observed in H-2^k CBA/J mice (Fig. 1A) was induced in another strain of mice BALB/c mice on H-2^d haplotype (data not shown). This phenomenon is dose dependent, because full reversion of EC induced suppression was observed when 100 ng of LPS per animal was used (Fig. 2, group C) and completely disappeared when mice were EC treated with 3 ng of LPS (Fig. 2, group F). Doses of LPS as high as 100 μg per patch were not more effective in reversion of EC induced suppression than 100 ng (data not shown).

EC immunization with Ag and LPS induces Tcs cells

The data presented above showed that EC immunization with TNP-Ig plus LPS reverses suppression of CS. However, the data did not answer whether exposure to TNP-Ig and LPS induces cells that could block suppression or whether this maneuver just allows for induction of immune response without interference with T suppressor cells. However, our already published data (10) showed

that mice EC treated with TNP-Ig plus LPS without TNP-Cl sensitization did not develop CS response, suggesting that EC treatment with Ag plus LPS does not induce Th1 CS effector cells but rather regulatory cells that could protect the effector cells from the action of Ts cells. To test this hypothesis, we used "a transfer out" protocol. TNP-specific Th1 effector cells were incubated with LN and spleen cells from TNP-Ig plus LPS-treated mice and then incubated with Ts cells induced by EC treatment with TNP-Ig alone. The data presented in Fig. 3 (group D) show that EC immunization with TNP-Ig and LPS induces regulatory cells, Tcs cells that could block suppression of CS response. However, Tcs added to T effector cells without Ts cells did not change the level of immune response (group C). Additionally, LN and spleen cells isolated from mice patched with TNP-Ig and LPS were not able to transfer CS response (group E). These data strongly suggest that EC treatment with protein Ag and TLR ligand induces regulatory Tcs cells that protect Th1 effector cells from Ts cells.

Prior exposure to Ag with LPS does not allow for induction of suppression via EC immunization with Ag alone

We tried to answer the question of whether prior exposure to Ag plus LPS would allow induction of suppression after EC immunization with Ag alone and the other way round. Data presented in Fig. 4A show that EC immunization with TNP-Ig alone and subsequent EC treatment with TNP-Ig plus LPS reverses already established suppression (group D vs group B). However, mice patched with TNP-Ig plus LPS before EC exposure to TNP-Ig alone were not able to develop suppression of CS (group E vs group B).

Additionally, data presented in Fig. 4A were fully confirmed by a transfer experiment. We found that lymph node and spleen cells from mice EC immunized with TNP-Ig plus LPS blocked EC induced suppression when transferred into mice just before immunization via skin with TNP-Ig alone (Fig. 4B, group E vs group B). Moreover, LN and spleen cells from mice EC immunized with TNP-Ig alone did not

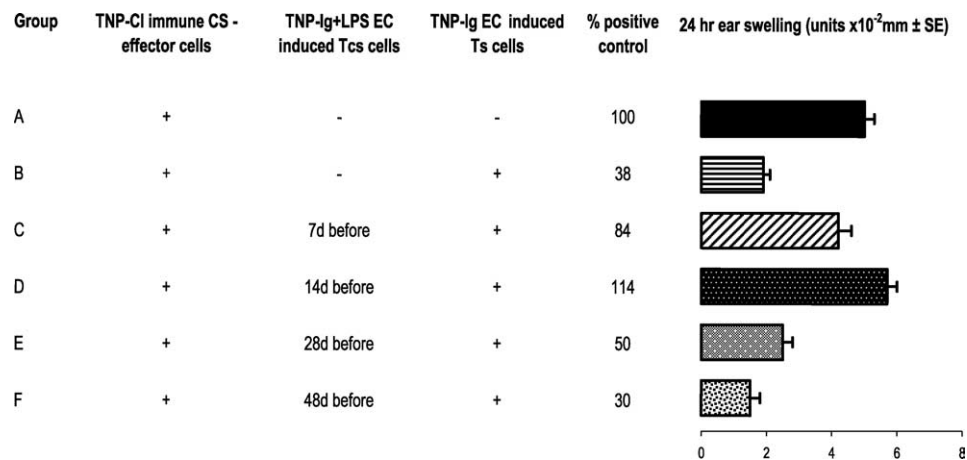


FIGURE 7. Skin-induced contrasuppression declines with time. To determine the duration of skin-induced contrasuppression, donor mice of the contrasuppressor cells were EC immunized with TNP-Ig plus LPS 1, 2, 4, or 6 wk before their use. On day 4 TNP-CI immune cells were incubated with Tcs cells isolated from mice EC immunized with TNP-Ig plus LPS at different time points (in days (d); groups C–F, respectively) and then with EC induced Ts cells. The resultant cell mixtures were then transferred into syngeneic recipients. Positive control recipients were transferred with immune cells alone (group A), whereas suppression controls received immune cells cotransferred with EC induced Ts cells (group B). All of the recipients were challenged and tested for CS. Statistical significance: $p < 0.001$ for group B vs A; $p < 0.01$ for group C vs B; $p < 0.01$ for group D vs B; and $p = \text{NS}$ for groups E and F.

disturb the induction of contrasuppression when transferred just before EC immunization with TNP-Ig plus LPS (Fig. 4B, group D vs B).

EC induced Tcs cells belong to the population of $\text{TCR}\alpha\beta^+$ CD4^+ lymphocytes

To determine the phenotype of Tcs cells, LN and spleen cells isolated from animals EC treated with TNP-Ig and LPS were treated with anti- $\text{TCR}\beta$ or anti- $\text{TCR}\delta$ mAb and RC (Fig. 5A, groups D and E respectively) or anti- CD4 or anti- CD8 mAb and RC (Fig. 5B, groups D and E respectively). The resultant cell populations were incubated with TNP-specific effector cells and then with EC induced Ts cells. In contrasuppression controls, LN and spleen cells were treated with RC alone before incubation with CS effector cells and Ts cells (Fig. 5, A and B, group C).

The data presented in Fig. 5A show that depletion of $\text{TCR}\alpha\beta$ cells abolished contrasuppressor activity of LN and spleen cells isolated from mice EC immunized with TNP-Ig and LPS (group D vs group C), whereas treatment with anti- $\text{TCR}\delta$ mAb and RC did

not affect contrasuppression (group E vs group C). Additionally, LN and spleen cells isolated from animals EC exposed to TNP-Ig and LPS lost their contrasuppressor activity after depletion of the CD4^+ cell population (Fig. 5B, group E vs group C). Treatment of Tcs cells with anti- CD8 mAb and RC did not interfere with their biological activity (Fig. 5B, group D vs group C).

Summing up, the presented data show that EC induced Tcs cells are among the population of $\text{TCR}\alpha\beta^+$ CD4^+ lymphocytes.

Tcs cells induced via EC immunization are Ag specific

Our previous work on skin-induced suppression showed that this phenomenon is Ag nonspecific. In our current work we tested Ag specificity of EC induced Tcs cells in a transfer out experiment using two non-crossreacting Ags, TNP-Ig and OX-Ig. The data presented in Fig. 6 show that Tcs cells induced by TNP-Ig and LPS could protect TNP-specific CS effector cells from EC induced Ts cells (Fig. 6, group C vs group B). However, when Tcs cells were induced by patching with OX-Ig and LPS they could not protect

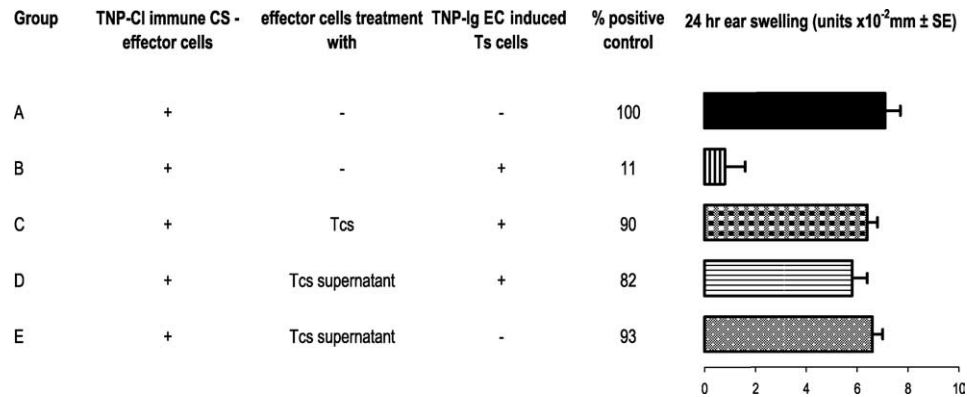


FIGURE 8. Tcs cells can be replaced by culture supernatant produced by LN cells isolated from mice EC exposed to TNP-Ig and LPS. To determine whether soluble factors produced by LN cells isolated from mice immunized with TNP-Ig plus LPS can replace Tcs lymphocytes, TNP-CI immune CS effector cells (7×10^7) were preincubated with either culture supernatant (group D) or 5×10^7 EC induced contrasuppressor cells (group C) for 30 min at 37°C before incubation with 5×10^7 EC induced suppressor cells. Positive control recipients received immune cells only (group A), whereas in suppression control immune cells were cotransferred together with EC induced Ts cells (group B). To determine whether the tested supernatant affects function of CS effector cells, on day 4 TNP-CI immune cells were incubated with supernatant alone for 30 min at 37°C before adoptive transfer (group E). After cell transfer, recipient mice were challenged with TNP-CI and tested for CS. Statistical significance: $p < 0.001$ for group B vs A; $p < 0.001$ for groups C and D vs B; and $p = \text{NS}$ for group E vs A.

Table I. EC immunization with TNP-Ig plus LPS results in secretion of proinflammatory cytokines by LN cells^a

Group	EC Immunization	TNF- α (pg/ml)	IL-6 (pg/ml)	IL-12 (pg/ml)	IFN- γ (pg/ml)	TGF- β (pg/ml)
A	PBS	62 $\pm$ 5	0 $\pm$ 0	560 $\pm$ 45	1209 $\pm$ 46	0 $\pm$ 0
B	TNP-Ig	28 $\pm$ 10	0 $\pm$ 0	880 $\pm$ 55	230 $\pm$ 22	50 $\pm$ 8
C	TNP-Ig+LPS	50 $\pm$ 10	40 $\pm$ 5	1750 $\pm$ 80	3442 $\pm$ 229	36 $\pm$ 20
D	LPS	54 $\pm$ 5	0 $\pm$ 0	580 $\pm$ 50	1438 $\pm$ 50	0 $\pm$ 0

^a To determine production of proinflammatory and suppressive cytokines, 3×10^6 LN cells from mice treated EC with PBS (group A), TNP-Ig (group B), TNP-Ig plus LPS (group C), or LPS alone (group D) were cultured in 1 ml of RPMI 1640 medium supplemented with 5% FCS in the presence of 100 μ g/ml TNP-Ig and were distributed in triplicate wells in flat 24-well Falcon plates. After 48 h, culture supernatants were collected. Then, TNF- α , IL-6, IL-12, IFN- γ , and TGF- β were measured. The presented data show that EC immunization with TNP-Ig and LPS results in increased production of IL-6 and IL-12 when compared with PBS- (group A), TNP-Ig-treated (group B), or LPS-treated (group D) mice, and there was a significant difference in TNF- α production between TNP-Ig-treated (group B) vs TNP-Ig plus LPS-treated (group C) animals. Additionally, TNP-Ig applied to the skin in the presence of LPS results in significant production of IFN- γ by LN cells when compared to TNP-Ig-, PBS-, or LPS alone-treated mice. However, we did not find any difference in TGF- β production by TNP-Ig and TNP-Ig plus LPS-treated mice. Each experimental group contained 4–5 mice and experiments were repeated three times. Statistical significance: $p < 0.05$ for TNF- α group C vs group B and $p = \text{NS}$ for group C vs groups A and D; $p < 0.001$ for IL-6 group C vs groups A, B, and D and $p < 0.001$ for IL-12 group C vs groups A, B, and D; and $p = \text{NS}$ for TGF- β group C vs group B.

TNP-specific CS effector cells from EC induced Ts cells (Fig. 6, group D vs group A).

Additionally, Tcs cells induced by OX-Ig and LPS could protect OX-specific CS effector cells from EC induced Ts cells (Fig. 6, group G vs group F). However, when Tcs cells were induced by patching with TNP-Ig and LPS they could not protect OX-specific CS effector cells from EC induced Ts cells (Fig. 6, group H vs group E). Moreover, our previous work on EC induced contrasuppression in an active model of immunization showed that nonspecific suppression can be abolished by Ag-specific Tcs cells, simultaneously leaving suppression to other Ags (10).

Skin-induced contrasuppression declines with time

To determine the duration of skin-induced contrasuppression, donor mice of the contrasuppressor T cells were EC immunized with TNP-Ig plus LPS 1, 2, 4, or 6 wk before their use. Four-day TNP-CI immune cells were incubated with Tcs cells isolated from mice EC immunized with TNP-Ig plus LPS at different time points (Fig. 7, groups C–F, respectively), and then treated with EC induced Ts cells before adoptive cell transfer. Fig. 7 shows that skin induced contrasuppression declines with time (groups C–F vs group B). Maximal protection of CS effector cells from suppression was observed when Tcs cells were used 1 or 2 wk after their induction (groups C and D).

Tcs cells can be replaced by culture supernatant produced by LN cells isolated from mice EC exposed to TNP-Ig and LPS

Our previous work on contrasuppression showed that the activity of Tcs cells induced by immune complexes can be replaced by culture supernatants produced by Tcs cells (19).

To determine whether a similar situation occurs in the currently studied model of EC induced contrasuppression, CS effector cells, before their exposure to Ts cells, were treated with culture supernatant produced by LN cells isolated from mice patched with TNP-Ig and LPS.

The data presented in Fig. 8 show that pretreatment of TNP-CI immune CS effector cells either with culture supernatant produced by LN cells isolated from animals EC immunized with TNP-Ig plus LPS (group D) or with Tcs cells (group C), before incubation with Ts cells reversed skin-induced suppression (groups D and C vs group B). Treatment of CS effector cells with culture supernatant alone did not affect the CS response (group E vs group A). Such contrasuppressor activity was not found in supernatants pro-

duced by lymph node cells isolated from mice patched with TNP-Ig, PBS, or LPS alone (data not shown).

Epicutaneous exposure to TNP-Ig and LPS induces an increased production of proinflammatory cytokines by LN cells

To determine whether cytokines are involved in the reversal of skin induced tolerance, we measured the level of the following

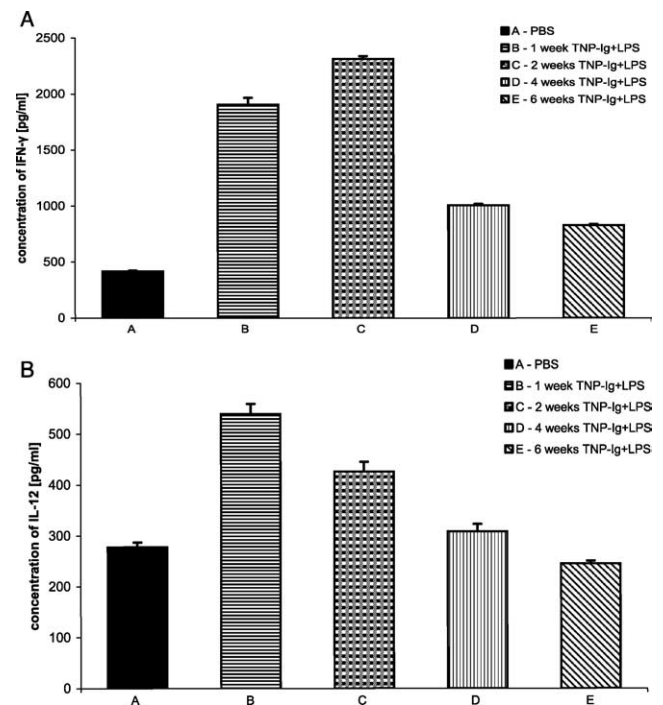


FIGURE 9. Time-dependent production of proinflammatory cytokines. To determine whether production of IFN- γ and IL-12 declines with time, 3×10^6 LN cells from EC PBS-treated controls (group A) or TNP-Ig plus LPS-treated mice 1, 2, 4, or 6 wk before harvest (groups B–E) were cultured in 1 ml RPMI 1640 medium supplemented with 5% FCS in the presence of 100 μ g/ml TNP-Ig and distributed in tetraplicate wells in flat 24-well Falcon plates. After 48 h, culture supernatants were collected. Then, IFN- γ and IL-12 were measured. A and B show that production of IFN- γ and IL-12, respectively, declines with time. Statistical significance: $p < 0.001$ for IFN- γ groups B and C vs group A; $p < 0.001$ for groups D and E vs B; $p < 0.001$ for groups D and E vs C; $p < 0.001$ for IL-12 groups B and C vs A; and $p = \text{NS}$ for groups D and E vs A.

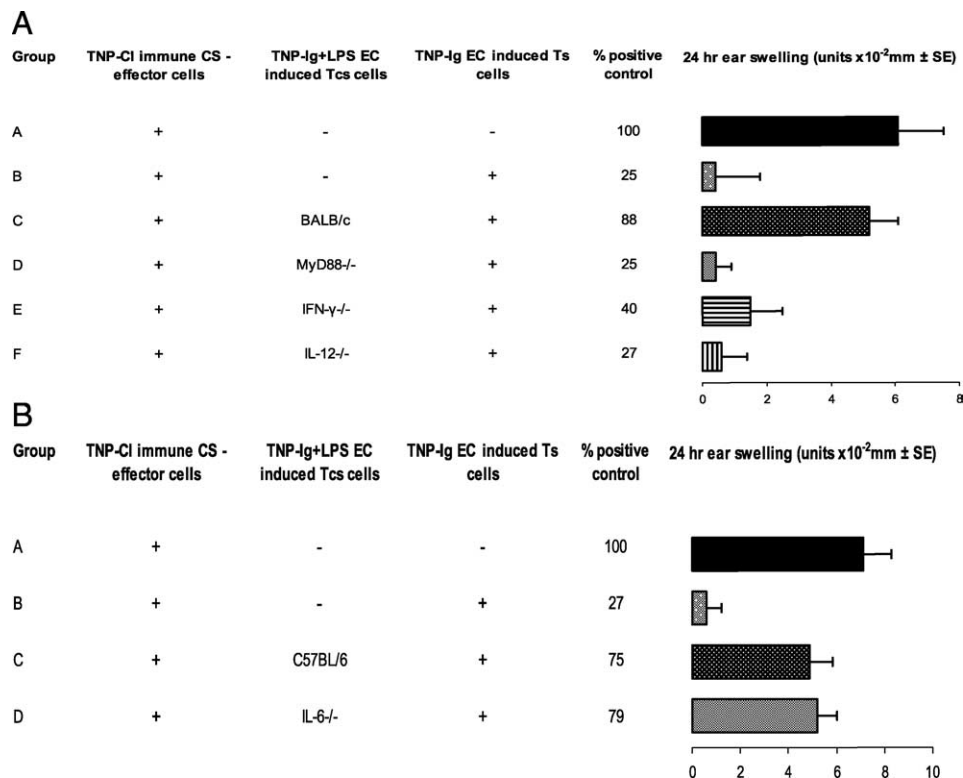


FIGURE 10. EC induced contrasuppression is MyD88, IFN- γ , and IL-12 dependent and IL-6 independent. **A**, EC induced contrasuppression is MyD88, IFN- γ , and IL-12 dependent. To determine the mechanism of EC induced contrasuppression, LN and spleen cells isolated from control BALB/c mice (group C), MyD88^{-/-} (group D), IFN- γ ^{-/-} (group E) or IL-12^{-/-} (group F) mice EC treated with TNP-Ig and LPS were incubated with TNP-specific CS effector cells and then with EC induced Ts cells. Positive control recipients were transferred with immune cells alone (group A), whereas suppression controls received immune cells cotransferred with EC induced Ts cells (group B). Then, the recipients were challenged and tested for CS. Statistical significance: $p < 0.05$ for group B vs A; $p < 0.05$ for group C vs B; $p < 0.01$ for group D vs C; $p < 0.05$ group E vs C; and $p < 0.01$ for group F vs C. **B**, IL-6 is not involved in EC induced contrasuppression. LN and spleen cells isolated from control C57BL/6J (group C) or IL-6^{-/-} (group D) mice EC treated with TNP-Ig and LPS were incubated with TNP-specific CS effector cells and then with EC induced Ts cells. Positive control recipients were transferred with immune cells alone (group A), whereas suppression controls received immune cells cotransferred with EC induced Ts cells (group B). Then, the recipients were challenged and tested for CS. Statistical significance: $p < 0.01$ for group B vs A; and $p < 0.01$ for groups C and D vs B.

proinflammatory cytokines: TNF- α , IL-6, IL-12, and IFN- γ . Additionally, in this experiment, we tested whether EC immunization with TNP-Ig plus LPS affects TGF- β production. The data presented in Table I show that EC immunization with TNP-Ig plus LPS (group C) induces significant production of IL-6 and IL-12 when compared with PBS (group A) or TNP-Ig- (group B) or LPS-treated (group D) mice. There was a significant difference in TNF- α production between TNP-Ig- (group B) vs TNP-Ig plus LPS-treated (group C) animals.

Additionally, TNP-Ig applied to the skin in the presence of LPS resulted in significant production of IFN- γ by LN cells when compared with TNP-Ig-, PBS-, or LPS alone-treated mice.

However, we did not find any difference in TGF- β production by both TNP-Ig and TNP-Ig plus LPS-treated mice.

Production of IFN- γ and IL-12 declines with time

Data presented in Fig. 7 show that EC induced contrasuppression declines with time whereas data shown in Table I indicate that EC immunization with TNP-Ig plus LPS induces production of proinflammatory cytokines such as IFN- γ and IL-12 that potentially might be involved in observed reversal of suppression. To determine whether production of proinflammatory cytokines also declines with time, mice were EC immunized with TNP-Ig plus LPS 1 (group B), 2 (group C), 4 (group D) or 6 wk (group E) before isolation of LN and their culture. In a control group, LN were collected from animals EC treated with PBS. Data presented in

Figs. 9, **A** and **B**, show that production of IFN- γ and IL-12 declines with time (groups B–E) and correlates with the gradual loss of contrasuppressor activity in vivo (Fig. 7).

EC induced contrasuppression is MyD88 and IFN- γ and IL-12 dependent

To determine the mechanism of EC induced contrasuppression, LN and spleen cells isolated from control BALB/c mice (group C), MyD88^{-/-} (group D), IFN- γ ^{-/-} (group E) or IL-12^{-/-} (group F) mice EC treated with TNP-Ig and LPS were incubated with TNP-specific CS effector cells and then with EC induced Ts cells, all on a BALB/c background, and subsequently transferred into naive BALB/c recipients. The data presented in Fig. 10A show that Tcs cells were not induced in MyD88^{-/-}, IFN- γ ^{-/-}, and IL-12^{-/-} mice when compared with wild-type BALB/c controls (Fig. 9A, groups D, E, and F vs group C).

In another experiment using IL-6^{-/-} mice, we tested whether IL-6 is involved in EC induced contrasuppression. LN and spleen cells isolated from control C57BL/6 (group C) or IL-6^{-/-} (group D) mice EC treated with TNP-Ig plus LPS were incubated with TNP-specific effector cells before incubation with EC induced Ts cells on the C57BL/6 background. Then, the cell mixtures were washed and transferred into naive C57BL/6 recipients. The data presented in Fig. 10B show that EC immunization of either C57BL/6 wild-type mice or IL-6^{-/-} mice with TNP-Ig plus LPS

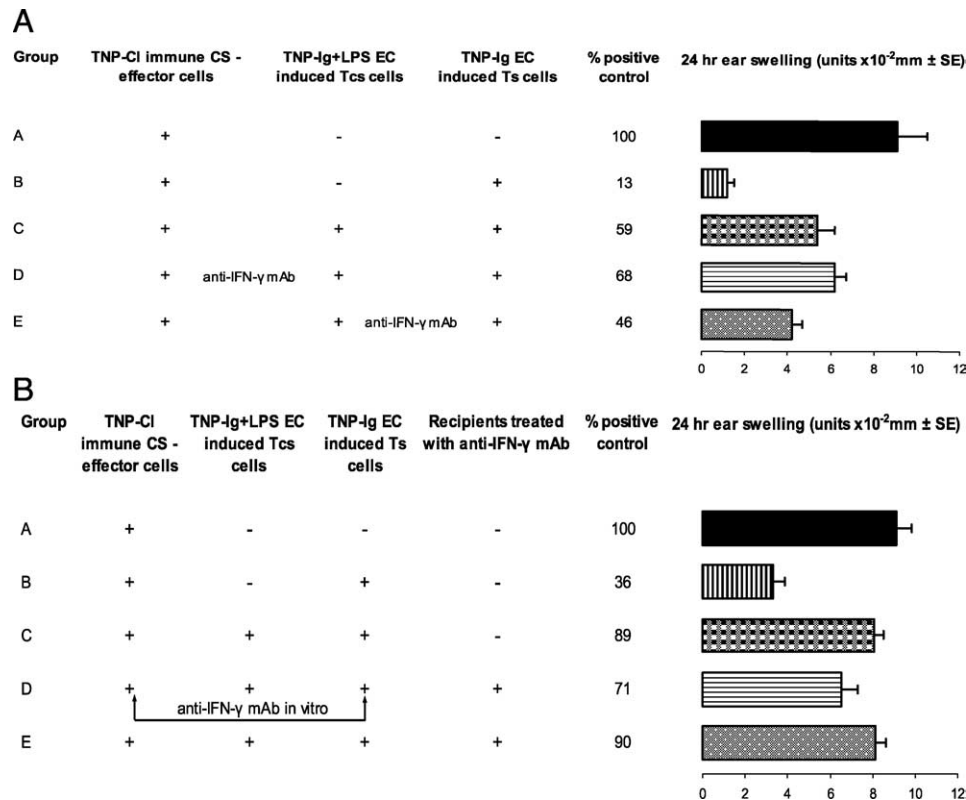


FIGURE 11. IFN- γ is not involved in the effector phase of contrasuppression. **A**, Neutralization of IFN- γ in vitro does not abolish contrasuppression. CS effector immune cells (7×10^7) from TNP-CI contact-sensitized donors were incubated for 30 min at 37°C with 5×10^7 Tcs with (group D) or without anti-IFN- γ neutralizing mAb (10 μ g/ml) (groups C and E). Then, cell mixtures were washed and incubated with 5×10^7 Ts cells with (group E) or without anti-IFN- γ neutralizing mAb (groups C and D) for another 30 min at 37°C. In a suppression control group TNP-specific CS effector cells were incubated with Ts cells before transfer (group B). Positive control contained immune cells only (group A). Statistical significance: $p < 0.001$ for group B vs A; $p < 0.001$ for groups C, D, and E vs B; and $p = \text{NS}$ for groups E and D vs C. **B**, Both in vitro and in vivo neutralization of IFN- γ does not affect EC induced contrasuppression. CS effector immune cells (7×10^7) from TNP-CI contact-sensitized donors were incubated for 30 min at 37°C with 5×10^7 Tcs with (group D), or without anti-IFN- γ neutralizing mAb (10 μ g/ml) (groups C and E). Then, cells were washed and incubated with 5×10^7 Ts cells with (group D) or without anti-IFN- γ mAb (groups C and E) for another 30 min at 37°C. Suppressor (group B) and positive controls (group A) were prepared according to the description in Fig. 10A. Then, cell mixtures were transferred into naive recipients (groups A–C), or mice treated with anti-IFN- γ mAb (250 μ g/mouse 1 day before and on the day of cell transfer) (groups D and E). Statistical significance: $p < 0.001$ for group B vs A; $p < 0.001$ for group C vs B; $p < 0.05$ for group D vs B; and $p = \text{NS}$ for groups D and E vs C.

results in induction of Tcs cells able to protect TNP-specific effector cells from the action of EC induced Ts cells (groups C and D vs group B).

These data suggest that Tcs cells induced via EC immunization with protein Ag and LPS in MyD88^{-/-}, IFN- γ , and IL-12^{-/-} mice are not capable of abrogating suppression. Additionally, experiment using IL-6^{-/-} mice shows that IL-6 does not play a crucial role in the described contrasuppression.

IL-12 but not IFN- γ is involved in the effector phase of contrasuppression

Our previous experiments using IFN- γ ^{-/-} mice and IFN- γ production by LN cells of EC immunized mice with TNP-Ig plus LPS suggest that IFN- γ could be involved in EC induced contrasuppression. To determine this, IFN- γ was neutralized with anti-IFN- γ mAb at the time of incubation of TNP-specific CS effector cells with Tcs cells before the addition of Ts cells or at the time of incubation of Ts cells with TNP-specific CS effector cells preincubated with Tcs cells. The data presented in Fig. 11A show that treatment with neutralizing anti-IFN- γ mAb at the time of incubation of T effector cells with Tcs cells or at the time of incubation of Ts cells with T effector cells preincubated with Tcs did not abolish contrasuppression (groups D and E vs group B and groups

D and E vs group C). Moreover, even adoptive transfer of in vitro treated T effector, Tcs, and Ts cells together with neutralizing mAb into recipients injected with anti-IFN- γ mAb did not affect contrasuppression (Fig. 11B, group D vs group B). Additionally, transfer of in vitro mock treated T effector, Tcs, and Ts cells into recipients treated with neutralizing IFN- γ mAb did not alter contrasuppression when compared with contrasuppression control (Fig. 11B, group E vs group C).

These data suggest that IFN- γ does not play a crucial role in the effector mechanisms of contrasuppression.

Both our in vitro and in vivo adoptive transfer experiments using Tcs cells induced in wild-type and IL-12^{-/-} mice, respectively, showed that IL-12 might be involved in contrasuppression induced via EC immunization with TNP-Ig and LPS. To confirm that this proinflammatory cytokine is indeed involved in the observed contrasuppression, the following experiments were conducted.

The data of the first experiment, presented in Fig. 12A, show that in vitro neutralization of IL-12 abolishes EC induced contrasuppression (group D vs group C).

Additionally, data of the second experiment presented in Fig. 12B show that treatment of TNP-specific CS effector cells with rIL-12 before exposure to Ts (group E vs group B) reverses

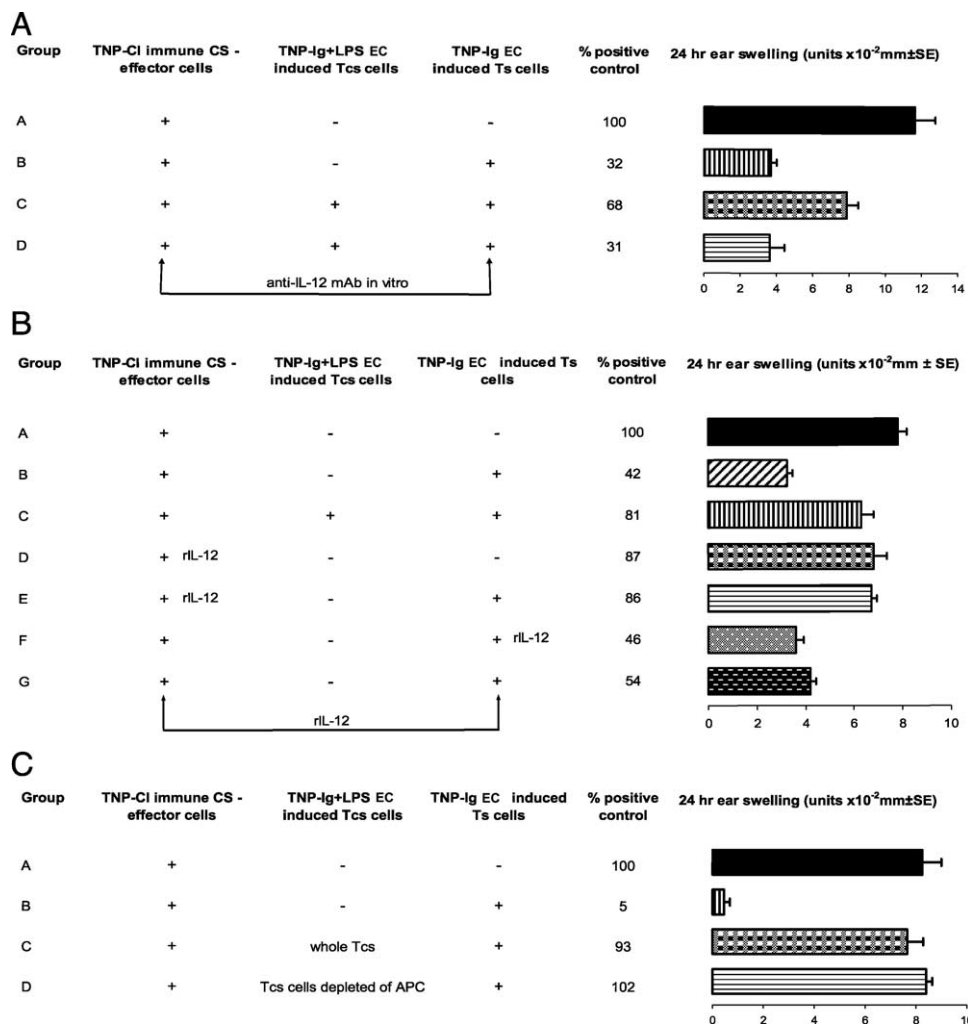


FIGURE 12. IL-12 protects CS effector cells from EC induced suppression. **A**, Neutralization of IL-12 in vitro abrogates EC induced suppression. CS effector cells (7×10^7) were incubated for 30 min at 37°C with 5×10^7 Tcs with (group D) or without anti-IL-12 neutralizing mAb ($10 \mu\text{g/ml}$) (group C). After that, cells were washed and incubated with 5×10^7 Ts cells with (group D) or without anti-IL-12 neutralizing mAb (group C) for another 30 min at 37°C . Positive control recipients were transferred with immune cells alone (group A), whereas suppression controls received immune cells cotransferred with EC induced Ts cells (group B). Then, the recipients were challenged and tested for CS. Statistical significance: $p < 0.001$ for group B vs A; $p < 0.001$ for group C vs B; $p < 0.05$ for group D vs C; and $p = \text{NS}$ for group D vs B. **B**, Exogenous IL-12 can replace Tcs cells. To determine whether exogenous IL-12 could replace Tcs cells, rIL-12 (50 ng/ml) was added to 7×10^7 CS effector cells (group E) or 5×10^7 Ts cells (group F) or coincubated CS effector cells with Ts cells (group G). Cells were incubated with rIL-12 for 30 min at 37°C . In a control group, CS effector cells were treated with rIL-12 alone (group D). In the contrasuppression control group, 7×10^7 CS effector cells were incubated for 30 min at 37°C with 5×10^7 Tcs and then the cell mixture was washed and incubated with 5×10^7 Ts cells for another 30 min at 37°C (group C) whereas in the suppression control group 7×10^7 CS effector cells were incubated with 5×10^7 Ts cells for 30 min at 37°C (group B). Positive control contained immune cells only (group A). Statistical significance: $p < 0.001$ for group B vs A; $p < 0.001$ for groups C and E vs B; and $p = \text{NS}$ for groups G and F vs B. **C**, EC induced Tcs cells depleted of APC are still able to abolish suppression. On day 4 TNP-Cl immune CS effector cells (7×10^7) were incubated for 30 min at 37°C with medium alone (positive control) (group A) or 5×10^7 EC induced Tcs (whole Tcs) before treatment with 5×10^7 Ts cells (group C). To determine involvement of APC in EC induced contrasuppression, 7×10^7 CS effector cells were incubated with an equivalent of 5×10^7 Tcs cells depleted of APC (for details see "Materials and Methods") prior to incubation with 5×10^7 Ts cells (group D). Suppression control contained immune cells incubated with Ts cells only (group B). Statistical significance: $p < 0.001$ for group B vs A; $p < 0.001$ for group C vs B; and $p = \text{NS}$ for group D vs C.

suppression. Similar observations were made when suppression was induced by EC immunization with OX-Ig (data not shown).

However, neither treatment of Ts cells before incubation with CS effector cells (group F) nor the addition of rIL-12 at the time of coincubation of CS effector cells with Ts cells (group G) reversed suppression. Treatment of CS effector cells with rIL-12 (group D) also did not affect their ability to transfer CS. Additionally, data presented in Fig. 12C show that EC induced Tcs cells depleted of APC can still express their protecting activity similarly to that of whole Tcs cells (groups D and C respectively).

These data show that IL-12 is indeed involved in EC induced contrasuppression and that this cytokine protects CS effector cells from Ts cells and does not act directly on Ts cells.

Discussion

We previously have shown that EC immunization with protein Ag induces an Ag -nonspecific suppression of CS responses to subsequent active sensitization (4, 5). This effect is dependent on TGF- β and transferable to naive animals by a non-Ag-specific $\text{CD4}^+ \text{CD8}^+ \text{TCR}\alpha\beta^+$ suppressor Ts cell population (6). We have

made a similar observation in animal models of multiple sclerosis and rheumatoid arthritis, where EC application of protein Ag reduced disease severity and disease incidence (7, 8, 20). In other work, using allogeneic skin grafts we showed that EC immunization with an uninvolved protein Ag delays graft rejection in an Ag-nonspecific manner (9).

The ease of induction and the potent non-Ag-specific effect of skin-induced Ts suggests that this may be a procedure that is applicable to the treatment of inflammatory responses occurring in many clinical cases, including autoimmunity. The fact that skin-induced tolerance did not show Ag specificity may suggest that such treatment could cause and maintain the status of Ag-nonspecific tolerance that can lead to a decreased ability to develop Ag-specific immune responses to pathogens. However, it is unlikely that this nonspecific suppression interferes significantly with immune response to microorganisms because, in contrast to the Ag commonly used in experimental models (keyhole limpet hemocyanin, TNP, SRBC), microbial products can activate amplification loops via TLR, which may help to overcome tolerance.

We have hypothesized that immunization with protein Ag through the skin, in the context of TLR ligands, can reverse the suppression of CS reactivity observed after EC immunization with protein Ag alone. Indeed, our previous work showed that EC immunization with protein Ag together with TLR2, TLR3, TLR4, or TLR9 ligands reverses skin induced suppression (10).

In this study we attempted to determine how TLR ligands reverse skin-induced suppression. For that purpose we have selected the TLR4 ligand LPS, because it is the most frequently found TLR ligand in the environment (21).

The elicitation of CS reactions by T effector cells is known to be under strict control by various regulatory cells. CS responses are negatively regulated by Ag specific, either $\text{TCR}\alpha\beta^+\text{CD8}^+$ (22, 23) or $\text{TCR}\gamma\delta^+$ Ts cells (24). Recently, it was found that CS responses also are negatively regulated by T regulatory $\text{CD4}^+\text{CD25}^+$ cells and that their mechanism of suppression differs from that of previously characterized Ts cells (25, 26).

In addition to such negative regulation, CS responses are positively regulated by contrasuppressor Tcs cells (27, 28). Contr suppression can be defined functionally as a regulatory activity that renders the targets of suppression (immune cells) resistant to suppressor cell signals and enables them to function in a suppressed environment (29). Two types of specialized Tcs cells that modulate CS, both of which are CD4^+ , have been described in mice. One is nonspecific and is present in both immune and nonimmune animals (30). The second Tcs cell is Ag specific, shields immune cells against Ag-specific CD8^+ Ts cells, and is triggered by Ag-Ab complexes (31, 32). Our present experiments add to this regulatory cell armamentarium a third type of Tcs. This cell protects immune cells in Ag-specific manner from the action of Ag-nonspecific $\text{CD4}^+\text{CD8}^+$ double positive Ts cells generated by EC application of protein Ag alone. This particular Tcs is generated by EC deposition of protein Ag in the presence of TLR ligands (10).

Our results can be summarized as follows. First, EC application of LPS together with TNP-Ig as Ag before skin sensitization with TNP-CI prevents mice from skin-induced suppression by TNP-Ig alone.

Second, the anti-suppressive effect of LPS is dose dependent and disappears after 4 wk.

Third, tolerogenic TNP-Ig and anti-tolerogenic TNP-Ig plus LPS, do not need to be applied on the skin at one time to reverse suppression. In both situations, when TNP-Ig is applied first and TNP-Ig plus LPS is applied 4 days later or the other way round, the animals do not show suppression.

Fourth, the generation of specialized cells blocking suppression was confirmed in transfer experiments. TNP-Ig plus LPS-induced lymphoid cells, when transferred to recipients, prevented suppression induced by EC administration of TNP-Ig in these animals. In the reverse situation, transferred TNP-Ig-induced cells did not interfere with induction of contrasuppression by TNP-Ig plus LPS in recipients.

Fifth, cells induced by EC applied TNP-Ig plus LPS and responsible for the observed prevention of suppression are $\text{TCR}\alpha\beta^+\text{CD4}^+$ lymphocytes, Ag specific, and can only prevent suppression to homologous Ag that was used for induction of suppression (i.e., TNP-Ig), leaving active other nonspecific suppressor cells (10).

Our previous work using TLR4 mutant mice (10) and our currently performed experiments on $\text{MyD88}^{-/-}$ mice show that the induction of Tcs via EC immunization with TNP-Ig plus LPS operates by a downstream pathway common to TLR ligands. MyD88 adaptor protein participates intracellularly in a chain of molecular interactions leading to activation of the transcription factor NF- κ B, which finally activates genes responsible for the secretion of proinflammatory cytokines (33).

The mechanism of nonspecific suppression was shown previously to be TGF- β dependent and IL-4 and IL-10 independent (6). The suppressive action of TGF- β seems to be temporary, only lasts as long as the cytokine is present, and can easily be reversed by proinflammatory cytokines produced by cells of the contrasuppressor circuit.

Our previous work on contrasuppression showed that Tcs cells induced by immune complexes mediate their biological activity via soluble molecules released into culture supernatant (19). To determine whether a similar situation occurs in the currently studied model of EC induced contrasuppression, CS effector cells, before exposure to Ts cells, were treated with culture supernatant produced by LN cells isolated from mice EC exposed to TNP-Ig and LPS. This experiment showed that the function of EC induced Tcs can be replaced by soluble molecules e.g., cytokines produced by LN cells. Additionally, we have found that the production of proinflammatory cytokines by LN cells isolated from mice EC immunized with TNP-Ig and LPS declines with time and correlates with the gradual decrease of CS *in vivo*. To determine whether cytokines are indeed involved in the reversal of skin induced tolerance, we measured the concentration of proinflammatory cytokines. We found increased production of IL-6, IL-12, and IFN- γ by LN cells isolated from animals patched with TNP-Ig and LPS. Experiments using knockout mice showed that IFN- γ and IL-12 are indeed involved in EC induced contrasuppression. In contrast to findings by other authors (34), our experiments did not seem to indicate a role of IL-6 in the effect of Tcs.

Two additional transfer experiments helped us to determine the role of IFN- γ and IL-12 in EC induced contrasuppression. We found that IFN- γ neutralization *in vitro* before adoptive transfer of CS does not abolish contrasuppression. These data, together with experiments using IFN- $\gamma^{-/-}$ mice, suggest that IFN- γ is required for the induction of Tcs cells whereas IFN- γ does not play a crucial role in the effector phase of contrasuppression. In contrast, CS effector cells treated with rIL-12 became resistant to negative signals of Ts cells and were able to successfully transfer CS. However, neither treatment of Ts cells with rIL-12 before incubation with CS effector cells nor the addition of this cytokine at the time of cocubation of CS effector cells with Ts cells restored the CS.

These data show that IL-12 is indeed involved in EC induced contrasuppression and that this cytokine protects CS effector cells from Ts cells and does not act directly on Ts cells. Additionally,

we showed that Tcs depleted of APC could still play their protective role. These data might suggest that EC induced Tcs cells could mediate their contrasuppressor activity via induction of either donor's or recipient's APC to produce IL-12.

This finding is in line with our previous work in a CS model where we found that IL-12 is involved in reversal of suppression (35–38).

Summing up, data presented in our manuscript suggest that IFN- γ is required for induction of Tcs cells, whereas IL-12 plays a role in the effector phase of EC induced contrasuppression.

If, however, the mechanism of reversal of suppression is so simple and depends on the presence of nonspecific proinflammatory cytokines produced by Tcs cells, the question can be posed as to why contrasuppression in this model is Ag specific, whereas that in excess of added IL-12 becomes nonspecific.

EC induced suppression is a generalized phenomenon that affects effector cells with different specificities. In contrast, contrasuppression induced by Ag plus LPS is a phenomenon that acts on effector T cells of a single specificity. This situation may suggest that contrasuppression requires direct interaction between Tcs and effector T cells. This is in line with our previous observation that contrasuppression demands direct cell-cell contact between Tcs and effector cells (39). If so, even minute amounts of soluble mediators, e.g., cytokines produced locally, may make T effector cells resistant to suppression.

Thus, we propose tentatively the following scenario of EC induced contrasuppression. In this contrasuppressor circuit at least three types of cells are involved: T effector cell as a target of regulation, Ag-specific Tcs cell, and IL-12 producing accessory cell e.g., dendritic cell. Tcs cells presumably activate accessory cell to secrete IL-12 that protects T effector cells from the action of Ts cells. Such a type of interaction between Tcs and accessory cells is not unexpected, because similar interactions were found in other regulatory circuits (40).

We have regarded it as unlikely that the ease of induction in an experimental situation of nonspecific Ts cells by EC deposition of Ag could lead under standard conditions to a decreased ability to produce a specific immune response to relevant Ags, because microbes and/or their products present on the skin could activate amplification loops by TLR ligation and thus prevent down-regulation of immunity. Our present experiments suggest that these two conflicting signals i.e., tolerogenic and contrasuppressive, can be separated without affecting the overall ability to mount the immune response. It is likely that skin areas particularly rich in bacterial flora, like auxiliary or inguinal regions, may be important in such a mechanism as a source of the important ligands. The presence of skin microbial flora can thus be regarded as a safety valve to prevent suppression against Ags deposited on the skin and thus safeguard the homeostasis of the immune system. The immunoregulatory role of skin bacteria is not unique. A similar phenomenon of microbial regulation was observed in mucosa-associated immune responses of T cells that function in the contrasuppressor pathway, are present in Payer's patches, and are responsible for allowing immunity to persist in gut mucosa in the face of systemic suppression (41, 42). In germ-free animals, participation of Th2 lymphocytes and IgE synthesis is dependent on colonization of the digestive tract of these animals by commensal flora. In summary, the presence of bacteria or their products on body surfaces (i.e., on skin or mucosa) exposed to potentially tolerogenic antigenic signals seems to have a decisive final effect by either tilting the balance toward positive humoral or cell-mediated immunity or, if the TLR signal is inadequate, may lead to establishing the state of tolerance.

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Disclosures

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