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Epicutaneous (EC) immunization with type II collagen (COLL II) induces CD4$^+$ CD8$^+$ T suppressor cells that protect from collagen-induced arthritis (CIA)

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ABSTRACT

Background: We have shown previously that epicutaneous (EC) immunization with protein antigen induces T suppressor cells that alleviate inflammatory response in contact hypersensitivity reactions, in an animal model of multiple sclerosis, and in TNBS-induced colitis.
Methods: DBA/1 mice were EC immunized with type II collagen (COLL II) spread over a gauze patch on days 0 and 4. On day 7, patches were removed and mice were intradermally (*id*) immunized with COLL II in CFA to induce collagen-induced arthritis (CIA).
Results: Our work shows that EC immunization with 100 μg of COLL II prior to CIA induction reduces disease severity as determined by macroscopic evaluation. Reduced disease severity after EC immunization with COLL II correlates with milder histological changes found in joint sections. Experiments with the three non-cross-reacting antigens COLL II, ovalbumin (OVA) and myelin basic protein (MBP) showed that skin-induced suppression is antigen non-specific. Transfer experiments show that EC immunization with COLL II induces suppressor cells that belong to the population of CD4$^+$ CD8$^+$ double positive lymphocytes. Flow cytometry experiments showed increased percentage of CD4$^+$ CD8$^+$ RORγt$^+$ cells in axillary and inguinal lymph nodes isolated from mice patched with COLL II.
Conclusion: Maneuver of EC immunization with a protein antigen that induces suppressor cells to inhibit inflammatory responses may become an attractive, noninvasive, needle-free therapeutic method for different clinical situations.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects about 1% of the adult population and occurs twice as frequently among women than men [1]. The onset may appear at any age, but the peak of incidence comes in the 25–55 age range [2]. Because the disease affects persons in economically productive age ranges, it presents an ever-increasing economic and social problem affecting the quality of life of the patients and their ability to work.

Non-steroid anti-inflammatory drugs commonly used in RA therapy through their anti-inflammatory and analgesic activity, principally relieve the symptoms of RA, with minimal impact on the disease process, and concurrent side effects [3]. Additionally, the treatment of RA includes drugs modifying the disease process, *e.g.* sulfasalazin, methotrexate and cyclosporin A. These drugs

Abbreviations: Ab, antibody; ALNC, axillary and inguinal lymph node cells; anti-CPP, Anti-cyclic citrullinated peptide; CFA, complete Freund's adjuvants; CHS, contact hypersensitivity reactions; CIA, collagen-induced arthritis; COLL II, type II collagen; EAE, experimental autoimmune encephalomyelitis; EC, epicutaneous; FBS, fetal bovine serum; *id*, intradermally; IFA, incomplete Freund's adjuvants; Ig, immunoglobulin; IL, interleukin; *ip*, intraperitoneally; *iv*, intravenous; LPS, lipopolysaccharide; MBP, myelin basic protein; MPO, myeloperoxidase; NK, natural killer; NKT, natural killer T cell; OVA, ovalbumin; PBS, phosphate buffered saline; RA, rheumatoid arthritis; RC, rabbit complement; TGF-β, transforming growth factor beta; Tc, T cytotoxic cell; Th, T helper cell; TNBS-induced colitis, the 2,4,6-trinitrobenzene sulfonic acid-induced colitis; TNP-Ig, TNP conjugated mouse immunoglobulins; Ts, T suppressor cell.

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apart from their therapeutic effects may cause a number of severe side effects [4]. Finally, RA therapy uses biological drugs, which can interfere with the immune mechanisms underlying the RA pathology, such as, *e.g.* cytokine inhibitors. These drugs face barriers, *e.g.* limited availability, high price and inducement of non-specific immunosuppression [5].

Therefore, there is huge need to develop new therapies, non-invasive and free of side effects, specifically eliminating the undesired inflammatory reaction accompanying RA. Throughout the world, efforts have been made to develop a "vaccine" which would prevent the onset of autoimmune diseases, including RA.

Our previous work showed that epicutaneous (EC) immunization of mice with different protein antigens applied on the skin in the form of a gauze patch or cream emulsion induces a state of subsequent tolerance that inhibits both Th1 and Tc1-mediated contact hypersensitivity (CHS) [6–9]. Further study showed that maneuver of EC immunization with protein antigen also suppressed NK cell dependent CHS [10]. This was also found in an experimental autoimmune encephalomyelitis (EAE) where EC immunization with myelin basic protein (MBP) reduced disease severity and decreased disease incidence [11,12]. Furthermore, using allogeneic skin graft experimental model, we showed that EC immunization with a protein antigen delays graft rejection [13]. Then we showed that EC immunization with protein antigen TNP-Ig can also alleviate TNBS-induced colitis in mice [14].

Our recent study shows that EC immunization with type II collagen (COLL II) reduces disease severity as determined macroscopically [15]. Decreased disease severity observed after EC immunization with COLL II was confirmed by reduced MPO (myeloperoxidase) activity in joint tissue and with decreased production of anti-citrullinated protein (anti-CPP) and anti-type II collagen IgG2a antibodies. Observed protection from disease was transferable with TCR$\alpha\beta$+ lymphocytes. Both *in vitro* and *in vivo* experiments show that IL-17A plays an important role in EC-induced suppression of CIA. Moreover, EC application of COLL II at the first signs of CIA also results in suppression of disease [15].

The current study shows that similarly to CHS and EAE, skin-induced suppression is antigen-non-specific and is mediated by $CD4^+$ $CD8^+$ double positive suppressor cells and that these cells express transcription factor RORγt.

Materials and methods

Mice

Male DBA/1 mice 8–12 weeks old were from the breeding unit of the Department of Medical Biology, Jagiellonian University, School of Medicine. Mice were fed autoclaved food, and water. All experiments were conducted according to guidelines of the Animal Use and Care Committee of the Jagiellonian University School of Medicine.

Reagents

Bovine type II collagen, complete (CFA) and incomplete (IFA) Freund's adjuvants were obtained from Chondrex Inc. (Redmond, WA, USA). LPS (from *Escherichia coli* 026:B6), OVA (Grade V) and guinea pig MBP were purchased from Sigma (St. Louis, MO, USA). RPMI 1640 and fetal bovine serum (FBS) were from Life Technologies (Grand Island, NY, USA). Low-tox rabbit complement (RC) was from Pel-Freeze Biologicals (Brown Deer, WI, USA). Protein A was from Pharmacia Fine Chemicals (Piscataway, NJ, USA), whereas Sepharose 4 Fast Flow was from Pharmacia LKB Biotechnology AB (Uppsala, Sweden).

Monoclonal antibodies and hybridoma

Protein A and Sepharose 4 Fast Flow were used to obtain purified IgG from supernatants of hybridoma cell cultures according to the directions of the manufacturer with our modifications [16]. The following protein A affinity purified rat anti-mouse monoclonal antibodies (mAb) were used: anti-CD4 (clone TIB 207) and anti-CD8 (clone TIB 105.3) from late Dr. C.A. Janeway, Jr., Yale University, New Haven, CT, USA.

Induction and gross assessment of CIA

Immunization and arthritis evaluation were performed as described previously [11]. DBA/1 mice were injected *id* at the base of the tail with 100 μg COLL II emulsified in CFA on day 0 and boosted with 100 μg COLL II in IFA on day 21 after the first immunization. To accelerate the development of arthritis, 40 μg of LPS in sterile PBS was intraperitoneally (*ip*) injected on day 28. Animals were observed daily for the presence of arthritis and the clinical severity of disease was scored for each paw on a scale of 0–4 [15]. The criteria for the grading were as follows: 0–no evidence of erythema and swelling; 1–mild erythema and swelling of the wrist or the ankle; 2–moderate erythema and swelling from the wrist to the metacarpal joints or from the ankle to the metatarsal joints; 3–severe erythema and swelling of the entire paw including digits; 4–maximal erythema and swelling of the paw.

Histology of the ankle joint

On day 67 after immunization, the mice were sacrificed and the hind paws were fixed in 10% buffered formalin, decalcified and embedded in paraffin. Joint sections (5–7 μm) were prepared and stained with hematoxylin and eosin (H&E). The histological evaluation was performed under the light microscope.

EC immunization with protein antigen

EC immunization was performed by applying to the shaved skin of the mouse dorsum a 1 cm^2 gauze patch soaked with a solution containing COLL II (100 μg/mouse) in a volume of 100 μl PBS on day 0. The patch was secured by adhesive tape wrapped around the midsection. Positive control mice were patched with PBS alone. The patch was left in place from day 0 until day 4, when it was replaced with a fresh patch [15]. On day 7, mice were *id* immunized with COLL II in CFA as described above.

To test antigen specificity of skin-induced suppression, mice were patched with COLL II or non-cross-reacting antigens OVA or MBP prior to CIA induction.

In some experiments, lymph organs isolated from donors EC immunized with COLL II were used as a source of suppressor cells.

Phenotype of suppressor cells

To determine the phenotype of EC-induced suppressor cells *in vivo*, axillary and inguinal lymph node cells (ALNC) isolated from mice EC treated with COLL II were incubated in PBS on ice with purified anti-CD4 or anti-CD8 mAbs (1 μg Ab/10^6 cells), or with PBS alone for 45 min. 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. Next, cells were counted and cell viability was assessed by trypan blue exclusion. 2.5×10^7 of EC-induced suppressor cells treated with RC alone (suppression control), or 2.5×10^7 of cell aliquots of EC-induced suppressor cells treated with appropriate mAb and RC were transferred intravenously (*iv*) into naive recipients that underwent CIA induction. Mice were observed daily and scored for arthritis as described above.