



Epicutaneous immunization with protein antigen induces antigen-non-specific suppression of CD8 T cell mediated contact sensitivity

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Abstract:

Background: Allergic contact dermatitis (ACD) resulting from exposure to low molecular weight chemicals is a common clinical condition in industrialized countries and can be mediated by either Th1 or Tc1 lymphocytes. The animal model of contact sensitivity (CS) is commonly used to study ACD in mice and helps to test new therapeutics.

We have previously shown that epicutaneous (EC) immunization with TNP-Ig prior to hapten sensitization inhibits Th1-mediated CS and observed that the suppression is mediated by TCR $\alpha\beta^+$ CD4 $^+$ CD8 $^+$ cells and is TGF- β dependent. More recently we have shown that EC immunization with DNP-BSA induces TCR $\alpha\beta^+$ CD4 $^+$ CD25 $^+$ FoxP3 $^+$ T regulatory (Treg) cells that suppress Tc1-mediated CS.

Methods: Animal model of contact sensitivity was used to study skin-induced suppression.

Results: Current work employing Tc1-mediated CS shows that skin-induced suppression is dose-dependent and declines with time. Experiments with the four non-cross-reacting antigens 2,4-dinitrophenylated bovine serum albumin (DNP-BSA), ovalbumin (OVA), myelin basic protein (MBP) and immunoglobulins conjugated with oxazolone (OX-Ig) employing models of active suppression, “transfer in” and “transfer out” protocols showed that EC immunization with any tested protein antigen inhibits CS response suggesting lack of antigen-specificity of the investigated phenomenon.

Conclusion: The ease of EC generation of antigen-non-specific regulatory cells may have important implications for designing therapeutic schemes aimed at modulating immune responses.

Key words:

contact sensitivity, suppression, skin, protein antigen, CD8 T cell mediated

Abbreviations: ACD – allergic contact dermatitis, Ag – antigen, ALNC – inguinal and axillary lymph node cells, CS – contact sensitivity, CTLA-4 – cytotoxic T-lymphocyte-associated antigen-4, DNFB – dinitrofluorobenzene, DNP-BSA – 2,4-dinitrophenylated bovine serum albumin, EAE – experimental autoimmune encephalomyelitis, EC – epicutaneous, FoxP3 – forkhead box P3, IFN- γ – interferon gamma, Ig – immunoglobulins, IL – interleukin, MBP – myelin basic protein, MHC – major histocompatibility complex, MLNC – mesen-

teric lymph node cells, OVA – ovalbumin, OX – oxazolone (4-ethoxymethylene-2-phenyloxazolone), OX-Ig – immunoglobulins conjugated with oxazolone, PBS – phosphate-buffered saline, SPF – specified pathogen free, SPLC – splenocytes, Tc 1 – T cytotoxic lymphocytes type 1, TCR – T cell receptor, Teff – effector T cells, TGF- β – transforming growth factor beta, Th – T helper lymphocytes, Thy – thymocytes, TNF – tumor necrosis factor, Treg – T regulatory cells, Ts – T suppressor cells

Introduction

Contact sensitivity is a classical form of *in vivo* T cell-mediated immunity induced by topical skin immunization with reactive chemical haptens. Contact sensitivity (CS) responses can be mediated by either CD4⁺ Th1, MHC class II-restricted cells locally releasing IFN- γ to recruit a characteristic inflammatory infiltrate [9, 24], or by CD8⁺ MHC class I-restricted Tc1 cells that can similarly release IFN- γ , but predominately mediate cytotoxic damage to local skin cells such as keratinocytes [1, 14]. It has been recently shown that also IL-17 producing Th17 cells can be involved in CS [28].

The typical CS response in humans is allergic contact dermatitis (ACD). Allergic contact dermatitis resulting from exposure to chemicals in the workplace, constitutes about 30% of all occupational diseases [7, 13] and can be mediated by either Th1 or Tc1 lymphocytes [5]. This type of reaction may occur in patients as a result of long-term exposure of the skin to low molecular weight substances, including heavy metals (e.g., nickel, chromium, cobalt), latex, turpentine, fragrances and preservatives in cosmetics, epoxy resins and their hardeners, as well as some drugs applied in ointments (e.g., neomycin).

Localized acute allergic contact dermatitis lesions are successfully treated with mid- or high-potency topical steroids. If ACD involves an extensive area of skin, systemic steroid therapy is often required [26]. Although this therapy offers relief within a short time it might also be associated with serious side effects. Therefore, it is important to develop new non-invasive and free of side effects therapeutic strategies alleviating the undesired inflammatory reaction accompanying ACD.

Employing a model of Th1-mediated CS response we showed previously that EC immunization of normal mice with different protein Ag applied on the skin in the form of a patch or cosmetic cream induced a state of subsequent unresponsiveness due to suppressor T cells (Ts) [17, 22]. This type of suppression was transferable by TCR $\alpha\beta$ ⁺ CD4⁺ CD8⁺ double positive lymphocytes harvested from lymphoid organs of EC immunized animals and was TGF- β dependent [22]. We made a similar observation in an animal model of multiple sclerosis where EC immunization with myelin basic protein reduced disease severity and decreased disease incidence [11, 23, 25]. Finally, our work showed that EC immunization with protein antigen delays skin graft rejection [10].

More recently we showed that EC immunization of normal mice with 2,4-dinitrophenylated bovine serum albumin (DNP-BSA) applied on the skin in the form of a patch induces a state of subsequent unresponsiveness due to regulatory T cells (Treg) that inhibited sensitization and elicitation of Tc1-mediated CS responses. Observed suppression is transferable *in vivo* by TCR $\alpha\beta$ ⁺ CD4⁺ CD25⁺ lymphocytes harvested from lymph nodes of skin patched animals [12]. Flow cytometry revealed that EC immunization with DNP-BSA increased TCR $\alpha\beta$ ⁺ CD4⁺ CD25⁺ FoxP3⁺ lymphocytes in subcutaneous lymph nodes suggesting that observed suppression was mediated by Treg cells. Further *in vitro* experiments showed that EC immunization with DNP-BSA prior to 1-fluoro-2,4-dinitrobenzene (DNFB) sensitization suppressed lymph node cell proliferation and inhibited production of TNF- α , IL-12 and IFN- γ . Using trans-well system that prevents from direct cell to cell contact or anti-CTLA-4 mAb we found that EC induced suppression required direct Treg-Teff cell contact and is CTLA-4 dependent [12].

In the current paper we show that skin-induced suppression of Tc1-mediated CS is dose-dependent, antigen-non-specific and declines with time.

Material and Methods

Mice

SPF female BALB/c (H-2^d) mice from the breeding unit of the Department of Medical Biology, Jagiellonian University, College of Medicine were used.

Mice were rested for at least 1 week before use, maintained under specific pathogen-free conditions, and used at 6–10 weeks of age in groups of 5–6. All experiments were conducted according to guidelines of Jagiellonian University College of Medicine.

Reagents

DNFB (1-fluoro-2,4-dinitrobenzene), OX (4-ethoxymethylene-2-phenyloxazolone), OVA (Grade V) and guinea pig MBP were obtained from Sigma (St. Louis, MO, USA). DNP-BSA was from Biosearch Technologies, Inc. (Novato, CA, USA). Mouse immunoglobulins were prepared from BALB/c mouse sera and conjugated with OX hapten [2]. A single preparation with the level of substitution 20 OX per immunoglobulin molecule (OX₂₀-Ig) was used throughout.

Induction and elicitation of CS

Mice were sensitized with 25 μ l of 0.5% DNFB in acetone : olive oil (4:1) applied by painting on the shaved skin. Five days later, CS responses were elicited by painting both ears with 5 μ l of 0.1% DNFB in acetone and olive oil (4:1) [3]. Resulting ear thickness was measured prior to testing, with a micrometer (Mitutoyo, Tokyo, Japan), by an observer, who was unaware of the experimental groups, and then, 24 h after challenge, was measured again. Background increase in ear thickness (± 20 μ m at 24 h) of littermate sham sensitized animals that were similarly challenged was subtracted from each experimental group to yield the net ear swelling expressed in μ m $\pm$ SE.

Epicutaneous immunization with protein antigen to induce tolerance

Animals were shaved on their backs with a razor blade before immunization. On day “0” 100 μ l of either DNP-BSA (graded dilutions of antigen starting at 1 mg/ml) in phosphate-buffered saline (PBS) or PBS alone was applied to gauze in the centre of an occlusive patch (DuoDerm Extra Thin, ConvaTec, Princeton, NJ, USA) which was then affixed to their backs [12, 22]. Patches were left intact for 4 days and then replaced with new antigen containing patches and kept on until day “7”. On day “7”, the patches were removed and mice were sensitized with DNFB and tested for CS or lymphoid cells were harvested and used as a source of regulatory cells. To determine antigen specificity of skin induced suppression, animals were EC immunized with DNP-non-cross-reacting antigens such as OVA, MBP or OX-Ig.

“Transfer in protocol”

Four 10^7 of inguinal and axillary lymph node cells (ALNC), splenocytes (SPLC), mesenteric lymph node cells (MLNC) or thymocytes (Thy) from mice EC tolerized with different protein antigens were transferred intravenously (*iv*) into naive recipients that were subsequently sensitized with 0.5% DNFB. Animals in positive control were sensitized with DNFB without prior injection of regulatory cells. Five days after sensitization mice were challenged and tested for CS 24 h later.

Adoptive cell transfer of CS and cell mixing assay to evaluate suppression – “transfer out protocol”

Donors of CS-immune effector cells were painted with 0.5% DNFB. Axillary and inguinal lymph nodes and spleens were harvested 5 days after DNFB sensitization and 7×10^7 immune cells were incubated for 30 min at 37°C in RPMI 1640 medium alone, washed and then injected *iv* into normal syngeneic recipients (positive transfer). For the cell mixing assay, 7×10^7 of the CS-effector immune cells from DNFB sensitized donors were incubated for 30 min at 37°C with 4×10^7 ALNC from mice tolerized by EC immunization with different protein antigens and harvested on day 7 [22]. After incubation, the cell mixture was transferred *iv* into naive recipients that were challenged with DNFB 30 min after cell transfer and tested for CS 24 h later.

FACS analysis

First, ALNC, SPLC, Thy and MLNC from mice EC treated with PBS or DNP-BSA were stained for surface markers with anti-CD4-PE-Alexa Fluor, anti-TCR β -FITC and anti-CD25-PE mAb (all from BD Bioscience, San Jose, CA, USA). Then, samples were stained for intracellular FoxP3 expression using the mouse Treg staining kit (eBioscience) according to manufacturers instructions.

Cells were analyzed with a FACSCalibur (BD Biosciences) and data were analyzed using Cell Quest software.

Statistics

Data in graphs are shown as the mean $\pm$ SE. ANOVA followed by Student's *t*-test was used for multiple comparisons. Statistical significance was set at $p < 0.05$.

Results

Dose dependence of skin induced tolerance

BALB/c mice were EC immunized twice with graded doses of DNP-BSA (100, 30, 10, 3, 1 or 0.3 μ g/animal) or exposed to PBS alone as described in Materials and Methods, Figure 1A. On day 7, patches were removed and mice were sensitized with 0.5% DNFB

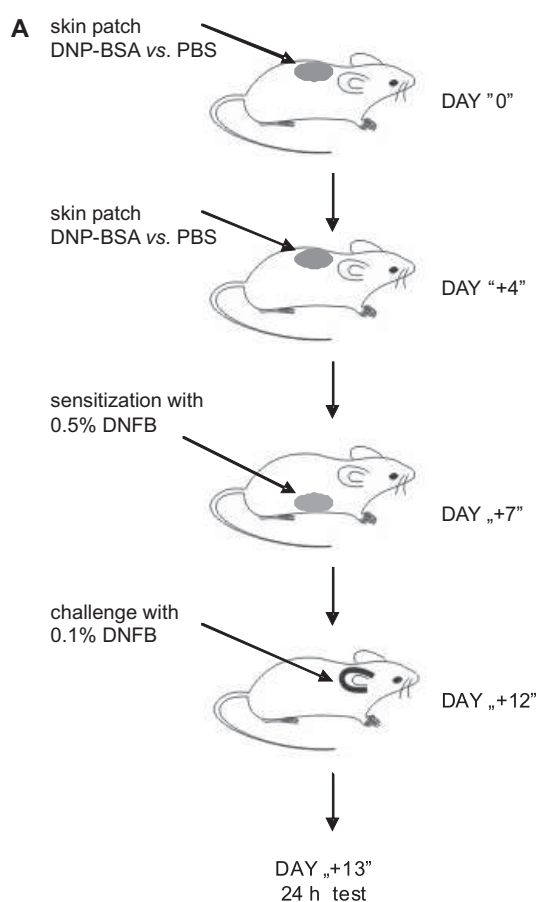
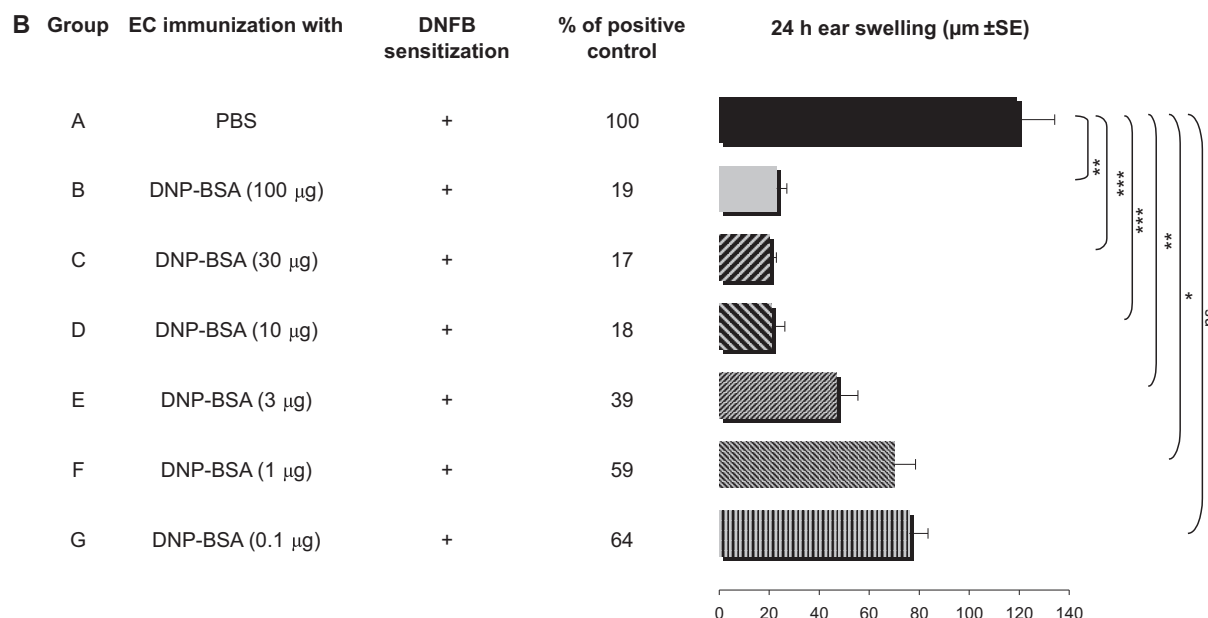


Fig. 1. Epicutaneous immunization with DNP-BSA inhibits Tc1-mediated CS. **(A)** Skin-induced suppression *via* EC immunization with hapten-conjugated protein antigen. BALB/c mice were EC immunized with 100 μ g of DNP-BSA or PBS alone applied in a gauze patch on days "0" and "+4". On day "+7" patches were removed and both groups of animals were contact sensitized by abdominal skin painting with 0.5% DNFB. Five days later (day "+12") mice were challenged by painting of both ears with 5 μ l of 0.1% DNFB in vehicle and 24 h later (day "+13") animals were tested for CS. **(B)** Skin-induced suppression is dose dependent. BALB/c mice were EC immunized *via* patches with graded doses of DNP-BSA (100, 30, 10, 3, 1 or 0.1 μ g/animal) (Groups B–G, respectively) or exposed to PBS alone (Group A) as described in Figure 1A. On day "+7" patches were removed, mice were actively immunized with 0.5% DNFB and then challenged and tested for CS. Background increase in ear thickness (± 20 μ m 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 μ m $\pm$ SE, $n = 10$; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$



and then tested 5 days later for elicitation of CS. Figure 1B shows that mice exposed to doses of DNP-BSA between 100 to only 1 μ g/animal prior to hapten sensitization develop significantly decreased CS in a dose-response manner, ranging from 19 to

59% compared to positive controls (Fig. 1, Groups B–F vs. Group A). The lowest dose of DNP-BSA at 0.1 μ g/animal still suppressed CS but observed inhibition was non-significant when compared to positive control (Groups F and G vs. Group A). These data

strongly suggest that EC immunization with related antigen DNP-BSA prior to skin sensitization with DNFB significantly reduces CS responses and that the strength of suppression is dependent on the tolerizing dose of antigen applied to the skin.

Skin-induced tolerance is caused by regulatory cells

We determined if skin-induced tolerance can be transferred with cells of the immune system, in a classical

A Donors of regulatory cells

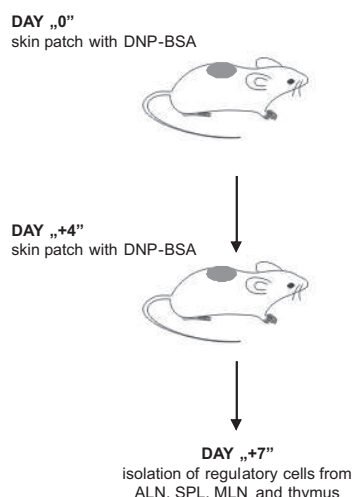
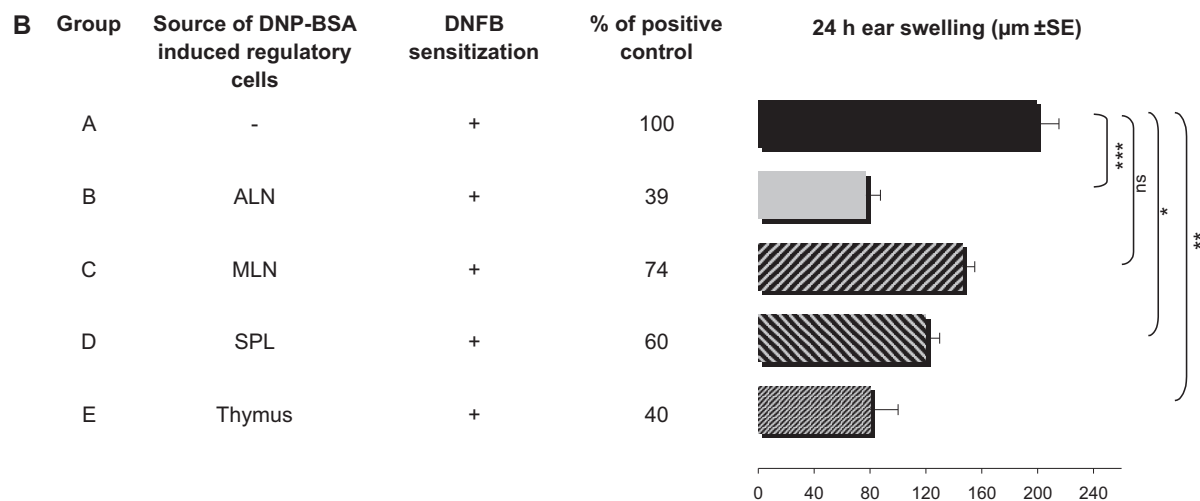
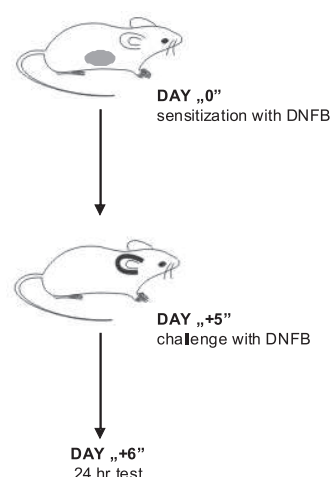


Fig. 2. Skin-induced suppression can be transferred with lymphoid cells **(A)** Transfer of skin-induced suppression – transfer “in protocol”. Animals were EC immunized with DNP-BSA for one week as described in Materials and Methods. On day “+7” patches were removed, mice sacrificed and lymphoid organs isolated (axillary and inguinal lymph nodes, spleens, mesenteric lymph nodes and thymus). Prepared suspensions of 4×10^7 ALNC, SPLC, MLNC or Thy were injected *iv* into naive recipients that were sensitized with 0.5% DNFB (day “0”), challenged five days later (day “+5”) and tested for CS 24 h later (day “+6”). **(B)** Compartmentalization of skin-induced suppression. 4×10^7 ALNC (Group B), MLNC (Group C), SPLC (Group D) or Thy (Group E) isolated from mice that were EC immunized with DNP-BSA and transferred into syngeneic recipients. All transferred recipients and one group of naive BALB/c mice used as a positive control (Group A) were sensitized with DNFB and tested for CS five days later. Background increase in ear thickness ($\pm 20 \mu\text{m}$ 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 $\mu\text{m} \pm \text{SE}$, $n = 10$; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Recipients of regulatory cells



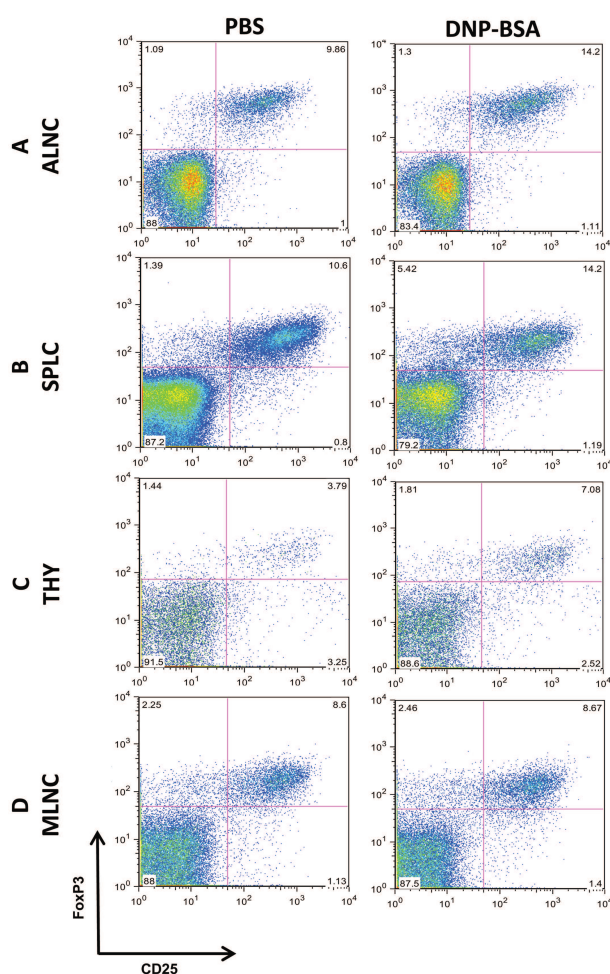


Fig. 3. Epicutaneous immunization with DNP-BSA induces Treg cells. ALNC, SPLC, Thy and MLNC from BALB/c mice EC treated with PBS or DNP-BSA first were stained for surface markers with anti-CD4-PE-Alexa Fluor, anti-TCR β -FITC and anti-CD25-PE and then for intracellular FoxP3 expression using the mouse Treg staining kit (eBioscience). Resultant cells were analyzed by flow cytometry. The plots were made after gating on CD4 $^{+}$ TCR β $^{+}$ cells

“transfer in” experiment (Fig. 2A). Four $\times 10^7$ ALNC, MLNC, SPLC or Thy isolated from mice that were skin tolerized with DNP-BSA were transferred into naive syngeneic recipients that were sensitized with DNFB and tested for CS. Figure 2B shows that ALNC, SPLC and Thy from tolerized donors were able to suppress CS in actively sensitized mice (Groups B, D and E vs. Group A). Suppression was not observed when mice received MLNC prior to induction of CS (Group C vs. Group A).

EC immunization with DNP-BSA induces Treg cells

To determine whether maneuver of EC immunization induces Treg cells, we stained for FoxP3 expression in ALNC, SPLC, Thy and MLNC isolated from animals EC immunized with DNP-BSA or treated with PBS. Data presented in Figure 3 show that EC immunization with DNP-BSA indeed induced a significant increase of CD25 $^{+}$ FoxP3 $^{+}$ cells in ALN, SPL and Thy when compared to mice EC treated with PBS alone (Fig. 3A–C). Skin immunization with DNP-BSA did not affect percentage of CD25 $^{+}$ FoxP3 $^{+}$ cells in MLN (Fig. 3D).

EC induced suppression is antigen non-specific

Our previous work on skin induced suppression showed that this phenomenon was antigen-non-specific both in Th1 contact sensitivity response and animal model of multiple sclerosis [22, 23]. In our current work, we tested the antigen specificity of EC induced suppression of Tc1-mediated CS response (Fig. 4A Groups B, C and D vs. Group A and Fig. 4B Groups B and C vs. Group A, respectively). To confirm the data of active tolerization we employed a “transfer in” protocol where mice were transferred with lymph node cells from mice tolerized with three non-cross-reacting antigens prior to DNFB sensitization. Presented data show that transfer of lymph node cells from mice tolerized with different antigens such as DNP-BSA, MBP (Fig. 5A Groups B and C vs. Group A) or OX-Ig (Fig. 5B Group C vs. Group A) prior to sensitization with DNFB suppresses CS response. Finally, using the “transfer out” protocol described in Figure 6A, we showed that investigated suppression is indeed antigen non-specific as DNP-BSA, OVA, MBP (Fig. 6B Groups B, C and D vs. Group A) and OX-Ig (Fig. 6C Group C vs. Group A) induced regulatory cells affected function of DNP-specific CS-effector cells.

Skin-induced suppression declines with time

To determine the duration of skin-induced tolerance, BALB/c mice were EC immunized with 100 μ g/ani-

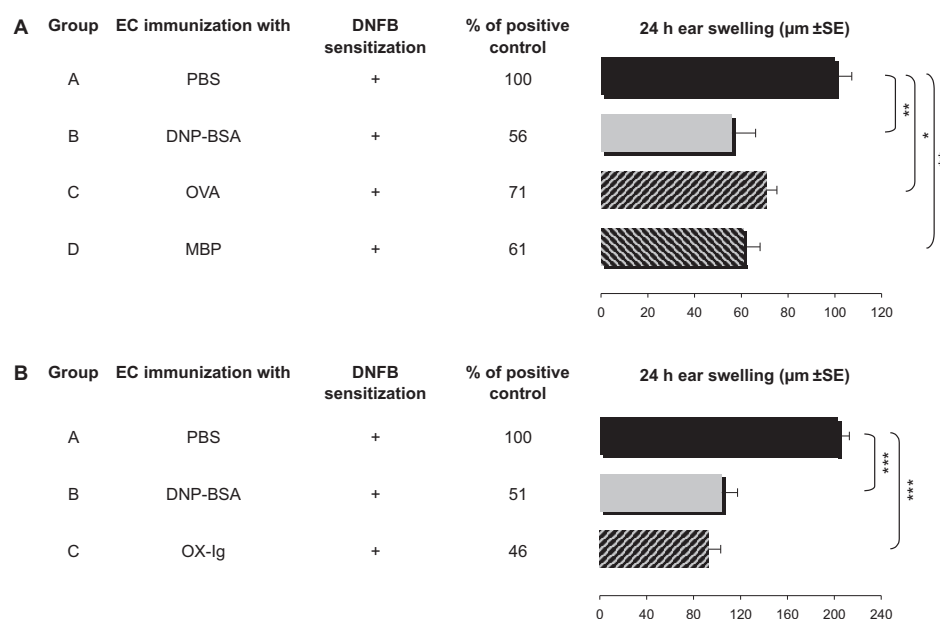


Fig. 4. Skin-induced suppression is antigen non-specific. **(A)** EC immunization with non-cross-reacting protein antigens prior to DNFB sensitization inhibits CS. Animals were EC immunized with DNP-BSA (Group B), OVA (Group C) or MBP (Group D) for one week as described in Materials and Methods. On day "+7" patches were removed and mice were sensitized with 0.5% DNFB and tested for CS. Mice in positive control were patched with PBS alone before DNFB sensitization (Group A). Background increase in ear thickness ($\pm 20 \mu\text{m}$ at 24 h) of littermate non-sensitized animals that were similarly challenged was subtracted from each experimental group to yield the net ear swelling expressed in $\mu\text{m} \pm \text{SE}$, $n = 10$; * $p \leq 0.05$, ** $p \leq 0.01$. **(B)** EC immunization with OX-Ig suppresses CS to DNFB. Mice were EC immunized with DNP-BSA (Group B) or OX-Ig (Group C) or patched with PBS alone for seven days. On day "+7" patches were removed, animals underwent DNFB sensitization and were tested for CS five days later. Background increase in ear thickness ($\pm 20 \mu\text{m}$ 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 $\mu\text{m} \pm \text{SE}$, $n = 10$; ***, $p \leq 0.001$

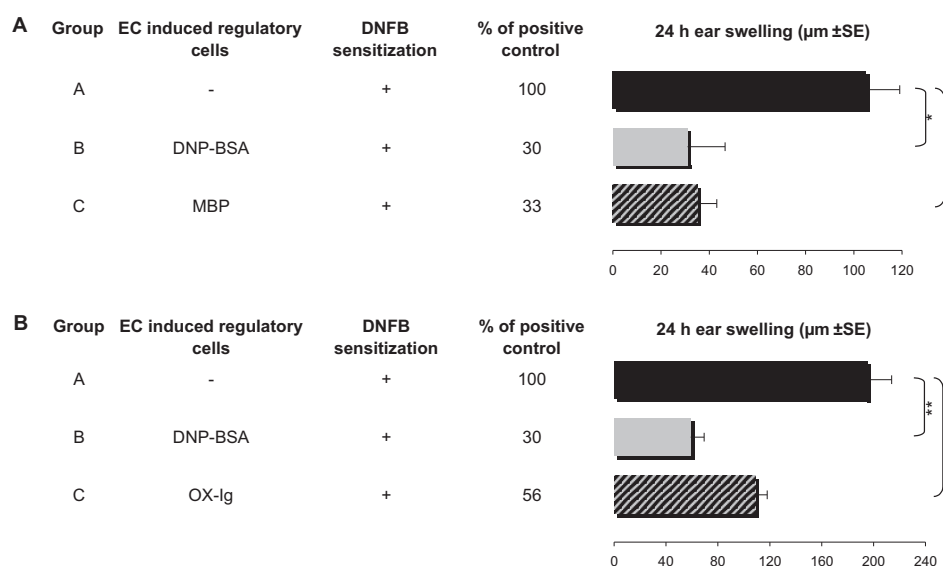


Fig. 5. Lack of antigen specificity of skin-induced suppression – “transfer in” protocol. **(A)** Transfer of MBP-induced regulatory cells inhibits DNFB-specific CS. ALNC (4×10^7) isolated from mice that were EC immunized with DNP-BSA (Group B) or MBP (Group C) and transferred into syngeneic recipients that were sensitized with DNFB and tested for CS five days later. Animals in positive control did not receive any cells prior to DNFB sensitization (Group A). Background increase in ear thickness ($\pm 20 \mu\text{m}$ 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 $\mu\text{m} \pm \text{SE}$, $n = 10$; * $p \leq 0.05$, ** $p \leq 0.01$. **(B)** Adoptive transfer of OX-Ig induced regulatory cells inhibits CS to DNFB. ALNC (4×10^7) isolated from animals EC immunized with DNP-BSA (Group B) or OX-Ig (Group C) were transferred into syngeneic recipients that underwent DNFB sensitization and subsequent challenge and test. Mice in positive control were sensitized only (Group A). Background increase in ear thickness ($\pm 20 \mu\text{m}$ 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 $\mu\text{m} \pm \text{SE}$, $n = 10$; ** $p \leq 0.01$

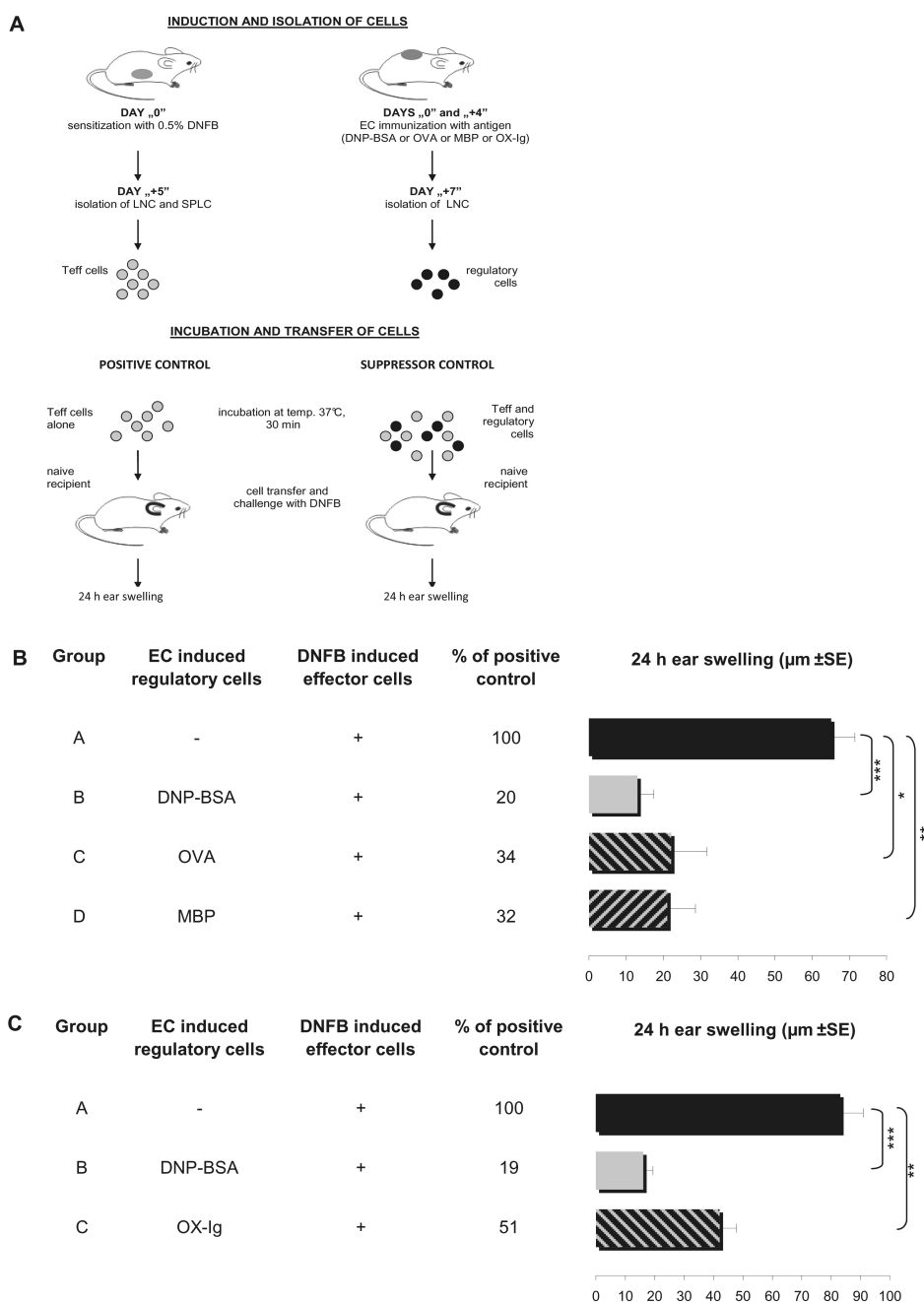


Fig. 6. EC immunization with protein antigen induces antigen non-specific regulatory cells – “transfer out” protocol. **(A)** Scheme of “transfer out” protocol. Donors of CS-effector cells (Teff) were sensitized with 0.5% DNFB. Immune ALNC and SPLC were harvested on day 5 and 7×10^7 CS-effector cells were incubated for 30 min at 37°C in RPMI 1640 medium alone, washed and then injected *iv* into naive syngeneic recipients – positive control (Group A). In suppressor control (Group B), 7×10^7 of the CS-effector cells from DNFB sensitized donors were incubated for 30 min at 37°C with ALNC from mice EC immunization with protein antigen and harvested on day “+7” (regulatory cells). After incubation the cell mixture was transferred *iv* into naive recipients that were challenged with DNFB and tested for CS 24 h later. Mice in positive control received DNFB immune cells only (Group A). Background increase in ear thickness ($\pm 20 \mu\text{m}$ 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 $\mu\text{m} \pm \text{SE}$, $n = 10$; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. **(B)** DNFB immune CS-effector cells are suppressed by regulatory cells induced via EC immunization with protein antigen. DNFB immune cells (7×10^7) were incubated with 4×10^5 of ALNC isolated from syngeneic animals EC immunized with DNP-BSA, OVA or MBP (Groups B–D, respectively) before cell transfer into naive recipients that were ear challenged with 0.1% DNFB and tested for CS 24 h later. Mice in positive control were transferred with DNFB immune cells only (Group A). Background increase in ear thickness ($\pm 20 \mu\text{m}$ 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 $\mu\text{m} \pm \text{SE}$, $n = 10$; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. **(C)** EC induced regulatory cells inhibit CS-effector cells to non-cross-reacting hapten. DNFB specific CS-effector cells (7×10^7) were incubated with 4×10^5 of ALNC isolated from mice EC immunized with DNP-BSA (Group B) or OX-Ig (Group C) before *iv* injection into naive recipients that were challenged with DNFB and tested for CS 24 h later. Mice in positive control received DNFB specific CS-effector cells only (Group A). Background increase in ear thickness ($\pm 20 \mu\text{m}$ 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 $\mu\text{m} \pm \text{SE}$, $n = 10$; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

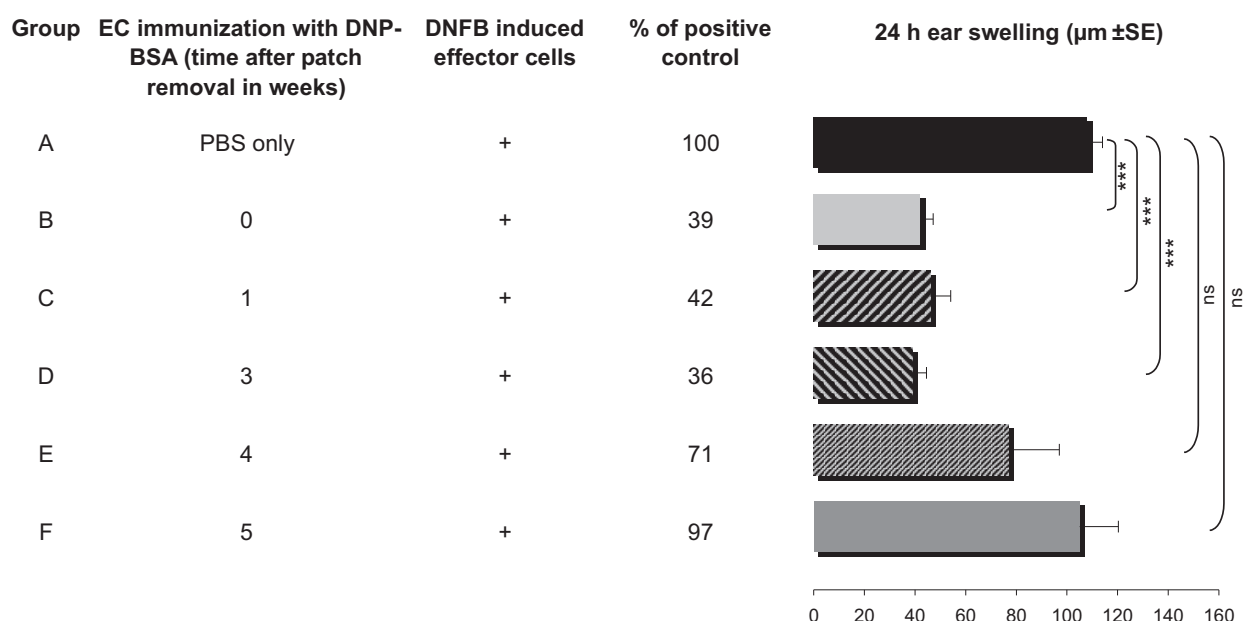


Fig. 7. Skin-induced suppression declines with time. BALB/c mice were EC immunized with 100 $\mu\text{g}/\text{ml}$ animal of DNP-BSA or exposed to PBS alone (Group A) as described in Materials and Methods. On day "+7" patches were removed and mice were sensitized with 0.5% DNFB immediately (Group B), or 1, 3, 4 or 5 weeks later (Groups C–F, respectively). Mice from all experimental groups were challenged and tested for CS. Background increase in ear thickness ($\pm 20 \mu\text{m}$ 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 $\mu\text{m} \pm \text{SE}$. $n = 10$; *** $p \leq 0.001$

mal of DNP-BSA or treated with PBS alone as a control. On day 7, patches were removed and then mice were sensitized with 0.5% DNFB immediately after patch removal or 1, 3, 4 or 5 weeks later. Mice from all experimental groups were challenged and tested for CS at the same time. Figure 7 shows that skin-induced suppression of CS declines with time (Groups B–F vs. Group A) and persisted up to 3 weeks after patch removal (Group D vs. Group A). Suppression of CS was still observed 4 weeks after patch removal, however, it was not significant (Group E).

Discussion

It is well known that immunization with an antigen *via* the digestive tract or nasal mucosa leads both to a local immune response and a state of profound immunosuppression in the periphery [27]. This suppression seems to play an important role in avoiding the development of immune responses to non-pathogenic antigens.

Although the skin is considered an organ where immune responses are easily induced [4], little attention

has been given to skin induced tolerance [21]. Because skin and mucosa play similar function in our bodies (i.e., as a barrier to external pathogens) it is possible that epicutaneous (EC) application of a protein antigen, apart from inducing a strong immune response, may also induce peripheral tolerance.

Our previous work proved that EC immunization with protein antigen inhibits Th1-mediated CS [17, 22]. Observed suppression was transferable by $\text{TCR}\alpha\beta^+ \text{CD4}^+ \text{CD8}^+$ double positive lymphocytes and was TGF- β dependent [22]. Additionally, our work in animal model of multiple sclerosis (EAE) showed that maneuver of EC immunization with MBP protects from disease [11, 23, 25]. Our further work in an animal model of skin graft rejection showed that skin application of a protein antigen prior to allogeneic skin graft prolonged graft survival [10]. These results suggested that EC induced suppression might inhibit not only Th1 but Tc1 mediated immune response as well. To prove this hypothesis we employed Tc1-mediated CS to DNFB. Our recently published paper showed that EC immunization with protein antigen DNP-BSA prior to hapten sensitization indeed inhibits CS in mice [12]. Observed suppression was transferred with $\text{TCR}\alpha\beta^+ \text{CD4}^+ \text{CD25}^+ \text{FoxP3}^+$ [12].

The aim of current work was to characterize EC induced suppression of Tc1-mediated CS more precisely.

Data presented in Figure 1B show that EC immunization with DNP-BSA prior DNFB sensitization results in significant reduction of DNP-specific CS. The optimal dose of antigen that induces this phenomenon is between 100 and 1 $\mu\text{g}/\text{mouse}$. This observation was in line with our previous finding in Th1-mediated CS [22]. Investigated skin-induced suppression caused by low doses of hapten-conjugated protein antigen resembles previously described low zone tolerance (LZT) provoked by immunization with subsensitizing doses of hapten [16]. However, the regulatory cells involved in LZT and skin-induced suppression are different. As we showed previously, suppression induced *via* EC immunization with hapten-conjugated protein antigen DNP-BSA is mediated by $\text{TCR}\alpha\beta^+ \text{CD4}^+ \text{CD25}^+ \text{FoxP3}^+$ Treg cells [12], whereas LZT is caused by CD8^+ T cells [18]. Additionally, the mechanism of suppression seems to differ for both phenomena. EC induced suppression is CTLA4-dependent and cytokine independent [12], whereas LZT depends on IL-10 [15].

Then, “transfer in” experiment showed that maneuver of EC immunization with a protein antigen induces regulatory cells which are present in ALN, SPL and Thy (Fig. 2B). Transfer of MLNC isolated from EC tolerized donors did not inhibit CS. This data suggest that lack of regulatory cells in MLN might result from inaccessibility of antigen deposited on the skin. This presumption was confirmed by FACS analysis of ALNC, SPLC, Thy and MLNC stained for the presence of Treg cell markers. Data presented in Figure 3 show that EC application of DNP-BSA indeed induced a significant increase of $\text{CD25}^+ \text{FoxP3}^+$ cells in ALN, SPL and Thy (Fig. 3A–C) but not in MLN (Fig. 3D) when compared to animals EC treated with PBS alone.

To determine antigen specificity of EC induced suppression we employed three different experimental models: active suppression (animals patched with protein antigen prior to DNFB sensitization, Fig. 4), “transfer in” (EC induced regulatory cells were transferred into mice that were sensitized with DNFB, Fig. 5) and “transfer out” (EC induced regulatory cells were co-transferred with CS-effector cells, Fig. 6). The study of the Ag specificity of skin induced tolerance showed suppression of CS responses in mice immunized EC with four different non-cross reacting antigens like DNP-BSA, OX-Ig, OVA and MBP prior to contact sensitization. These results show that the final mediation of suppressor T cell ac-

tivity is Ag non-specific (Fig. 4A and Fig. 4B, respectively). These data were fully confirmed by “transfer in” (Fig. 5A, Fig. 5B) and “transfer out” experiments (Fig. 6B and Fig. 6C). Thus, EC immunization with any tested protein antigen induced strong antigen-non-specific suppression of Tc1-mediated CS. This finding is in line with our previous studies both in Th1-mediated CS and EAE [17, 22, 25].

In classic T cell mediated suppression both suppressor and effector cells often are specific for the same antigen, i.e., the suppression is antigen specific and limited to that antigen. The non-specific tolerance observed in our experiments possibly is akin to the phenomenon known as “bystander suppression” or determinant spreading [8] in which suppressor cells elicited by a particular antigen secrete antigen non-specific cytokines that down-regulate the immune responses of other antigen-specific T cells. In the well studied model of oral tolerance, orally administered antigen appears to interact with gut associated lymphoid tissue to generate T suppressor cells that produce TGF- β , IL-4 and IL-10 [19], and also mediate bystander suppression [8]. It was shown both *in vivo* and *in vitro*, that orally induced suppression is dependent on antigen-specific triggering of the T suppressor cells. Once antigen-specific triggering occurs, suppression is mediated by non-specific suppressive cytokines produced by the T suppressor cells [6]. In the case of our skin induced tolerance of Tc1-mediated CS, immune response to one antigen could be inhibited by all tested antigens used to induce skin tolerance. Thus, the presence of the antigen responsible for inducing T suppressor cells is not required during elicitation of the T suppressor effect [20].

This might suggest that skin induced suppressor cells work either *via* different mechanisms than are active in bystander suppression in mucosa oral tolerance, or an original antigen persists for a long period in peripheral lymph organs.

Finally, our study shows that skin-induced suppression of Tc1-mediated CS declines with time and persisted up to 3 weeks after patch removal (Fig. 7, Group D). Suppression of CS was still observed 4 weeks after patch removal, however, it was not significant (Fig. 7, Group E). This might result from either limited lifespan of regulatory cells or disappearance of antigen from the organism. Observed attenuation of EC induced suppression suggests that for prolonged therapeutic effect a booster treatment might be required.

The ease of EC generation of antigen-non-specific regulatory cells may have important implications for designing therapeutic schemes aimed at modulating immune responses to self antigens involved in autoimmune diseases.

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