Role of TLR ligands in epicutaneously induced contrasuppression

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Abstract:

Our previous work showed that epicutaneous (EC) immunization in mice with protein antigen (Ag) induced an Ag-independent unresponsiveness mediated by suppressor CD4⁺8⁺ T cells (Ts), which inhibited contact hypersensitivity (CS). Simultaneous EC immunization with Ag and various Toll-like receptor (TLR) ligands reversed skin-induced suppression. Our present study shows that this process activates Ag-specific T contrasuppressor (Tcs) cells and leads to the protection of CS effector T cells from suppression. Epicutaneous immunization with Ag and the TLR4 ligand lipopolysaccharide (LPS) led to a significant increase in IFN- γ production by lymph node and spleen cells. Ag and TLR ligands, like LPS, CpG or lipoteichoic acid did not need to be applied concomitantly to the skin. An identical contrasuppressive effect was observed when the Ag and TLR ligands were deposited on distant skin areas, suggesting that both the generation of Ts and Tcs are independent. To corroborate this finding, we used a model system that uses macrophages (Mf) as Ag-presenting cells. Mf labeled *in vitro* with Ag (Mf-Ag) induced, upon intravenous (*iv*) administration, an unresponsiveness reaction that was mediated by Ts cells. When treated simultaneously with LPS-treated Mf (Mf-Ag-LPS), a TLR-ligand could induce CS. Both the Ag and the LPS signal could be uncoupled i.e., Mf-Ag and Mf-LPS given at separate time points (with an 1 h interval between injections) induced immunity. We also found that LPS-treated Mf also produced significant amounts of IL-12, a cytokine that has well-known anti-tolerogenic properties. Our experiments suggest that reversal of EC-induced suppression by TLR-ligands may be a potential tool to increase the immunogenicity of weakly immunogenic antigens.

Key words:

epicutaneous immunization, macrophages, Toll like receptor, reversal of suppression, contrasuppression

Abbreviations: Ag – antigen; APC – antigen presenting cells, CIA – collagen induced arthritis, CpG – oligodeoxynucleotide, CS – contact sensitivity, EAE – experimental autoimmune encephalomyelitis, EC – epicutaneous, FCS – fetal calf serum, IFN – interferon, Ig – immunoglobulins; IL – interleukin; *iv* – intravenous; LC – Langerhans cells; LNC – lymph nodes cells, LPS – lipopolysaccharide, LTA – lipoteichoic acid, MBP –

myelin basic protein, Mf – macrophages, OVA – ovalbumin, PAMP – pathogen associated molecular pattern, PBS – phosphate buffered saline, Spl – spleen, Tc1 – T cytotoxic cell type 1, Tcs – T contrasuppressor cell, Th1 – T helper cell type 1, TLR – Toll-like receptor, TNP – 2,4,6-trinitrophenyl, TNP-Cl – 2,4,6-trinitrophenyl chloride, Ts – T suppressor cells

Introduction

Both the skin and mucosa are constantly exposed to many antigens and play a crucial role in protecting the body from a variety of distinct pathogens. The development of an immune responses to innocuous antigens is, at best, not helpful and often leads to a harmful allergic reaction. It is well known that immunization with an antigen (Ag) *via* the digestive tract or nasal mucosa leads to both a local immune response and to a state of profound immunosuppression at the periphery [27]. This suppression seems to play an important role in the attenuation of immune responses towards non-pathogenic Ags.

While the skin is considered an organ where immune responses are easily induced [2], little attention has been given to skin-induced tolerance [19]. Because the skin and the mucosa have a similar function in our bodies (i.e., as a barrier to external pathogens), it is possible that epicutaneous (EC) application of Ag, in addition to inducing a strong immune response, may also induce peripheral tolerance.

Indeed, our previous work showed that similar to the mucosa, EC immunization of mice with different protein Ags applied to the skin (in the form of a patch or cream emulsion) induced a state of subsequent Ag-independent suppression, a process mediated by suppressor T cells (Ts) that inhibit contact hypersensitivity [18]. This 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 [22].

We found similar results in an animal model of multiple sclerosis (EAE) and collagen-induced arthritis (CIA) where EC immunization with myelin basic protein (MBP) or collagen reduced disease severity and disease incidence respectively [12, 23–25]. Furthermore, our work employing allogeneic skin grafts showed that EC immunization with a protein antigen delayed graft rejection [11].

Recently, we showed that EC immunization with protein antigen TNP-Ig and Toll-like receptor (TLR) ligands reversed skin-induced suppression [16]. This finding suggests that EC immunization with an antigen in combination with TLR ligand administration might play an important role in immunopotentiality. Such a maneuver may potentially be effective for new vaccines and anti-cancer therapy.

The aim of current work was to precisely determine the mechanism of EC induced reversal of skin-

induced suppression (immunopotentiality, contrasuppression).

Materials and Methods

Mice

Male CBA/J mice 6–8 week-old were obtained from the breeding unit of the Department of Human Developmental Biology, Jagiellonian University, College of Medicine. Mice were fed autoclaved food and water. All experiments were conducted according to guidelines of the Jagiellonian University College of Medicine.

Reagents

2,4,6-Trinitrophenyl chloride (TNP-Cl) was purchased from Chemica Alta (Edmonton, Canada). Ovalbumin (OVA, Grade V) and LPS (from *Escherichia coli* 026:B6) were obtained from Sigma Chemical Co. (St. Louis, MO). The CpG oligodeoxynucleotide TCCATGACGTTCTGACGTT was prepared by the HHMI/Keck Oligonucleotide Synthesis Facility at Yale University. Lipoteichoic acid (LTA) from *Staphylococcus pulvereri* was extracted as described [6, 9], then fractionated on a gel filtration column (Bio-Gel P100) with phosphate buffered saline (PBS) as the eluant. LTA was donated by Dr. A. Gamian from the Institute of Immunology and Experimental Therapy, Polish Academy of Science, Poland.

Mouse immunoglobulins (Ig) were prepared from CBA/J mouse sera and conjugated with TNP hapten [8, 13]. A single preparation of conjugate with a ratio of 40 TNP molecules per Ig molecule (TNP₄₀-Ig) was used throughout the study. To measure the levels of IL-12p40 and IFN- γ in culture supernatants, mouse IL-12p40 and mouse IFN- γ ELISA kits were used (BD Pharmingen, San Diego, CA).

Additionally, horseradish peroxidase conjugated streptavidin (Vector Laboratories, Burlingame, CA), and *o*-phenylenediamine, hydrogen peroxide (Sigma, St. Louis, MO) were used.

Epicutaneous immunization with TNP-Ig and TLR ligands

EC immunizations were performed by applying to the shaved skin of the mouse dorsum a 1-cm² gauze patch

soaked with a solution containing 100 µg TNP-Ig and 100 ng of LPS in a volume of 100 µl PBS on day 0. The patch was secured by adhesive tape, which was wrapped around 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 0–4, where it was replaced by a fresh patch. In one experiment mice were EC immunized with 100 µg TNP-Ig mixed with 100 µg of CpG or 100 µg of LTA in a volume of 100 µl PBS. On day 7, mice were actively sensitized with TNP-Cl.

Active sensitization and measurement of contact sensitivity (CS) *in vivo*

Mice were actively sensitized by a topical application of 0.15 ml of 5% TNP-Cl in an acetone-ethanol mixture (1:3) to both 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 with 10 µl of 0.4% TNP-Cl in an olive oil-acetone mixture (1:1) applied to both sides of the ears. The positive control group consisted of animals that were only skin-sensitized. The subsequent increase in ear thickness was measured 24 h later with an engineer's micrometer (Mitutoyo, Tokyo, Japan) and expressed in units of 10^{-2} mm $\pm$ SE [5]. Background increase in ear thickness (~ 2 units at 24 h), of non-sensitized, litter-mate animals that were similarly challenged was subtracted from each experimental group to yield the net ear swelling expressed in units of 10^{-2} mm $\pm$ SD. 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. Immune axillary and inguinal lymph nodes (LNC) 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 intravenously 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 lymphoid cells that were harvest on day “+7” from mice

that were tolerized by epicutaneous immunization with TNP-Ig [10]. After incubation, the cell mixture was intravenously transferred into naive recipients (suppression control). To test if EC immunization with both TNP-Ig and LPS induced regulatory cells that could inhibit the 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 lymphoid cells that were harvested on day “+7” from mice that were EC immunized with TNP-Ig plus LPS. The resultant cells were washed and incubated for 30 min at 37°C with 5×10^7 lymphoid cells harvest on day “+7” from mice tolerized by EC immunization with TNP-Ig. After the final incubation, the cell mixture was transferred intravenously into naive recipients (contrasuppression control). Mice were challenged with antigen within 30 min after cell transfer and tested for CS at 24 h as described above.

Peritoneal macrophages and induction of immunity

CBA/J mice were injected intraperitoneally with 2 ml of Markol 52 oil. Four days later, Mf were washed out with 5 ml of phosphate buffered saline (PBS) containing 5 U heparin per ml. Macrophages were washed three times and subsequently labeled with 2,4,6-trinitrobenzene sulfonic acid (10 mM TNBSA/ 10^7 cells) as previously described [21] or exposed to 100 ng of LPS/ 5×10^5 Mf for 30 min at 37°C. In some experiments Mf were labeled with TNP and subsequently incubated with LPS. After each step, Mf were washed three times and then injected intravenously into the retro-orbital plexus in naïve CBA/J mice. In separate groups, each mouse received 6×10^6 TNP-Mf or TNP-Mf treated with LPS. In two other groups, mice received both 6×10^6 TNP-Mf and 6×10^6 LPS-Mf or alternatively, 6×10^6 non-treated Mf. The mice were tested for the contact sensitivity reaction seven days later as described above.

IFN- γ production by lymph node and spleen cells

In total, 3×10^6 lymph node or spleen cells from mice that were 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 µg/ml TNP₄₀-Ig as the antigen. Cells were seeded in triplicate wells in flat 24 well Falcon plates, and, 48 h later, the culture supernatants

Fig. 1. EC immunization with TNP-Ig and the TLR4 ligand LPS reverses skin induced tolerance. 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 alone (Group A) or LPS alone (Group D). On day “+7,” the patches were removed and the mice were subsequently immunized with 5% TNP-CI and tested for CS. Results were expressed in units of 10^{-2} mm $\pm$ SE. Each experimental group consisted of 5 or 6 mice. Statistical significance: Group B vs. A $p < 0.001$ and Group C vs. B $p < 0.001$

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

IL-12 p40 production by peritoneal macrophages

In total, 5×10^5 Mf incubated in the presence or absence of LPS (see above) were cultured at 37°C in 1 ml RPMI-1640 medium supplemented with 5% FCS in triplicate wells of a flat 24-well Falcon plate. After 48 h, the culture supernatants were collected. The concentration of IL-12 was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using Corning Easy Wash™ plates (Corning, Corning N.Y.) as described previously [16]. Recombinant murine cytokines were used as standards.

Statistics

Statistics were performed using the paired two-tailed Student *t*-test, and $p < 0.05$ was taken as the level of significance.

Results

Skin induced suppression is reversed by the TLR4 ligand LPS

Our previous work showed that EC immunization with protein antigen (i.e. TNP-Ig) induced antigen independent suppression of CS sensitivity [18, 22].

The aim of this experiment was to determine if EC immunization with protein antigen in the presence of PAMP could reverse skin-induced suppression. Indeed, we show that EC immunization with the protein antigen TNP-Ig plus the TLR4 ligand LPS reversed skin induced suppression (Fig. 1, Group C vs. Group B). Epicutaneous exposure to LPS before TNP-CI sensitization did not affect CS (Group D vs. Group A).

Additionally, by employing a “transfer-out” protocol, we found that EC immunization with the protein antigen TNP-Ig plus LPS induced cells that protect TNP-CI immune CS-effector cells (Fig. 2, “Group C”) from EC induced T suppressor (Ts) cells (Fig. 2, “Group B”). We call the cell population that expresses protective activity “T contrasuppressor (Tcs)” cells.

Tcs cells induced *via* EC immunization are antigen specific

Our previous work on skin induced suppression showed that this phenomenon was antigen-independent [18, 22]. In our current work, we tested the antigen specificity of EC induced Tcs cells. Antigen specificity was tested both in actively EC immunized mice and in adoptive cell transfer experiments.

Data presented in Figure 3A show that previously induced suppression with TNP-Ig could be reversed by subsequent EC immunization with the corresponding TNP-Ig antigen and LPS, but not by immunization with a non-cross-reacting OVA antigen in combination with LPS. (Group D vs. Group B and Group E vs. Group B, respectively).

Fig. 2. EC immunization with antigen and LPS induces T contrasuppressor (Tcs) cells that protect from suppression. To determine if EC immunization with TNP-Ig and LPS induces regulatory cells (Tcs) that could reverse skin induced suppression, 7×10^7 of the CS-effector cells from TNP-Cl sensitized donors were incubated for 30 min at 37°C with 5×10^7 lymphoid cells collected on day “+7” from mice that were EC immunized with TNP-Ig plus LPS. The resultant cells were then washed and incubated for 30 min at 37°C with 5×10^7 lymphoid cells (Ts) harvested on day “+7” from mice tolerized by EC immunization with TNP-Ig. After the final incubation, the cell mixtures were transferred intravenously into naïve recipients (contrasuppression control) (Group D). In the positive control group, recipients received only immune cells (Group A), whereas in the suppression control, immune cells were transferred together with EC induced Ts cells (Group B). All recipient mice were subsequently challenged with 0.4% TNP-Cl and tested for CS. Statistical significance: Group B vs. A $p < 0.01$; Group D vs. B $p < 0.001$

Fig. 3. EC-induced contrasuppression is antigen specific. **(A)** Test for Ag-specificity of contrasuppression in a model of active immunization. CBA/J mice were EC immunized with TNP-Ig first (Groups D and E) and then with TNP-Ig + LPS (Group D) or OVA + LPS (Group E). Positive control animals were patched with PBS alone (Group A) whereas in suppression control groups, mice were EC immunized with TNP-Ig (Group B) or OVA (Group C) alone. All mice were subsequently sensitized with TNP-Cl and tested for CS. Statistical significance: Group B vs. A $p < 0.001$; Group C vs. A $p < 0.01$; Group D vs. B $p < 0.001$ and Group E vs. D $p < 0.001$. **(B)** Test for Ag-specificity of contrasuppression – transfer out protocol. To confirm the results presented in Figure 3A, we used a transfer-out protocol. TNP-Cl immune CS-effector cells (7×10^7) harvested at four days were incubated for 30 min at 37°C with medium alone (positive control) (Group A) or with 5×10^7 contrasuppressor cells induced via EC immunization with either TNP-Ig + LPS (Group C) or OVA + LPS (Group D). The cell mixtures (Groups C and D) were then incubated for 30 min at 37°C with 5×10^7 suppressor cells induced via TNP-Ig patching and transferred intravenously into naïve recipients that were challenged with TNP-Cl; the CS reactions was measured 24 h later. In the suppressor control group, immune cells were co-transferred together with EC induced Ts cells (Group B). Statistical significance: Group B vs. A $p < 0.001$; Group C vs. B $p < 0.001$ and Group D vs. C $p < 0.001$

Fig. 4. PAMPs applied to the skin together with or separately from protein antigen reverse skin-induced suppression. CBA/J mice were EC treated on their back (b) with PBS (Fig. 4A–C; Group A), TNP-Ig alone (Fig. 4A–C; Group B), TNP-Ig with LPS (Fig. 4A; Group C), LTA (Fig. 4B; Group C) or CpG (Fig. 4C; Group C). In Group D, animals were patched with TNP-Ig on the back (b) and LPS, LTA or CpG on the chest (ch) (Fig. 4A–C, respectively). Seven days later, the animals were sensitized with TNP-CI and tested for CS. Results were expressed in units of 10^{-2} mm $\pm$ SE. Each experimental group consisted of 5 or 6 mice. Statistical significance for Fig. 4A: Group B vs. A $p < 0.001$; Groups C vs. B $p < 0.01$ and Group D vs. B $p < 0.001$. Statistical significance for Fig. 4B: Group B vs. A $p < 0.001$; Groups C vs. B $p < 0.01$ and Group D vs. B $p < 0.001$. Statistical significance for Fig. 4C: Group B vs. A $p < 0.001$; Groups C and D vs. B $p < 0.01$. **(A)** LPS applied to the skin together with or separately from the protein antigen reverses skin-induced suppression. **(B)** LTA applied to the skin together with or separately from protein antigen reverses skin-induced suppression. **(C)** CpG applied to the skin together with or separately from protein antigen reverses skin-induced suppression.

Employing a “transfer-out” model, we confirmed the antigen specificity of Tcs. We found that Tcs cells induced by TNP-Ig and LPS could protect TNP-specific CS effector cells from EC induced Ts cells (Fig. 3B, Group C vs. Group B). However, when Tcs cells were induced by patching the mice with OVA and LPS, the cells could not protect TNP-specific CS effector cells from EC induced Ts cells (Fig. 3B, Group D vs. Group A).

PAMPs applied to the skin in combination with or separately from protein antigen reverse skin induced suppression

The previous experiments showed that TNP-Ig applied to the skin together with TLR ligands (i.e., LPS) reversed skin induced suppression. We next examined whether EC exposure could still reverse suppression when TNP-Ig and PAMPs were applied to separate areas on the skin. Mice were patched with TNP-Ig and TLR ligand (LPS, LTA or CpG) applied either to the same place on the back or to separate areas, with TNP-Ig applied to the back and PAMPs applied to the chest before sensitization with TNP-Cl.

Data presented in Figure 4 show that both EC immunization with TNP-Ig and PAMPs applied to the same area (Back: Fig. 4A-4C, Group C vs. Group B) or to separate areas (Back and Chest: Fig. 4A-4C, Group D vs. Group B) reversed suppression. PAMPs applied to the skin in the absence of antigen before

Tab. 1. EC immunization with TNP-Ig and LPS results in increased IFN- γ production by lymph node and spleen cells

Group	EC treated with	IFN- γ [pg/ml]	
		LNC	Spl
A	PBS	1050 $\pm$ 32	0 $\pm$ 0
B	TNP-Ig	200 $\pm$ 22	0 $\pm$ 0
C	TNP-Ig + LPS	3500 $\pm$ 305	47117 $\pm$ 1885
D	LPS	1390 $\pm$ 60	0 $\pm$ 0

In total, 3×10^6 of lymph node or spleen cells from mice EC treated with PBS (Group A), TNP-Ig (Group B), TNP-Ig + LPS (Group C) or LPS alone (Group D) were cultured in 1 ml RPMI 1640 medium supplemented with 5% FCS in the presence of 100 μ g/ml TNP₄₀-Ig as an antigen and seeded in triplicate wells in flat 24 well plates. Forty-eight hours later, the culture supernatants were collected and tested for IFN- γ concentration by ELISA. Statistical significance: for LN: Group B vs. A $p < 0.001$; Group C vs. B $p < 0.001$ and Group C vs. A $p < 0.01$. For Spl.: Group C vs. B $p < 0.001$ and Group C vs. A $p < 0.001$

sensitization with TNP-Cl did not interfere with the CS response (Fig. 4A-4C, Group E vs. Group A).

EC immunization with TNP-Ig and LPS results in production of IFN- γ by lymph node and spleen cells

Our previously published data [16] showed that EC immunization with TNP-Ig plus LPS caused an increase in the production of IL-6 and IL-12 by periph-

Fig. 5. Induction of contact sensitivity by TNP-Mf. CBA/J mice were injected intraperitoneally with 2 ml of Markol 52 oil. Four days later, the Mf were washed out with 5 ml of PBS containing 5 U heparin/ml. Macrophages were washed three times and then labeled with 2,4,6-trinitrobenzene-sulfonic acid (10 mM TNBSA/ 10^7 cells) or exposed to 100 ng of LPS/ 5×10^5 Mf for 30 min at 37°C. In some groups, Mf were labeled with TNP and incubated with LPS. After each step, Mf were washed three times before use. Each mouse received 6×10^6 TNP-Mf (Group A) or TNP-Mf treated with LPS (Group B). In two other groups, the mice received both 6×10^6 TNP-Mf and 6×10^6 LPS-Mf (Group C) or, alternatively, 6×10^6 non-treated Mf (Group D). Seven days later, the mice were tested for the contact sensitivity reaction. Statistical significance: Groups B and C vs. A $p < 0.001$; Group D vs. Groups B and C $p < 0.001$

eral lymph organs. In the current work, we measured production of IFN- γ by LNC and spleen cells isolated from mice EC immunized with TNP-Ig and LPS. Data presented in Table 1 show that TNP-Ig applied to the skin in the presence of LPS resulted in a significant production of IFN- γ by LNC and spleen cells when compared to LNC and spleen cells isolated from mice treated with TNP-Ig plus PBS or with LPS alone.

Induction of contact sensitivity by TNP-Mf

Data presented in Figure 4A–C show that PAMPs, when applied to the skin together with or separate from protein antigen, reversed skin induced suppression. We hypothesized that, in the case of EC exposure to antigen and PAMP on separate body locations (e.g., chest and back), antigen presenting cells would be stimulated by antigen or PAMP, migrate to draining lymph organs, and subsequently induce Tcs cells that protect from EC induced suppression.

To verify our hypothesis, we treated mice with TNP-Mf alone, TNP-Mf and LPS-Mf, TNP-Mf treated with LPS or TNP-Mf, and naive Mf.

As shown in Figure 5, intravenous administration of 6×10^6 TNP-Mf induced no immunity. However, when the cells were treated with LPS, they became highly immunogenic when administered into mice (compare groups A and B). When TNP-Mf and LPS-Mf (6×10^6 each) were given in conjunction to the same recipient, a strong response was produced while injection of TNP-Mf together with naive Mf produced no immunological reaction (compare groups C and D).

Additionally, data presented in Table 2 showed that macrophages treated with LPS *in vitro* produced higher levels of IL-12p40 compared to naive macrophages (Group B vs. A).

Tab. 2. Enhanced production of IL-12p40 by LPS stimulated macrophages

Group	Mf treated with	IL-12 p40 [pg/ml]
A	Non treated	1762 $\pm$ 27
B	LPS	3727 $\pm$ 267

In total, 5×10^5 Mf were incubated in 1 ml of medium with (Group B) or without 100 ng of LPS (Group A) and cultured at 37°C in triplicate in flat 24-well plates. After 48 h, culture supernatants were collected and analyzed for the levels of IL-12 by ELISA. Statistical significance: Group B vs. A $p < 0.01$

Discussion

We have previously shown that EC immunization of mice with protein Ag applied on the skin in the form of a patch induced a state of subsequent Ag-independent unresponsiveness as a result of activation of suppressor T cells (Ts) that inhibit sensitization and elicitation of effector T cell responses [18]. This suppression was transferable *in vivo* by TCR $\alpha\beta^+$ CD4 $^+$ CD8 $^+$ double positive lymphocytes harvested from lymphoid organs of skin-patched animals. Both CD25 $^+$ and CD25 $^-$ CD4 $^+$ CD8 $^+$ T cells were capable of suppressing the adoptive transfer of Th1 (T helper cell type 1) effector cells that mediate contact hypersensitivity. Furthermore, *in vitro* experiments showed that TGF- β played a crucial role in EC induced suppression [22]. Similar observations we made in CD8 $^+$ Tc1 mediated (T cytotoxic cell type 1) CS [24].

We also showed that application of MBP to the skin prior to the induction of EAE by immunization with MBP protected the mice from developing disease. The therapeutic effect was transferable to naive recipients containing lymph node cells from MBP-treated mice. These Ts cells were found to be non-specific with regards to antigen [12, 23]. Experiments employing knockout mice and adoptive transfer of negatively selected Ts cells showed that the EC induced suppressor cells that could protect mice from the disease belonged to the TCR $\alpha\beta^+$ CD4 $^+$ CD8 $^+$ double positive population of lymphocytes [25].

Additionally, employing an animal model of arthritis, we found that EC immunization with collagen protected from disease induction and ameliorated disease severity [24].

By employing an experimental model of graft rejection, we also found that EC pretreatment of female C57BL/6 mice with OVA prolonged the survival of the Y-H-miss matched male skin grafts; the observed suppression of transplantation reactivity was found to decline with time [11].

The ease of induction and the potent non-Ag specific effect of skin induced Ts cells suggest that this procedure may be applicable to the treatment of autoimmune diseases.

It is important to note that the antigen independent nature of the skin induced suppression raises the possibility that EC exposure to antigens could prevent the development of immune responses to novel antigens such as pathogen-associated antigens, which would be

an unacceptable scenario. However, in contrast to the experimental models discussed here, pathogen antigens are usually presented with pathogen-associated molecules that are recognized by innate immune receptors, i.e., Toll-like receptors (TLR), which may help to overcome tolerance. We therefore hypothesized that immunization with a protein antigen in the presence of TLR ligands through the skin could reverse the EC induced suppression of T-cell mediated immunity.

Our recent paper showed that indeed EC immunization with protein antigen induced antigen-independent suppression that could be reversed by crude bacterial material as well as purified TLR-2, TLR-3, TLR-4, and TLR-9 ligands [16]. 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 [16].

In the current work employing a CS model, we show that reversal of skin induced suppression by EC immunization with LPS and TNP-Ig was transferable using lymph node cells isolated from inguinal and auxiliary lymph nodes (Fig. 2). Additionally, the adoptive cell transfer experiments showed that spleen cells isolated from mice that were EC immunized with TNP-Ig plus LPS could protect CS-effector cells from the action of EC induced Ts cells (data not shown).

Our previous work on skin induced suppression showed that this phenomenon was antigen independent [12, 24]. In the current paper, we attempted to determine the antigen specificity of EC induced contrasuppression. Employing two non-cross reacting antigens, TNP-Ig and OVA, we showed in an active model of CS that reversal of skin-induced suppression was antigen specific (Fig. 3A). These data were confirmed in an adoptive transfer model of CS as only the lymph node cells isolated from mice that were EC immunized with TNP-Ig and LPS could protect TNP-specific CS-effector cells. Such protection was not found when donors of contrasuppressor cells were EC exposed to OVA and LPS (Fig. 3B).

Of particular interest was the finding that to induce contrasuppressor cells, the tolerogenic (TNP-Ig) and anti-tolerogenic (TLR ligands) signals could be delivered on separate skin regions. This effect was observed with all the ligands tested: lipoteichoic acid (TLR2), LPS (TLR4) or CpG (TLR9) thus confirming our previous assumption that all these ligands operate by common downstream pathways.

In our previous paper, we reasoned that skin LCs, which are immature under conventional conditions, preferentially induce Ts cells when challenged with protein antigen alone following migration to local lymph nodes [16]. In the simultaneous presence of TLR ligands, LCs undergo maturation, release increased amounts of cytokines and become competent to activate both Tcs cells and Ts cells [16, 22]. The present experiments showing the efficacy of separate application of reagents suggest that both the generation of Ts and the generation of Tcs cells are independent events that can occur at distant sites. To confirm this hypothesis, we used a well-controlled model system that utilizes peritoneal Mf as Ag-presenting cells [4]. TNP-substituted Mf (TNP-Mf) administered intravenously into mice, induced a state of unresponsiveness, a process that was mediated by Ts cells [4]. When challenged with a TLR ligand or stimulated by other means (e.g., by immune complexes), Mf acquire the ability to induce immunity due to the simultaneous generation of Th1 effector cells and Tcs cells [4]. Macrophages exposed to LPS undergo functional polarization from the M2 phenotype, which is characterized by IL-10 and TGF- β production, to an M1 phenotype, which is defined by the production of IL-12, IL-6, TNF- α , and numerous chemokines and costimulatory molecules (CD80, CD86) that are responsible for the interaction with lymphocytes. M2 cells are most likely responsible for the induction of Ts cells while M1 cells appear to be instrumental in triggering Tcs lymphocytes [3]. When mice are treated with consecutive injections of TNP-Mf and LPS-treated Mf, the mice produce a significant immune response. This system approximates the physiological situation whereby the protein antigen and the TLR ligand are applied separately on the skin. We propose that the common denominator in these two systems is the production of cytokines by Mf, or by LC plus keratinocytes stimulated by LPS, which leads to the formation of a cytokine environment that shifts the balance towards a positive response [15].

Our previously published data showed that EC immunization with TNP-Ig plus LPS caused increased production of IL-6 and IL-12 by peripheral lymphoid cells [16]. These pro-inflammatory cytokines have been shown to play a crucial role in the regulation of the immune response [1]. In the current work, we measured the production of IFN- γ by LNC and spleen cells isolated from mice that were EC immunized with TNP-Ig and LPS. Data presented in Table 1 show

that TNP-Ig applied to the skin in the presence of LPS resulted in a significant production of IFN- γ by LNC and spleen cells when compared to the production of these cytokines by LNC and spleen cells from mice treated with TNP-Ig or with PBS or LPS alone. In particular, the high production of IFN- γ by splenocytes may be the result of an accumulation of regulatory cells in the spleen.

On the basis of these data, we postulate that the increase in the production of IFN- γ by LNC and splenocytes following EC immunization with TNP-Ig and LPS may play an important role in contrasuppression by activating macrophages to release IL-12. This, in turn, favors a positive regulatory feedback loop that leads to the activation of Th1 lymphocytes. As we previously found with a number of experimental models, IL-12 could reverse the suppression of cell-mediated immune response [7, 14, 17, 20, 26].

If EC immunization with antigen in the presence of TLR ligands is capable of actively reversing suppression and is not limited to a particular animal species in the laboratory, then this type of immunization may represent a means of enhancing the immune response in situations when it is required. Thus, this finding suggests that EC immunization with an antigen together with TLR ligands might play an important role in immunopotential and that such a strategy may be effective for enhancing the immune system in response to new vaccines or for the use in anti-cancer therapy.

Acknowledgments:

This work was supported by grant No. N401 3553 33 from the Ministry of Science and Higher Education to MS and a grant No. N 401 000936 from Ministry of Science and Higher Education to MM and grant No. K/ZBW/000172 from Jagiellonian University College of Medicine to MM.

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Received:

November 4, 2008; in revised form: May 12, 2009.