



Review article

Transdermal immunotherapy: Past, present and future

Marian Szczepanik^{*}, Monika Majewska-Szczepanik

Department of Medical Biology, Jagiellonian University Medical College, Kraków, Poland

ARTICLE INFO

Article history:

Received 8 December 2015

Received in revised form 6 April 2016

Accepted 13 April 2016

Available online 25 April 2016

Keywords:

Epicutaneous immunization

Immunopotentialization

Allergy

Autoimmunity

Contact sensitivity

ABSTRACT

The human skin plays an important role in thermal regulation, the maintenance of the correct water and electrolyte balance; it is also the main source of the vitamin D for the organism. Moreover, the skin forms an effective barrier against microorganisms, protecting the organism against xenobiotics and harmful external factors. It must also be mentioned that the skin is an essential organ involved in immunological mechanisms. This article summarizes current knowledge about skin-induced suppression induced *via* epicutaneous (EC) application of an antigen and is described in both Th1- and Tc1-mediated contact sensitivity (CS) in mice. The skin-induced suppression is also considered in the regulation of experimental models of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA) and *colitis ulcerosa*. Furthermore, the efficacy of treatment of multiple sclerosis (MS) patients with EC immunization is discussed. Second part of this review introduces new subject of immunopotentialization induced *via* EC immunization with an antigen in the presence of various pathogen-associated molecular patterns (PAMPs). Finally, the review describes dermal immunization in the therapy of allergic diseases.

© 2016 Institute of Pharmacology, Polish Academy of Sciences. Published by Elsevier Urban & Partner Sp. z o.o. All rights reserved.

Contents

Introduction	774
The skin immune system	774
The skin as the place of medicine administration	774
The suppression of immune response <i>via</i> dermal application of antigen in experimental conditions	775
MS therapy <i>via</i> dermal application of antigen	777
Immunopotentialization induced <i>via</i> transdermal immunization	778
Dermal immunization in the therapy of allergic diseases	778
The suppression of allergic reaction <i>via</i> EC immunization with an antigen and TLR ligand in experimental conditions	779
Coda	779
Conflict of interest	780
Acknowledgements	780
References	780

Abbreviations: AD, atopic dermatitis; AIT, allergen immunotherapy; CIA, collagen-induced arthritis; CNS, central nervous system; COLL II, type II collagen; CpG, unmethylated cytidine–guanosine dinucleotides; DC, dermal dendritic cells; DNFB, 2,4-dinitrofluorobenzene; EAE, experimental autoimmune encephalomyelitis; EPIT, epicutaneous immunotherapy; EPO, eosinophil peroxidase; IgE, immunoglobulin E; IL, interleukin; ILC, innate lymphoid cells; ILIT, intralymphatic immunotherapy; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MRI, magnetic resonance imaging; MS, multiple sclerosis; NK, natural killer cells; NOD1 and NOD2, NOD-like receptors 1 and 2; PAMPs, pathogen-associated molecular patterns; PC, phosphorylcholine; PLP, myelin proteolipid protein; PRR, pattern recognition receptors; RLR, RIG-I-like receptors; RIG-I, retinoic acid-inducible gene-I; SCIT, subcutaneous allergen-specific immunotherapy; SLIT, sublingual immunotherapy; Tc, T cytotoxic cell; TCR, T cell receptor; TGF, transforming growth factor; Th, T helper cell; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNP-Cl, 2,4,6-trinitro-1-chlorobenzene; Tr1, type 1 regulatory cells; TNP-Ig, 2,4,6-trinitrophenyl-conjugated mouse immunoglobulins; Treg, regulatory T lymphocytes; T_{RM}, resident memory T cells; TSLP, thymic stromal lymphopoietin.

^{*} Corresponding author.

E-mail addresses: mmszczep@cyf-kr.edu.pl, marian.szczepanik@uj.edu.pl (M. Szczepanik).

Introduction

The skin is the largest human organ with its weight making up approximately 12–15% of the total body mass. The total surface of human skin varies between 1.5 and 2.0 m², which makes this organ the second largest surface of the organism's contact with external environment, whilst the mucous membrane is the largest one. The most external part of the skin is the epidermis attached to the basal membrane, under which there is dermis and subcutaneous adipose tissue. In describing the skin composition, one must not forget about its microbiome. It has been proven that the epidermal corneal layer is the area populated by non-pathogenic microorganisms, such as bacteria, fungi, viruses and protozoa. As it was shown, the natural skin flora (microbiota) plays a significant role in the process of wound healing, defence against pathogenic microorganisms and also determines the correct development of the immune system.

The human skin plays also an important role in thermal regulation, the maintenance of the correct water and electrolyte balance; it is also the main source of the vitamin D for the organism. Moreover, the skin forms an effective barrier against microorganisms, protecting the organism against xenobiotics and harmful external factors. It must also be stressed that the skin is an essential organ involved in immunological mechanisms.

The skin immune system

Epidermis, in its 95%, is built of keratinocytes. The other epidermal cells are melanocytes, Merkel's cells and Langerhans cells. Keratinocytes play a key role in the skin defence mechanisms by means of their ability to recognize and eliminate pathogens. These cells have a wide repertoire of innate immunity receptors – PRR (pattern recognition receptors) such as Toll-like receptors (TLR), NOD-like receptors (NOD1 and NOD2), RIG-I-like receptors (RLR) and dectin-1 receptor (Dectin-1) [1]. As a result of the pathogen recognition by PRR receptors, keratinocytes release a wide spectrum of substances capable of killing microorganisms, such as β -defensins and RNases. It is worth observing that keratinocytes release also a number of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-18) as well as anti-inflammatory ones (IL-10 and TGF- β). These cytokines ensure proper immune response. Moreover, the chemokines (CXCL8, CXCL9, CXCL10, CXCL11 and CCL20) released by keratinocytes, play an important role in the recruitment of T lymphocytes, monocytes and neutrophils to the skin [2].

Langerhans cells are located mostly in the spinous layer of the epidermis and their dendrites reach the corneal layer of the epidermis, which allows these cells to detect microorganism on the most external layer of the skin. Langerhans cells, after the capture and uptake of antigen in the environment of the cytokines (TNF- α , IL-6) which are released by keratinocytes, migrate to dermis and then travel from there, along the lymphatic vessels to the local lymph nodes, where they present the antigen to T lymphocytes. For many years it was believed that Langerhans cells take part only in the induction of the immune response to various antigens, including the skin pathogens (*Candida albicans*, *Staphylococcus aureus*) promoting Th17-mediated immune response [3,4]. However, the recent studies have shown that Langerhans cells play also a significant role in inhibiting the immune response by depletion of T lymphocytes and the induction of regulatory T lymphocytes (Treg) [5].

The cells within the dermis are packed much more loosely than in the epidermis. The presence of blood vessels in the dermis guarantees the inflow of the cells of the immune system if there is such a need. On the other hand, the lymphatic vessels, which originate within the dermis, deliver the antigen to the local lymph

nodes. In dermis, there is a large variety of cells involved in the defence mechanisms, and, among them, various populations of dendritic cells, mastocytes, TCR $\alpha\beta$ lymphocytes and innate lymphoid cells (ILC). Within the dermis, there are also TCR $\gamma\delta$ lymphocytes, which are involved in the process of wound healing [6]. Moreover, TCR $\gamma\delta$ lymphocytes producing various cytokines play an important role during induction and effector phase of the immune response [7].

Mast cells play an important role in the defence mechanism and inflammatory reactions. For instance microorganism recognition via TLR expressed by mast cells leads to a release of various cytokines, chemokines, prostaglandins and leukotrienes [8]. The release of TNF- α and CCL3 chemokine allows for the recruitment of T lymphocytes, monocytes and neutrophils to the site of infection.

Within the dermis, there are two populations of dendritic cells – dermal dendritic cells (dDC) with the expression of CD1c or CD141 respectively [1]. These cells play the key role in the uptake and processing of the antigen and its transport to the local lymph nodes, where they interact with T lymphocytes. Moreover, the dermis contains numerous NK (natural killer) cells which belong to ILCs. Because of their lytic activity, and the ability to release IFN- γ , NK cells play a significant role in the anti-infectious immunity as well as in the anti-tumour response [1]. The number of these cells increases significantly in the case of psoriasis [9].

Moreover, the skin is populated with numerous TCR $\alpha\beta$ lymphocytes which, in majority, belong to the population of memory cells. The number of memory T cells in the skin exceeds twice the number of these cells in the peripheral blood [10]. These cells belong to the population of CD4⁺ T lymphocytes, among which there are resident memory T cells (T_{RM}) and circulating memory T lymphocytes, known also as effector T cells (T_{EM}). About 20% of CD4⁺ T cells in the skin are made up by Treg FoxP3⁺ lymphocytes that belong to the population of resident regulatory T lymphocytes [11]. This population of Treg cells mostly inhabits the areas in the vicinity of hair follicles and thus the areas of the skin with larger amount of hair can be characterized with a larger number of Treg lymphocytes in comparison with the skin with less hair [12]. Currently, the cause of Treg lymphocyte location in the area of hair follicles is not known. One of the reasons might be the accumulation of natural flora in the hair follicles, playing a significant role in the interaction between the immune system and microbiome. Additionally, it is speculated that Treg cells are preferentially recruited to immune privileged hair follicles to prevent autoimmune attack [12]. Moreover, such location of Treg lymphocytes may be connected with the recruitment of the above population of cells to the place that is immunologically privileged, such as hair follicles, which could protect the organism against an autoimmunological attack.

The skin as the place of medicine administration

The first reports concerning the attempt to deliver medicinal products onto the skin originate from the ancient China and date from 2000 BC. This method was used for the therapy of skin lesions and consisted in the application of herbal extract in a form of a paste (emplastra) smeared on a fragment of a fabric and fixed onto the skin with a rubber band [13]. Later on, Galen (130–200 AD) applied cream (cold cream) containing herbal extract for the treatment of skin diseases for the first time [14]. Another stage in the drug administration was the application of ointment containing mercury (*unguentum hydrargyri*) for the treatment of syphilis in the 15th century [15].

The first administration of a medical product onto the skin in the therapy of remote organs took place in the 1940s. Zondek showed, for the first time, that dermal administration of a disinfectant, named chloroxylenol, in a form of ointment, was

effective in the treatment of the infections of urogenital system [16]. The further progress in dermal administration of drugs, making it possible to control the dose, was the administration of a drug in a form of a gauze dressing, which is a part of a patch. This method of dermal application of medications was introduced in the 1970s [17,18]. Currently, on the world market, there are many patches containing medical products, applied, among others, in hormone replacement therapy (Oestradiol) [19], pain relief (Fentanyl) [20], alleviation of nausea and the treatment of car motion sickness (Granisetron and Scopolamine) [21], or facilitating quitting smoking (Nicotine) [22]. The place which is the most frequently used for the drug administration is the arm, which is related with the easiness of applying next patches onto the same place and allows maintaining a similar concentration of the drug in the blood serum.

The application of a drug onto the skin makes it possible to omit the oral route, so the substance is not disintegrated chemically or enzymatically in gastrointestinal tract or liver. This is especially important in the case of drugs which are proteins or peptides, which are widely used in immunotherapy. It must be pointed out that the dermal application of proteins and peptides can be some kind of challenge in accordance with the “principle of 500 Daltons”: the particles larger than 500 Da cannot pass the skin barrier [23]. To increase skin permeability many approaches are used. That is why, many methods increasing the skin permeability and thus the substance penetration, were worked out. These methods comprise the removal of epidermis corneal layer with a sticky tape (tape stripping) [24–26], the use of liposomes [27,28], transferosomes [29] or membranes equipped with microneedles [30]. The penetration enhancement mechanism of liposomes is thought to be through disruption of the stratum corneum [27,28]. Transferosomes, are more recent class of modified liposomes described as deformable, highly deformable, elastic or ultra-flexible liposomes or vesicles. Transferosomes are claimed to be able to squeeze through conduits one-tenth the diameter of the vesicles, allowing them to spontaneously penetrate the stratum corneum [29]. The membranes equipped with microneedles have tiny needles with the length of which is 500–1000 μm , made of silicone, metal or biodegradable gel and allow for the delivery of the medicine into the area of dendritic cells of dermis, making it possible to stimulate the immune system [31,32]. It should be also pointed out that the administration of a protein or peptide in a form of a patch on an undamaged skin allows for the antigen penetration by the epidermis. In this case, an increased penetration of antigen by the epidermis is observed, which is the result of an increased perspiration under the dressing [33,34]. The changes in epidermal permeability take place already 4–10 h after the application of a patch. It is currently believed that the degree of epidermal damage before the administration of an antigen may determine the character of the induced immune response. Superficial damage of the epidermis, leading to the release of anti-inflammatory cytokines (TSLP, IL-25, IL-33) promotes the induction of Th2 and Treg lymphocytes [35,36]. It is suggested that Langerhans cells play an important role in promoting anti-inflammatory reaction, as they are reached by the antigen after the subtle damage of the epidermis. A more intensive damage of the epidermis in turn, leads to the release of pro-inflammatory cytokines (IL-1 α , IL-6, TNF- α), and the delivery of the antigen to deeper-lying dermal dendritic cells, which stimulates mostly the development of Th1-dependent immune response [37].

The suppression of immune response via dermal application of antigen in experimental conditions

The studies of dermal application induced suppression were inspired by earlier observations showing that epicutaneous (EC) application of protein antigen on the skin in a form of a gauze

dressing (patch method) induces the synthesis of IL-4 and IL-13, which may potentially inhibit the immune response mediated by CD4⁺ Th1 lymphocytes [38,39]. In the studies the model of Th1-dependent contact sensitivity reaction (CS) in mice was used (Fig. 1) [40]. In order to verify whether the EC application of protein antigen may inhibit the progress of inflammatory reaction with the participation of Th1 cells, 2,4,6-trinitrophenyl-conjugated mouse immunoglobulins (TNP-Ig) were administered on days 0 and 4 in a form of a gauze dressing with an attached patch, onto the shaved skin on the back of the mice. After one week, the dressings with the antigen were removed and the animals were sensitized with TNP-Cl hapten, and then CS reaction was tested (Fig. 2). This simple experiment allowed to prove that EC application of protein antigen before induction of CS reaction inhibits the Th1-mediated inflammatory response in the skin. Further studies showed that anti-inflammatory cytokines, such as IL-4, IL-10 and TGF- β play a significant role in the induction of skin-induced suppression. Then, negative selection experiments with proper mAb and complement, cell sorting experiments, use of NKT cell-deficient J α 18^{-/-} mice and *in vitro* cytokine neutralization revealed that EC induced suppressor cells belong to the rare population of TCR $\alpha\beta$ ⁺ CD4⁺ CD8⁺ cells, which release suppressive TGF- β [41]. CD4⁺ CD8⁺ T cells in naïve mice are less than 1% of peripheral lymphocytes and their origin is unclear. These cells may represent CD4⁺ CD8⁺ T cells that have prematurely escaped from the thymus or CD4⁺ CD8⁻ T cells that re-express CD8 after activation or exposure to cytokines e.g. IL-4, or are present due to prior “natural” suppressive events. In our case we believe that CD4⁺ CD8⁺ T cells with regulatory activity originate from prematurely escaped CD4⁺ T cells that acquire CD8 co-receptor. Our hypothesis is based on the observed increase in CD4⁺ CD8⁺ T cells in lymph nodes 7 days post EC immunization, and their subsequent decrease to the level observed in naïve mice within a week. These data can also suggest that these immature CD4⁺ CD8⁺ suppressor cells then may become single positive CD4⁺ T lymphocytes [41]. To avoid cumbersome and inconvenient patch method we developed another technique based on daily application of neutral cosmetic Nivea cream containing different protein antigens as additives on the shaved skin. Similarly to patch method EC immunization with an antigen emulsified in cream caused strong inhibition of CS response [42]. Similar observations were made also in CS reaction mediated by CD8⁺ Tc1 lymphocytes. It was observed that patching with the protein antigen labelled with DNFB hapten (2,4-dinitrofluorobenzene) before DNFB sensitization significantly inhibits Tc1-mediated inflammatory reaction in the skin [43,44]. However, in this experimental pattern, the cell that was responsible for inhibiting the CS reaction was Treg lymphocyte with TCR $\alpha\beta$ ⁺ CD4⁺ CD25⁺ FoxP3⁺ phenotype [45]. Moreover, our study in Tc1-mediated CS showed that the EC induced tolerance was not mediated by immunosuppressive cytokines as we could not detect IL-10 and TGF- β , or Th2 type cytokines including IL-4 and IL-13 [45]. Our *in vivo* experiments using the cells after incubation in a trans-well permeable system that permits for exchange of soluble substances between tested compartments but precludes direct cell membrane contact showed that EC induced suppression may require direct cell to cell contact [45]. Additionally, our work showed that the EC induced suppression is mediated by CTLA-4, as blocking CTLA-4 abolished the EC induced suppression [45]. It must be stressed that EC application of protein antigen after inducing CS reaction was also effective in inhibiting inflammatory reaction in the skin, which pointed to the potential possibility of the use of this method in the therapy of CS reactions [45].

The effectiveness of the method of EC immunization with protein antigen in inhibiting CS reaction triggered by T lymphocytes, encouraged the researchers to verify whether the manoeuvre of EC immunization turns out to be effective in the treatment of autoimmune diseases. For this study, an animal model of murine

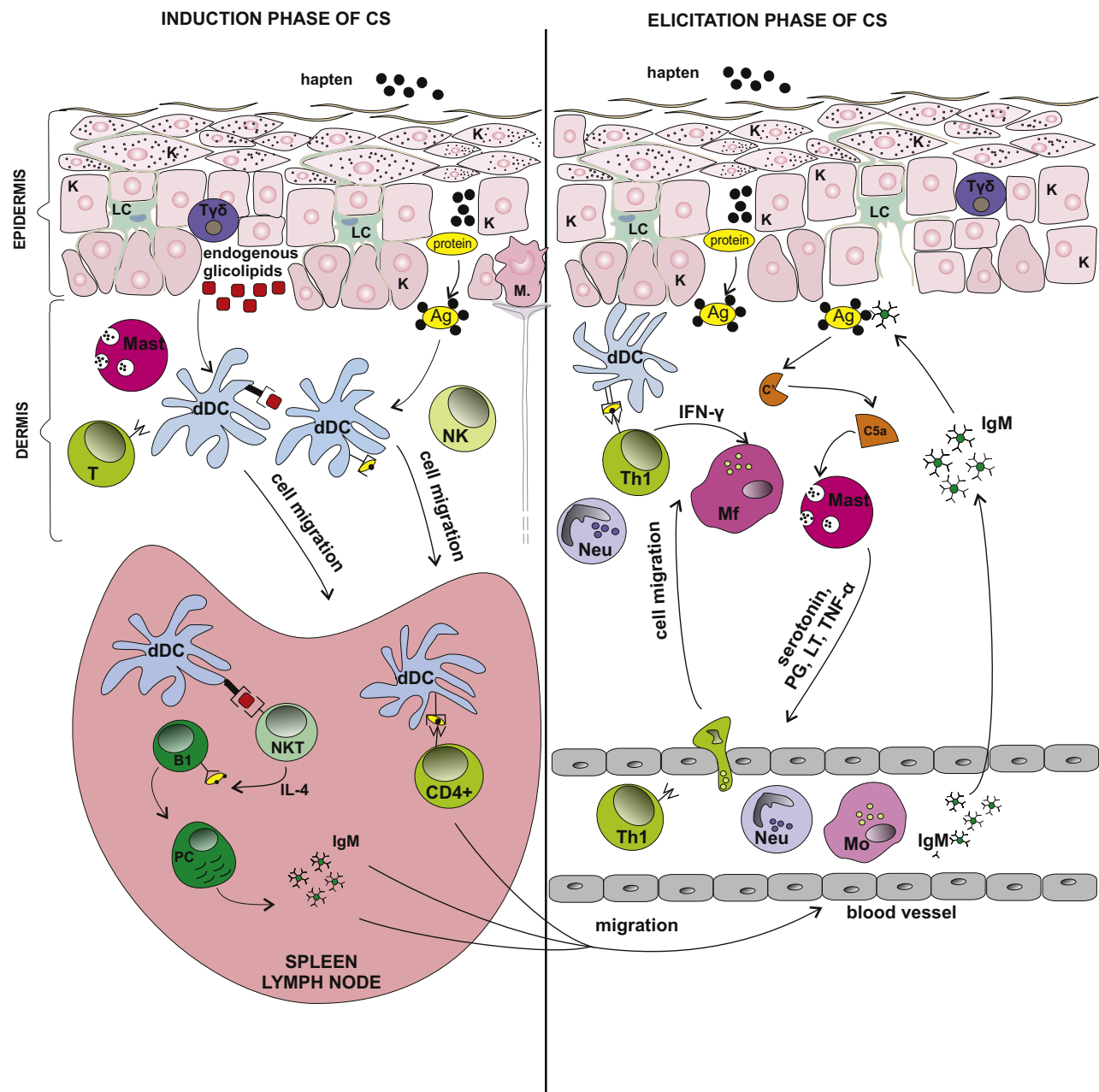


Fig. 1. Interaction between cells engaged in the contact sensitivity (CS). Hapten 2,4,6-trinitrophenyl chloride (TNP-Cl) binds to self-proteins which are processed to self-antigen peptides, which bind MHC molecules on dermal dendritic cells. Dermal dendritic cells migrate to lymph nodes to generate CS-effector Th1 cells by day 4 after immunization. Subsequently, the effector T cells circulate and can be recruited locally at the site of secondary hapten challenge at another skin site. T-cell recruitment is via the action of the CS-initiating B-1 cell-derived anti-hapten IgM antibodies, which bind challenge antigen to form antigen-IgM immune complexes. These immune complexes locally activate complement, generating C5a, which then triggers mast cells via C5a receptors (C5aR) to release TNF- α , prostaglandins, leukotrienes and serotonin. This increases endothelial permeability and expression of adhesion molecules allowing circulating CS-effector Th1 cells to adhere and pass into the tissues. Recruited Th1 cells then interact with peptide-antigen-MHC complexes on antigen presenting cells e.g. dermal dendritic cells and produce cytokines, which in turn recruit the inflammatory infiltrate of 24-h CS responses.

Ag – antigen; B1 – B1 lymphocyte; C – complement; dDC – dermal dendritic cells; K – keratinocyte; LC – Langerhans cell; LT – leukotriene; Mast – mast cell; Mf – macrophage; Mo – monocyte; Neu – neutrophil; NK – natural killer cells; NKT – NKT cells; PC – plasma cell; PG – prostaglandin; T – T cell; Th1 – T helper 1 cell; Tyδ – TCRγδ lymphocyte.

multiple sclerosis was used (EAE – experimental autoimmune encephalomyelitis). It was observed that EC application of myelin basic protein (MBP) before the induction of the disease significantly delays its development and alleviates its severity. The observed alleviation of the disease was mediated by TCR $\alpha\beta^+$ CD4 $^+$ CD8 $^+$ suppressor lymphocytes which inhibited the inflammatory reaction in the central nervous system via the released TGF- β [46–48]. From the clinical point of view, it is important to have the possibility to inhibit the ongoing disease. It is worth noting that the EC administration of MBP at the first signs

of EAE, significantly alleviated the course of the disease. This proves the effectiveness of this method in the treatment of ongoing disease [46]. The effectiveness of skin-induced suppression was also studied in collagen-induced arthritis (CIA) in mice, which is the animal model of rheumatoid arthritis in humans. These studies showed that EC application of type II collagen (COLL II) before inducing of CIA significantly inhibits the severity of the disease [49,50]. Moreover, the administration of COLL II at the first signs of CIA also results in disease suppression [49]. Similarly Strid et al. showed that EC immunization with COLL II effectively inhibits CIA

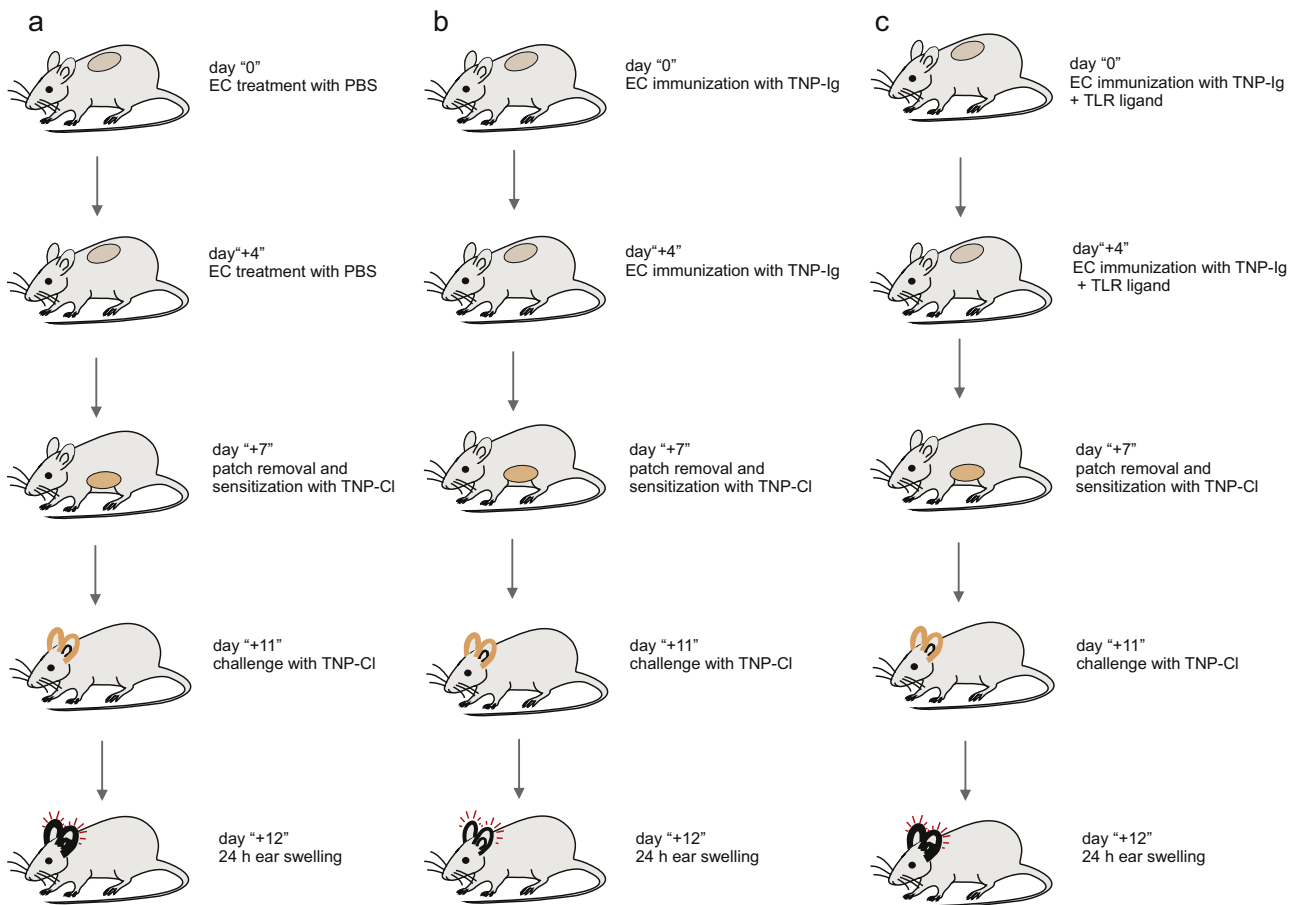


Fig. 2. Contact sensitivity and its regulation *via* epicutaneous (EC) immunization. (a) Induction of contact sensitivity. Mice were EC treated with PBS applied in a gauze patch on days 0 and 4. On day 7 patches were removed prior to TNP-Cl sensitization and test. (b) Suppression of CS *via* EC application of protein antigen. Mice were EC treated with 2,4,6-trinitrophenyl-conjugated mouse immunoglobulins (TNP-Ig) applied in a gauze patch on days 0 and 4. On day 7 patches were removed prior to TNP-Cl sensitization and test. (c) Reversal of skin-induced suppression of CS reaction *via* EC application of a protein antigen with TLR ligand. Mice were EC immunized with TNP-Ig and TLR ligand applied in a gauze patch on days 0 and 4. On day 7 patches were removed prior to TNP-Cl sensitization and test.

in DBA/1-TCR β Tg mice [51]. It is interesting that the method of skin-induced suppression turned out also to be effective in inhibiting inflammatory reaction in the intestines in the animal model of *Colitis ulcerosa* [52].

In summary, manoeuvre of EC immunization with hapten-conjugated protein antigen significantly suppresses T cell-mediated CS response in mice. Additionally, our findings suggest that CD4 Th1 and CD8 Tc1 cell-mediated immune responses are negatively regulated by different populations of EC induced regulatory cells that accomplish their regulatory function by distinct mechanisms [41,45]. Furthermore, EC immunization with protein antigen inhibits CD4-mediated inflammatory reaction in autoimmunity *via* similar mechanisms described in CD4-mediated CS reaction. Further work is required to investigate the effectiveness of transdermal immunization in humans.

Observations made in animal models of CS reactions and autoimmune disorders suggest that EC immunization with a selected antigen may become a versatile and potentially unlimited method for inducing tolerance and consequent control of unwanted immune responses. This assumption became a starting point for clinical trials in the group of patients suffering from multiple sclerosis (MS).

MS therapy *via* dermal application of antigen

The pathogenesis of MS is thought to result from antigen-specific autoimmunity where autoreactive immune cells, T and B

lymphocytes, directed at myelin-related peptides mediate the destruction of myelin within the central nervous system (CNS) [53–55]. Immunosuppressants, which block harmful immune response to self-antigens, are widely used in the therapy of MS and other autoimmune diseases originating from the improper reaction of the immune system. The disadvantage of currently used therapeutics is their lack of antigen-specificity. This treatment in addition to blocking unwanted immune response to self-antigens also inhibits the immunity to microbial antigens, which may lead to the development of serious infections. It is also worth noting that patients treated with such immunosuppressants are at higher risk for cancer [56–58]. In contrast, antigen-specific therapy aims to selectively target cells specific for a given antigen, thus disabling only a small part of the immune system responsible for an autoimmune response, which for MS includes antigens associated with the myelin sheath.

In many laboratories worldwide there are intensive studies aimed to develop an antigen-specific therapy which is free of side effects. Thus, the development of efficient, antigen-specific and at the same time free of side effects therapy for treatment of autoimmune diseases is an essential challenge in modern medicine. It is believed that, in MS, the immune system recognizes and responds to various antigens in CNS, such as myelin basic protein (MBP), myelin proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). A double-blind, placebo-controlled study in a group of 30 patients was carried out in order to verify whether, similarly to animal models, the EC application of

myelin antigens in patients with MS alleviates the disease symptoms. The patients were divided into three groups: the control group receiving placebo and the groups receiving the mix of myelin peptides (MBP 85-99, PLP 139-151 and MOG 35-55) in a dose of 1 mg or 10 mg. The mixture of peptides and placebo were applied onto the patient's arm in a form of a gauze dressing with a patch attached to it. In the first month the patients were treated with peptides or placebo once a week and in the next following months, they received the treatment once per month. The results of the MRI examination and clinical tests proved the effectiveness of the EC therapy with myelin peptides in relapsing-remitting form of MS [59]. This is of particular interest in light of the results with previous trials with antigen-specific therapy in MS, where despite induction of immune tolerance the clinical efficacy was limited.

Moreover, the method of EC induced tolerance does not manifest adverse effects. Additionally, immunological studies showed that EC application of myelin peptides (MBP 85-99, PLP 139-151 and MOG 35-55) leads to the development of immunological tolerance to these peptides. The procedure of dermal immunization with these peptides led to the activation of Langerhans cells in the skin, induction of a unique population of granular dendritic cells and IL-10 producing Tr1 regulatory cells in local lymph nodes. Moreover, transdermal immunization with myelin peptides reduced lymphocyte proliferative response to the peptides and suppressed IFN- γ synthesis [60].

Our work shows that EC exposure to an antigen results in the induction of regulatory cells. The ease by which regulatory cells can be generated through EC immunization may have important implications for designing new needle-free vaccines aimed at modulating immune responses to self-antigens involved in autoimmune diseases.

Immunopotential induced *via* transdermal immunization

The positive effects of experimental and clinical trials pointing to the possibility of eliciting the state of immunological tolerance through the skin directed the attention of researchers to further studies. The question which was asked was whether the application of poorly immunogenic antigen will make it possible to induce an immunological reaction. Contrary to the studied system where the pure protein antigen was used for immunization, the microorganisms, apart from immunogenic determinants are equipped also with so-called pathogen-associated molecular patterns (PAMPs) which are recognized by pattern recognition receptors of innate immunity which most likely may play the key role not only in the induction of immune response, but also in breaking the state of tolerance [61]. The research carried out in animal model of CS reaction have shown that EC application of protein antigen with TLR ligands led to breaking the immunological tolerances induced by the application of the same antigen alone (Fig. 2). The observed phenomenon of breaking the state of tolerance is mediated by TCR $\alpha\beta$ ⁺CD4⁺ lymphocytes and requires the presence of IFN- γ and IL-12 [62–64]. Moreover, it was shown that EC immunization with an antigen in the presence of ligands of PRR receptors other than TLR also allows for breaking the immune tolerance. It was observed that the stimulation of Dectin-1 receptor during EC immunization with protein antigen led to breaking the skin-induced suppression and this process is IL-17A dependent [65]. Breaking the state of tolerance was also observed with EC immunization with a protein antigen in the presence of ligand stimulating NOD2 receptor, with IFN- γ playing a significant role in the described process [66]. The above findings prove that EC immunization with protein antigen in the presence of a wide range of PRR ligands leads to breaking the state of immunological tolerance, which might suggest that this method might be effective in designing vaccines. To verify the effectiveness of EC vaccination,

the murine model of pneumonia was used. In these studies the animals were EC immunized with phosphorylcholine (PC), which is one of the main antigens present in the wall of *Streptococcus pneumoniae* together with unmethylated cytidine-guanosine dinucleotides (CpG), which is the ligand of TLR9 receptor prior to the intra-tracheal administration of live *S. pneumoniae*. The described procedure significantly alleviated the symptoms of pneumonia, which suggested that this method may be potentially useful for the preparation of vaccines [67]. It is believed that the core of protective role of EC immunization in this experimental model lies in the increased release of pro-inflammatory cytokines, such as IFN- γ , IL-17A and TNF- α [67]. The results of the animal studies, quoted here point to the fact that depending on the immunization method, i.e. either with the antigen alone or with the antigen in the presence of PRR ligands, the skin may be the place for the induction of immunological tolerance or immunopotential.

Dermal immunization in the therapy of allergic diseases

Allergic diseases are the result of immune response to innocuous environmental antigens and currently affect about 25% of population [68,69]. It is believed that Th2 lymphocytes releasing IL-4, IL-5 and IL-13 play a key role in the synthesis of IgE antibodies and the development of allergy [70]. Allergy treatment includes allergen avoidance and pharmacotherapy. Commonly used symptomatic therapy of allergic diseases includes corticosteroids, antihistamines and inhaled β 2-adrenoreceptor agonists, which efficiently ameliorate IgE-mediated symptoms. This type of treatment, however, does not treat disease per se, as pharmacotherapy benefits are not sustained after treatment is discontinued [69]. Thus, numerous efforts have been made to develop a treatment able to control allergy-associated immune responses in a specific manner.

Allergen immunotherapy (AIT) induces symptomatic relief not only during treatment but after its discontinuation as well [71]. The goal of AIT is to promote Th1-dependent response with the production of IFN- γ with a simultaneous inhibition of the response with the participation of Th2 lymphocytes, an increase of IL-10 and TGF- β production and the activation of Treg lymphocytes. This type of immunotherapy is also aimed at the synthesis of IgG antibodies specific for the allergen [72].

AIT includes subcutaneous allergen-specific immunotherapy (SCIT) and sublingual immunotherapy (SLIT). It is believed that subcutaneous or sublingual immunization with an antigen favours the production of Th1 cytokines such as IFN- γ over Th2 cytokines and activates IL-10 and TGF- β producing Treg cells [73]. Further efforts to find safer and more effective AIT have led scientists to study alternative routes of immunization. Both animal and clinical studies showed the effectiveness of – intralymphatic (ILIT) administration of allergens. It was shown that ILIT strengthened the efficacy of immunization causing production of high levels of allergen-specific IgG2a antibodies. Moreover, ILIT increased production of IL-2, IFN- γ , IL-4, and IL-10 when compared to SCIT, suggesting that ILIT does not polarize the response towards Th1, Th2 or Treg cells but generates overall stronger immune response [74,75].

The beginnings of the immunotherapy specific for the allergen go back to 1920s when Philips began the therapy of the patients with hay fever [76]. He observed that subcutaneous injections of the grass extracts significantly alleviated the symptoms of the disease. The currently used SCIT is based on Philips's observations and consists of several dozen subcutaneous injections of the allergen with an increasing dose within the period of a few years. The need for many injections and the risk of systemic side-effects limit broad patient acceptance of SCIT [77–79]. Another method of

immunotherapy, SLIT, consists of the sublingual administration of the allergen [80]. This method, similarly as SCIT requires the administration of many doses of allergen and may lead to local allergic reactions within the oral cavity. Currently allergen specific epicutaneous immunotherapy (EPIT) gains a lot of interest. This method of immunization is attractive, as it allows for a needleless administration of an antigen to the surface of the epidermis containing Langerhans cells. An additional advantage of this immunization method is the lack of vasculature in the place of antigen application, which prevents the systemic reaction to occur. The presence of Langerhans cells and the lack of vasculature in the place of antigen application make the epicutaneous immunization effective and safe. The attractiveness of EC immunotherapy resulted in the fact that some attempts of the treatment of allergic diseases with the EPIT method were taken up [81]. In order to increase the epidermis permeability for the antigen, the surface of the epidermis was delicately damaged by adhesive tape stripping. This procedure, not only removes the corneal layer, but also acts as a “physical adjuvant” activating keratinocytes to release IL-1, IL-6, IL-8, TNF- α and IFN- γ , thus contributing to the maturation of DC cells and their migration from the skin to the lymph nodes [82]. The clinical trials showed that the application of pollen extract in a dressing applied on the adequately prepared skin leads to the alleviation of the symptoms of hay fever in comparison with the placebo group. This therapy resulted solely in some skin lesions.

The suppression of allergic reaction via EC immunization with an antigen and TLR ligand in experimental conditions

Within the last ten years many clinical trials have been carried out in which CpG, being TLR9 ligand, were used as an adjuvant in the therapy of oncological diseases, allergies and as an ingredient of vaccines [83]. The therapeutic effects of CpG were observed in many animal studies, proving its efficiency with many routes of administration, including injection, inhalation, oral and even intravaginal route [83]. The numerous clinical studies showed that the administration of allergen together with CpG increased the efficiency of vaccinations. Some promising results were obtained also in a group of patients with prostate cancer and with melanoma [84,85]. It must be pointed out that the clinical trials in a group of patients with hay fever showed that subcutaneous administration of allergen with CpG alleviates clinical symptoms in comparison with the placebo group [86]. It must be noted that the CpG injections frequently lead to the occurrence of many local and systemic adverse effects, whose intensity depends on CpG dose. Local symptoms comprise pain, skin flushing, oedema and pruritus [83]. Systemic symptoms are more severe, including headache, myalgia, fever, nausea and vomiting [83].

In order to eliminate the adverse symptoms with the preservation of the therapeutic effect some attempts of EC administration of antigen together with CpG were made in order to treat the murine atopic dermatitis (AD) (Fig. 3). These studies showed that EC application of antigen with CpG inhibits the production of antigen specific IgE antibodies with the simultaneous increase of the synthesis of IgG2a antibodies [87]. It is worth pointing out that in mice EC treated with allergen together with CpG, a significantly lower activity of eosinophil peroxidase (EPO) in the skin was observed. It must be stressed that EC administration of antigen with CpG during an ongoing allergic reaction led to a significant alleviation of the course of the disease [87]. The observed changes in the synthesis of antibodies and EPO activity were accompanied with inhibited production of Th2 type cytokines such as IL-4, IL-5, IL-10 and IL-13 [87]. The inhibition of allergic reaction was mediated by T lymphocytes releasing significant quantities of IFN- γ and IL-17A [87]. The positive results of the studies evoked a great interest among clinicians.

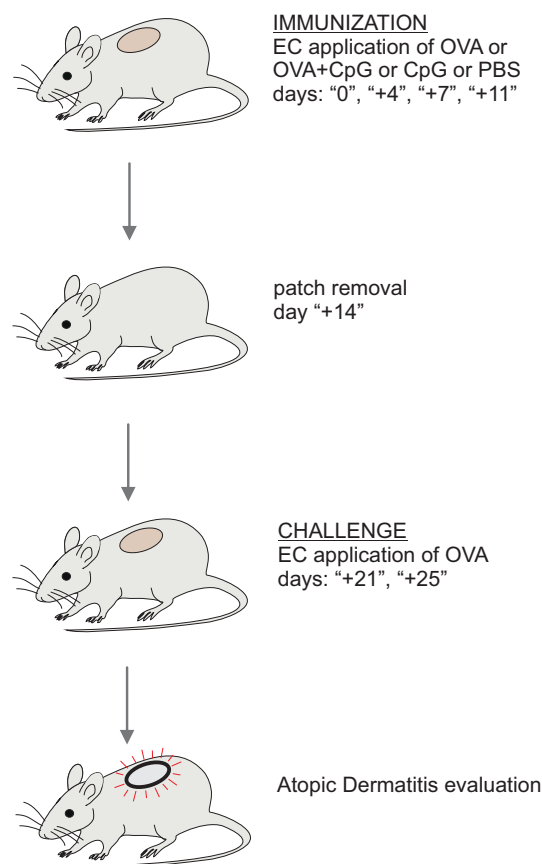


Fig. 3. Study design to evaluate atopic dermatitis. Mice were EC immunized for 2 weeks with OVA alone or OVA plus CpG or CpG alone or PBS. After a 1-week interval, all animals were challenged with OVA for 1 week before bleeding and skin sample collection.

Coda

The skin constantly exposed to many antigens plays a crucial role in body protection from different pathogens present in the external world. While development of the immune response to pathogens is of vital importance to the macroorganism, response to innocuous antigens is not required and even more, sometimes it can be harmful leading to allergy or autoimmunity. Thus, discrimination between pathogen-associated antigens and innocuous antigens, in order to induce either an effector immune response or tolerance, is of utmost importance in the skin. This supports the possibility of the using the skin as a focus of strategies to induce immunopotentiality or tolerance of selected antigens.

Our work shows that EC exposure to an antigen, results in the induction of regulatory cells that inhibit T cell-mediated immune responses. Additionally, our work shows that EC immunization with an antigen in the presence of PAMPs reverses skin-induced

Table 1
Experimental and clinical application of trans-dermal immunotherapy.

Disease	Tested	
	Mouse	Human
CD4 ⁺ -mediated CS	Yes [41,42]	No
CD8 ⁺ -mediated CS	Yes [44,45]	No
Multiple sclerosis (EAE)	Yes [46–48]	Yes [59,60]
Arthritis (CIA)	Yes [49–51]	No
Colitis ulcerosa	Yes [52]	No
Pneumococcal pneumonia	Yes [67]	No
Atopic dermatitis	Yes [87]	No

tolerance suggesting that skin may be the place for the induction of immunopotentiality. Finally, our work shows that EC immunization with allergen in the presence of TLR9 ligand inhibits allergic reaction. The ease by which regulatory cells can be generated through EC immunization may have important implications for designing new needle-free vaccines aimed at modulating immune responses (Table 1).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by grant from the National Science Center 2012/05/B/NZ6/00997 to MS and 2011/03/B/NZ6/00821 to MM-S and grant from Jagiellonian University Medical College K/ZDS/004650 to MS and K/ZDS/004665 to MM-S.

References

- Heath WR, Carbone FR. The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells. *Nat Immunol* 2013;14:978–85.
- Nestle FO, Di Meglio P, Qin JZ, Nickoloff BJ. Skin immune sentinels in health and disease. *Nat Rev Immunol* 2009;9:679–91.
- Igyártó BZ, Haley K, Ortner D, Bobr A, Gerami-Nejad M, Edelson BT, et al. Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses. *Immunity* 2011;35:260–72.
- Haley K, Igyártó BZ, Ortner D, Bobr A, Kashem S, Schenten D, et al. Langerhans cells require MyD88-dependent signals for *Candida albicans* response but not for contact hypersensitivity or migration. *J Immunol* 2012;188:4334–9.
- Igyártó BZ, Kaplan DH. Antigen presentation by Langerhans cells. *Curr Opin Immunol* 2013;25:115–9.
- Toulon A, Breton L, Taylor KR, Tenenhaus M, Bhavsar D, Lanigan C, et al. A role for human skin-resident T cells in wound healing. *J Exp Med* 2009;206:743–50.
- Matsue H, Cruz Jr PD, Bergstresser PR, Takashima A. Profiles of cytokine mRNA expressed by dendritic epidermal T cells in mice. *J Invest Dermatol* 1993;101:537–42.
- Majewska M, Szczepanik M. The role of Toll-like receptors (TLR) in innate and adaptive immune responses and their function in immune response regulation. *Post Hig Med Dosw* 2006;60:52–63.
- Batista MD, Ho EL, Kuebler PJ, Milush JM, Lanier LL, Kallas EG, et al. Skewed distribution of natural killer cells in psoriasis skin lesions. *Exp Dermatol* 2013;22:64–6.
- Clark RA, Chong B, Mirchandani N, Brinster NK, Yamanaka K, Dowgiert RK, et al. The vast majority of CLA⁺ T cells are resident in normal skin. *J Immunol* 2006;176:4431–9.
- Seneschal J, Clark RA, Gehad A, Baecher-Allan CM, Kupper TS. Human epidermal Langerhans cells maintain immune homeostasis in skin by activating skin resident regulatory T cells. *Immunity* 2012;36:873–84.
- Leavy O. Human skin residency. *Nat Rev Immunol* 2014;14:136–7.
- Pastore MN, Kalia YN, Horstmann M, Roberts MS. Transdermal patches: history, development and pharmacology. *Br J Pharmacol* 2015;172:2179–209.
- Bender GA, Thom RA. Great moments in medicine and pharmacy: a history of medicine and pharmacy in pictures. Detroit, MI: Northwood Institute Press; 1966.
- Cole HN, Schreiber N, Sollmann T. Mercurial ointments in the treatment of syphilis: their absorption as measured by studies on excretion. *Arch Derm Syphilol* 1930;21:372–93.
- Zondek B. The excretion of halogenated phenols and their use in the treatment of urogenital infections. *J Urol* 1942;48:747–58.
- Beckett AH, Gorrod JW, Taylor DC. Comparison of oral and percutaneous routes in man for the systemic administration of 'ephedrine'. *J Pharm Pharmacol* 1972;24(Suppl.):65–70.
- Riegelman S. Pharmacokinetics. Pharmacokinetic factors affecting epidermal penetration and percutaneous adsorption. *Clin Pharmacol Ther* 1974;16:873–83.
- Laufer LR, DeFazio JL, Lu JK, Meldrum DR, Eggena P, Sambhi MP, et al. Estrogen replacement therapy by transdermal estradiol administration. *Am J Obstet Gynecol* 1983;146:533–40.
- Sebel PS, Barrett CW, Kirk CJ, Heykants J. Transdermal absorption of fentanyl and sufentanil in man. *Eur J Clin Pharmacol* 1987;32:529–31.
- Graybiel A, Knepton J, Shaw J. Prevention of experimental motion sickness by scopalamine absorbed through the skin. *Aviat Space Environ Med* 1976;47:1096–100.
- Buchkremer G, Bents H, Horstmann M, Opitz K, Tölle R. Combination of behavioral smoking cessation with transdermal nicotine substitution. *Addict Behav* 1989;14:229–38.
- Bos JD, Meinardi MM. The 500 Dalton rule for the skin penetration of chemical compounds and drugs. *Exp Dermatol* 2000;9:165–9.
- He R, Oyoshi MK, Jin H, Geha RS. Epicutaneous antigen exposure induces a Th17 response that drives airway inflammation after inhalation challenge. *Proc Natl Acad Sci U S A* 2007;104:15817–22.
- Jin H, Oyoshi MK, Le Y, Bianchi T, Koduru S, Mathias CB, et al. IL-21R is essential for epicutaneous sensitization and allergic skin inflammation in humans and mice. *J Clin Invest* 2009;119:47–60.
- Strid J, Hourihane J, Kimber I, Callard R, Strobel S. Epicutaneous exposure to peanut protein prevents oral tolerance and enhances allergic sensitization. *Clin Exp Allergy* 2005;35:757–66.
- Betz G, Aeppli A, Menshutina N, Leuenberger H. In vivo comparison of various liposome formulations for cosmetic application. *Int J Pharm* 2005;296:44–54.
- El Maghraby GM, Barry BW, Williams AC. Liposomes and skin: from drug delivery to model membranes. *Eur J Pharm Sci* 2008;34:203–22.
- Mishra D, Dubey V, Asthana A, Saraf DK, Jain NK. Elastic liposomes mediated transcutaneous immunization against Hepatitis B. *Vaccine* 2006;24:4847–55.
- Kaushik S, Hord AH, Denson DD, McAllister DV, Smitra S, Allen MG, et al. Lack of pain associated with microfabricated microneedles. *Anesth Analg* 2001;92:502–4.
- Prausnitz MR. Microneedles for transdermal drug delivery. *Adv Drug Deliv Rev* 2004;56:581–7.
- Donnelly RF, Majithiya R, Singh TR, Morrow DI, Garland MJ, Demir YK, et al. Design, optimization and characterisation of polymeric microneedle arrays prepared by a novel laser-based micromoulding technique. *Pharm Res* 2001;28:41–57.
- Tan G, Xu P, Lawson LB, He J, Freytag LC, Clements JD, et al. Hydration effects on skin microstructure as probed by high-resolution cryo-scanning electron microscopy and mechanistic implications to enhanced transcutaneous delivery of biomacromolecules. *J Pharm Sci* 2010;99:730–40.
- Mondoulet L, Dioszeghy V, Ligouis M, Dhelft V, Dupont C, Benhamou PH. Epicutaneous immunotherapy on intact skin using a new delivery system in a murine model of allergy. *Clin Exp Allergy* 2010;40:659–67.
- Cevikbas F, Steinhoff M. IL-33: a novel danger signal system in atopic dermatitis. *J Invest Dermatol* 2012;132:1326–9.
- Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Honey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002;3:673–80.
- Swamy M, Jamora C, Havran W, Hayday A. Epithelial decision makers: in search of the 'epimuneome'. *Nat Immunol* 2010;11:656–65.
- Wang LF, Lin JY, Hsieh KH, Lin RH. Epicutaneous exposure of protein antigen induces a predominant Th2-like response with high IgE production in mice. *J Immunol* 1996;156:4077–82.
- Herrick CA, MacLeod H, Glusac E, Tigelaar RE, Bottomly K. Th2 responses induced by epicutaneous or inhalational protein exposure are differentially dependent on IL-4. *J Clin Invest* 2000;105:765–75.
- Askenase PW, Szczepanik M, Itakura A, Kiener C, Campos RA. Extravascular T-cell recruitment requires initiation begun by Vα14 + NKT cells and B-1 B cells. *Trends Immunol* 2004;25:441–9.
- Szczepanik M, Bryniarski K, Tutaj M, Ptak M, Skrzeczynska J, Askenase PW, et al. Epicutaneous immunization induces αβ T-cell receptor CD4 CD8 double-positive non-specific suppressor T cells that inhibit contact sensitivity via transforming growth factor-β. *Immunology* 2005;115:42–54.
- Ptak W, Szczepanik M, Bryniarski K, Tutaj M, Ptak M. Epicutaneous application of protein antigens incorporated into cosmetic cream induces antigen-non-specific unresponsiveness in mice and affects the cell-mediated immune response. *Int Arch Allergy Immunol* 2002;128:8–14.
- Majewska-Szczepanik M, Strzēpa A, Dorożyńska I, Motyl S, Banach T, Szczepanik M. Epicutaneous immunization with hapten – conjugated protein antigen alleviates contact sensitivity mediated by three different types of effector cells. *Pharmacol Rep* 2012;64:919–26.
- Zemelka-Wiącek M, Majewska-Szczepanik M, Ptak W, Szczepanik M. Epicutaneous immunization with protein antigen induces antigen – non-specific suppression of CD8T cell mediated contact sensitivity. *Pharmacol Rep* 2012;64:1485–96.
- Majewska-Szczepanik M, Zemelka-Wiącek M, Ptak W, Wen L, Szczepanik M. Epicutaneous immunization with DNP-BSA induces CD4⁺ CD25⁺ Treg cells that inhibit Tc1-mediated CS. *Immunol Cell Biol* 2012;90:784–95.
- Szczepanik M, Tutaj M, Bryniarski K, Dittel BN. Epicutaneously induced TGF-β-dependent tolerance inhibits experimental autoimmune encephalomyelitis. *J Neuroimmunol* 2005;164:105–14.
- Tutaj M, Szczepanik M. Epicutaneous (EC) immunization with myelin basic protein (MBP) induces TCRαβ⁺ CD4⁺ CD8⁺ double positive suppressor cells that protect from experimental autoimmune encephalomyelitis (EAE). *J Autoimmun* 2007;28:208–15.
- Majewska M, Zajac K, Srebro Z, Sura P, Ksiazek L, Zemelka M, et al. Epicutaneous immunization with myelin basic protein protects from the experimental autoimmune encephalomyelitis. *Pharmacol Rep* 2007;59:74–9.
- Marcinińska K, Majewska-Szczepanik M, Maresz KZ, Szczepanik M. Epicutaneous immunization with collagen induces TCRαβ suppressor T cells that inhibit collagen-induced arthritis. *Int Arch Allergy Immunol* 2015;166:121–34.
- Marcinińska K, Majewska-Szczepanik M, Lazar A, Kowalczyk P, Biała D, Woźniak D, et al. Epicutaneous (EC) immunization with type II collagen (COLL II) induces CD4⁺(+)CD8⁺(+) T suppressor cells that protect from collagen-induced arthritis (CIA). *Pharmacol Rep* 2016;68:483–9.

- [51] Strid J, Tan LA, Strobel S, Londei M, Callard R. Epicutaneous immunization with type II collagen inhibits both onset and progression of chronic collagen-induced arthritis. *PLoS ONE* 2007;2:e387.
- [52] Majewska-Szczepanik M, Góralska M, Marcińska K, Zemelka-Wiącek M, Strzepa A, Dorożyńska I, et al. Epicutaneous immunization with protein antigen TNP-Ig alleviates TNBS-induced colitis in mice. *Pharmacol Rep* 2012;64:1497–504.
- [53] Codarri L, Fontana A, Becher B. Cytokine networks in multiple sclerosis: lost in translation. *Curr Opin Neurol* 2010;23:205–11.
- [54] Franciotta D, Salvetti M, Lolli F, Serafini B, Aloisi F. B cells and multiple sclerosis. *Lancet Neurol* 2008;7:852–8.
- [55] Hafler DA, Slavik JM, Anderson DE, O'Connor KC, De Jager P, Baecher-Allan C. Multiple sclerosis. *Immunol Rev* 2005;204:208–31.
- [56] Brinkmann V, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, et al. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat Rev Drug Discov* 2010;9(11):883–97.
- [57] Giacomini PS, Darlington PJ, Bar-Or A. Emerging multiple sclerosis disease-modifying therapies. *Curr Opin Neurol* 2009;22:226–32.
- [58] Coles AJ, Wing MG, Molyneux P, Paolillo A, Davie CM, Hale G, et al. Monoclonal antibody treatment exposes three mechanisms underlying the clinical course of multiple sclerosis. *Ann Neurol* 1999;46:296–304.
- [59] Walczak A, Siger M, Ciach A, Szczepanik M, Selmaj K. Transdermal application of myelin peptides in multiple sclerosis treatment. *JAMA Neurol* 2013;70:1105–9.
- [60] Juryńczyk M, Walczak A, Jurewicz A, Jesionek-Kupnicka D, Szczepanik M, Selmaj K. Immune regulation of multiple sclerosis by transdermally applied myelin peptides. *Ann Neurol* 2010;68:593–601.
- [61] Szczepanik M. Skin-induced tolerance and its reversal by Toll-like receptor ligands. *Arch Immunol Ther Exp (Warsz)* 2007;55:161–72.
- [62] Ptak W, Bryniarski K, Ptak M, Majewska M, Gamian A, Lobo FM, et al. Toll-like receptor ligands reverse suppression of contact hypersensitivity reactions induced by epicutaneous immunization with protein antigen. *Int Arch Allergy Immunol* 2006;139:188–200.
- [63] Ptak W, Majewska M, Bryniarski K, Ptak M, Lobo FM, Zajac K, et al. 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 sensitivity. *J Immunol* 2009;182:837–50.
- [64] Majewska M, Bryniarski K, Ptak M, Zajac K, Zemelka M, Lobo FM, et al. Role of TLR ligands in epicutaneously induced contrasuppression. *Pharmacol Rep* 2009;61:539–49.
- [65] Majewska-Szczepanik M, Strzepa A, Marcińska K, Wen L, Szczepanik M. Epicutaneous immunization with TNP-Ig and zymosan induces TCRαβ+ CD4+ contrasuppressor cells that reverse skin-induced suppression via IL-17A. *Int Arch Allergy Immunol* 2014;164:122–36.
- [66] Majewska-Szczepanik M, Dorożyńska I, Strzepa A, Szczepanik M. Epicutaneous immunization with protein antigen TNP-Ig and NOD2 ligand muramyl dipeptide (MDP) reverses skin-induced suppression of contact hypersensitivity. *Pharmacol Rep* 2014;66:137–42.
- [67] Majewska-Szczepanik M, Yamamoto N, Askenase PW, Szczepanik M. Epicutaneous immunization with phosphorylcholine conjugated to bovine serum albumin (PC-BSA) and TLR9 ligand CpG alleviates pneumococcal pneumonia in mice. *Pharmacol Rep* 2014;66:570–5.
- [68] Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature* 2008;454:445–54.
- [69] Holgate ST, Polosa R. Treatment strategies for allergy and asthma. *Nat Rev Immunol* 2008;8:218–30.
- [70] van Panhuys N, Le Gros G, McConnell MJ. Epigenetic regulation of Th2 cytokine expression in atopic diseases. *Tissue Antigens* 2008;72:91–7.
- [71] Marogna M, Spadolini I, Massolo A, Canonica GW, Passalacqua G. Long-lasting effects of sublingual immunotherapy according to its duration: a 15-year prospective study. *J Allergy Clin Immunol* 2010;126:969–75.
- [72] Senti G, von Moos S, Kündig TM. Epicutaneous allergen administration: is this the future of allergen-specific immunotherapy. *Allergy* 2011;66:798–809.
- [73] Broide DH. Immunomodulation of allergic disease. *Annu Rev Med* 2009;60:279–91.
- [74] Cox L, Compalati E, Kundig T, Larche M. New directions in immunotherapy. *Curr Allergy Asthma Rep* 2013;13:178–95.
- [75] Johansen P, von Moos S, Mohanan D, Kündig TM, Senti G. New routes for allergen immunotherapy. *Hum Vaccin Immunother* 2012;8:1525–33.
- [76] Phillips EW. Relief of hay-fever by intradermal injections of pollen extract. *JAMA* 1926;86:182–4.
- [77] Frew AJ. Allergen immunotherapy. *J Allergy Clin Immunol* 2010;125(2 Suppl. 2):S306–13.
- [78] Cox L, Calderon MA. Subcutaneous specific immunotherapy for seasonal allergic rhinitis: a review of treatment practices in the US and Europe. *Curr Med Res Opin* 2010;26:2723–33.
- [79] Cox LS, Larenas Linnemann D, Nolte H, Weldon D, Finegold I, Nelson HS. Sublingual immunotherapy: a comprehensive review. *J Allergy Clin Immunol* 2006;117:1021–35.
- [80] Pajno GB. Sublingual immunotherapy: the optimism and the issues. *J Allergy Clin Immunol* 2007;119:796–801.
- [81] Senti G, von Moos S, Tay F, Graf N, Sonderegger T, Johansen P, et al. Epicutaneous allergen-specific immunotherapy ameliorates grass pollen-induced rhinoconjunctivitis: a double-blind, placebo-controlled dose escalating study. *J Allergy Clin Immunol* 2012;129:128–35.
- [82] Nickoloff BJ, Naidu Y. Perturbation of epidermal barrier function correlates with initiation of cytokine cascade in human skin. *J Am Acad Dermatol* 1994;30:535–46.
- [83] Krieg AM. CpG still rocks! Update on an accidental drug. *Nucleic Acid Ther* 2012;22:77–89.
- [84] Fourcade J, Kudela P, Andrade Filho PA, Janjic B, Land SR, Sander C, et al. Immunization with analog peptide in combination with CpG and montanide expands tumor antigen-specific CD8+ T cells in melanoma patients. *J Immunother* 2008;31:781–91.
- [85] Karbach J, Neumann A, Atmaca A, Wahle C, Brand K, von Boehmer L, et al. Efficient in vivo priming by vaccination with recombinant NY-ESO-1 protein and CpG in antigen naïve prostate cancer patients. *Clin Cancer Res* 2011;17:861–70.
- [86] Creticos PS, Schroeder JT, Hamilton RG, Balcer-Whaley SL, Khattignavong AP, Lindblad R, et al. Immunotherapy with a ragweed-toll-like receptor 9 agonist vaccine for allergic rhinitis. *N Engl J Med* 2006;355:1445–55.
- [87] Majewska-Szczepanik M, Askenase PW, Lobo FM, Marcińska K, Wen L, Szczepanik M. Epicutaneous immunization with ovalbumin and CpG induces TH1/TH17 cytokines which regulate IgE and IgG2a production. *J Allergy Clin Immunol* 2016. <http://dx.doi.org/10.1016/j.jaci.2015.11.018>.