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Review article

Skin-induced tolerance as a new needle free therapeutic strategy

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ABSTRACT

This article summarizes current knowledge about a new subject called "skin induced tolerance". Suppression is induced *via* epicutaneous (EC) immunization with a protein antigen and is described in Th1, Tc1 and NK mediated contact hypersensitivity (CHS) reactions. The subject of skin-induced suppression is also described in the regulation of experimental models of autoimmune diseases like experimental autoimmune encephalomyelitis (EAE), collagen induced arthritis (CIA) and inflammatory bowel disease (IBD) and finally in an animal model of graft rejection. The potential clinical use of this approach to regulate human diseases is also discussed.

Contents

Introduction

Both the skin and mucosa are constantly exposed to numerous antigens and play a crucial role in protecting the body from various pathogens present in the external world. While development of immune responses to pathogens is of vital importance to the macroorganism, response to innocuous antigens is, at best, not helpful and often leads to harmful allergy. 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 [31,50]. Mucosal tolerance seems to play an important role in avoiding the development of immune responses to non-harmful antigens.

It was already shown that certain types of regulatory T cells are preferentially induced in mucosa to maintain tolerance [47]. Moreover, it is believed that a special mucosal milieu may create tolerogenic dendritic cells that induce different populations of regulatory cells.

Although the skin is considered an organ where immune responses are easily induced [3], little attention has been given to skin induced tolerance [37]. Because skin and mucosa play similar function in our bodies (*i.e.* as a barrier to external pathogens) it is

Abbreviations: CD, Crohn's disease; CHS, contact hypersensitivity; CIA, collagen-induced arthritis; CNS, central nervous system; COLL II, bovine type II collagen; DNFB, dinitrofluorobenzene; DNP, 2,4-dinitrophenol; DTH, delayed type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; EC, epicutaneous; HAI, histological activity index; IBD, inflammatory bowel disease; IFN-γ, interferon gamma; IL, interleukin; KLH, keyhole limpet hemocyanin; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MPO, myeloperoxidase; MRI, magnetic resonance imaging; OVA, ovalbumin; OX, oxazolone; PLP, myelin proteolipid protein; RA, rheumatoid arthritis; TGF-β, transforming growth factor beta; Th1, T helper 1; Th2, T helper 2; Th17, T helper 17; TNBS, trinitrobenzene sulfonic acid; TNP-Cl, 2,4,6-trinitrobenzene sulfonic acid; TNP-Ig, TNP conjugated mouse immunoglobulins; Treg, T regulatory cell; Ts, T suppressor cell; UC, ulcerative colitis.

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possible that epicutaneous (EC) application of antigen, apart from inducing a strong immune response, may also induce suppression in the periphery. It was shown previously that EC immunization with the protein antigen ovalbumin (OVA) resulted in allergic dermatitis accompanied with the induction of IL-4 and IL-13 synthesis [43,45]. Further, it was found that EC immunization with OVA induced a Th2 mediated model of asthma in mice [15]. Finally, it was demonstrated that local administration of IFN-γ during the sensitization phase of protein antigen immunization suppresses development of Th2-mediated atopic dermatitis in mice [46].

These complex studies on EC immunization suggested to us that similar to mucosal immunization, application of protein antigens on the skin may also induce T lymphocytes producing anti-inflammatory cytokines that could inhibit Th1/Th17-mediated immune responses causing their suppression.

Epicutaneous immunization with hapten-conjugated protein antigen inhibits contact hypersensitivity in mice

Contact hypersensitivity (CHS) is a classical form of *in vivo* T cell-mediated immunity induced by topical skin immunization with haptens. CHS responses can be mediated by either $CD4^+$ Th1, MHC class II-restricted lymphocytes locally producing IFN-γ to recruit a typical inflammatory infiltrate [18], 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 [26]. Finally, the discovery by von Adrian's group proving that NK cells may act as effector cells in CHS in mice was a breakthrough in research on the mechanisms involved in CHS response [29]. Further our studies demonstrated that the NK cells able to adoptively transfer CHS response belong to CXCR6-expressing subset [32]. In contrast to Th1- and Tc1-mediated CS, NK cell-mediated CHS occurs independently of B-1 or NKT lymphocytes but is IFN-γ, IL-12 and IFN-α dependent [23].

CHS like all other immune responses are under strict control of regulatory mechanisms. It was shown previously that this type of immune response can be negatively regulated by T suppressor (Ts)/T regulatory (Treg) cells induced *via* intravenous injection [4] or oral deposition of high dose of antigen [10]. Our research showed for the first time that apart from intravenous and oral antigen administration also EC deposition of protein antigen inhibits CHS.

Work on skin-induced suppression was inspired by previous findings showing that EC immunization with protein antigen induces production of IL-4 and IL-13 that can potentially inhibit Th1-mediated immune response.

To test if EC immunization with protein antigen could inhibit cell-mediated immune responses we have developed the procedure presented below. The efficacy of EC induced suppression was tested in three different models of CHS.

First, we tested skin-induced suppression in Th1-mediated CHS. Mice were exposed to hapten-conjugated mouse immunoglobulins, TNP-Ig on days "0" and "+4" spread over the gauze patch. Then, on day "+7" patches were removed and animals were sensitized with TNP-Cl and tested for CHS four days later. This simple experiment proved that EC immunization with protein antigen results in strong decrease of Th1-mediated CHS [38]. 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 [34]. Similarly to patch method EC immunization with an antigen emulsified in cream caused strong inhibition of CHS.

Further experiments in TNP-Cl model showed that skin-induced suppression is dose dependent and optimal dose of antigen that induces this phenomenon is between 30 and 100 μg/animal [38]. Additionally we found that EC induced suppression lasts for about four weeks. Using both "transfer out" and "transfer in" protocols we showed that skin-induced suppression is transferable and that EC induced suppressor cells were very potent as they were able to significantly inhibit CHS effector cells when transferred into naïve recipients at a ratio of 1–10 [38]. Then, negative selection experiments with proper mAb and complement, cell sorting experiments and use of NKT cell-deficient $J\alpha18^{-/-}$ mice revealed that EC induced suppressor cells belong to the rare population of $TCR\alpha\beta^+$ $CD4^+$ $CD8^+$ cells. $CD4^+$ $CD8^+$ 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 [38].

Experiments with various non-cross reacting antigens such as TNP, oxazolone (OX), ovalbumin (OVA), keyhole limpet hemocyanin (KLH), elastin, collagen and keratin showed that the final mediation of suppressor T cell activity is antigen non-specific [34,38].

The association of antigen non-specific suppression with inhibitory cytokines led us to test if a specific cytokine milieu is required for induction of Ts cells *via* EC immunization, as observed in some other systems [33]. To determine whether a specific cytokine milieu is required to induce suppressor cells *via* EC immunization, mice were treated with TNP-Ig alone or with TNP-Ig plus anti-cytokine antibodies: anti-IL-4, anti-IL-10 or anti-TGF-β or control antibody on the skin prior to induction of CHS. All of the tested anti-cytokine mAbs significantly reduced suppression induced *via* EC immunization with TNP-Ig, suggesting that anti-inflammatory cytokines are indeed involved in the induction of suppressor cells [38]. Finally, *in vitro* studies with cytokine neutralizing antibodies showed that TGF-β but not IL-4 or IL-10 plays a crucial role in effector phase of skin-induced suppression [38].

To test whether EC immunization with an antigen can also inhibit CD8 dependent immune responses, we employed Tc1-mediated CHS to dinitrofluorobenzene (DNFB). This study showed that similarly to skin-induced suppression of Th1-dependent CHS, EC induced inhibition of Tc1-dependent CHS is dose dependent and optimal dose of antigen that gives suppression is between 1 and 100 μg/animal [52]. Epicutaneously induced suppression lasted at least three weeks since patch removal. Adoptive cell transfer experiments showed that skin-induced suppression can be transferred with lymphoid cells isolated from previously patched donors [25,52]. Negative selection experiments with proper mAb and complement, flow cytometry, MACS cell sorting experiments and use of $TCR\delta^{-/-}$, $\beta_2m^{-/-}$ and $CD1d^{-/-}$ mice showed that EC induced suppressor cells belong to the population $TCR\alpha\beta^+$ $CD4^+$ $CD25^+$ $FoxP3^+$ T regulatory (Treg) cells [25]. Additionally, our adoptive transfer experiments showed that $CD4^+$ $CD25^+$ Treg cells isolated from mice EC immunized with DNP-BSA could potently suppress CHS response by effector cells at a ratio of 1–35 (2×10^6 Treg cells *vs.* 7×10^7 effector cells) [25]. This was similar to our previous findings that very low numbers of skin-induced $CD4^+$ $CD8^+$ suppressor cells, such as 2×10^3 per mouse, were able to inhibit the effector function of 7×10^7 4-day TNP-Cl immune cells *in vivo* [38]. Further, our *in vitro* experiments showed that EC

induced Treg cells inhibited proliferation of CHS effector cells and suppressed TNF-α, IL-12 and IFN-γ production [25].

Our previous work showed that TGF-β was required for the suppressive function of EC induced suppressor cells in Th1-dependent CHS [38]. On the other hand our study in Tc1-mediated CHS showed that the EC induced tolerance was not mediated by immune regulatory cytokines as we could not detect IL-10 and TGF-β, or Th2 type cytokines including IL-4, IL-13 and IL-17E [25]. Our *in vivo* experiments using the cells after incubation in a transwell 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 [25]. Additionally, our work showed that the EC induced suppression is mediated by CTLA-4, as blocking CTLA-4 abolished the EC induced suppression [25]. Finally, experiment employing FoxP3-IRES-m-RFP (FIR) reporter mice showed that ear challenge has no influence on Treg cells distribution in lymphoid tissue. These data suggest that EC induced regulatory cells are distributed equally in lymph organs where they regulate T effector cell function. Experiments with four non-cross reacting antigens such as DNP, OX, OVA and myelin basic protein (MBP) employing three different models of CHS (active immunization, "transfer in" and "transfer out") showed that EC induced suppression is antigen non-specific [52].

We further investigated the timing of Treg cell action using adoptive transfer experiments. Our results suggested that Treg cells induced by EC immunization with DNP-BSA operated at both the afferent and efferent phases of Tc1-mediated CHS. This supports the recent finding showing that Treg cells suppressed both the sensitization and effector phases of CHS reaction [35]. However, we could not exclude the possibility that Treg cells transferred at the time of immunization may not affect induction of Tc1 effector cells, but persist in the recipients and suppress Tc1 cells only in the efferent phase, at the time of challenge. In other words, transferred Treg cells may play a more protective role. Although from clinical point of view treatment of ongoing inflammatory response is more important and realistic than prevention. Our previous work in animal model of multiple sclerosis showed that EC application of myelin basic protein at the time of disease onset could still ameliorate disease progression [39]. To test if it is possible to control ongoing inflammatory reaction in Tc1-mediated CHS, we adoptively transferred regulatory cells after CHS development and our results demonstrated that Treg cells could significantly inhibit inflammatory reaction. Finally, we showed that EC immunization with DNP-BSA one day after DNFB sensitization or even one day after 1st ear challenge that precede rechallenge could still efficiently suppress CHS reaction [25].

At present its unknown if NK cells are involved in CHS responses in humans. Moreover, there is no information about negative regulation of NK mediated CHS. Thus, in our study we decided to determine if EC immunization with a protein antigen could inhibit NK dependent CHS. Our recent work proved that indeed adoptive transfer of DNFB induced liver NK cells into previously DNP-BSA tolerized mice significantly inhibits CHS reaction [24].

In summary, maneuver of EC immunization with hapten-conjugated protein antigen significantly suppresses CHS response in mice mediated by three different types of effector cells such as Th1 and Tc1 lymphocytes and NK cells as well. 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 [25,38]. Further work is required to investigate mechanisms involved in EC induced suppression of NK dependent CHS responses.

Skin-induced tolerance and autoimmunity

Multiple sclerosis

Multiple sclerosis (MS) is an example when immune system responds to self-antigens present in the central nervous system (CNS). MS is a chronic inflammatory, devastating disorder of the brain and spinal cord. The inflammatory plaque, whether determined histopathologically or using magnetic resonance imaging (MRI), is the pathological hallmark of MS [11]. Studies demonstrating the presence of inflammatory cells and their products in the brain lesions of MS patients, and in animal models with induced experimental autoimmune encephalomyelitis (EAE), has led to the generally accepted hypothesis that this disease is mediated by pathogenic $CD4^+$ Th1/Th17 cell mediated responses against myelin antigens, followed by a broader neurodegenerative process [11].

Treatment modalities for MS are limited, with the most common treatments being steroids or anti-mitotic drugs acting nonspecifically on the immune system resulting in a general immunosuppression accompanied by many severe side effects [7]. Other drugs such as copaxone or IFN-β have therapeutic effect only in some patients. Thus numerous efforts have been made to develop a treatment able to control the autoimmune response in an antigen specific way.

One approach is based on the induction of antigen-specific tolerance. There are currently four different methods employed for inducing peptide-specific immune tolerance: altered peptide ligand induced tolerance, mucosal (oral–nasal)-induced tolerance, soluble-peptide-induced tolerance and tolerance induced *via* injection of ethylene carbodiimide peptide-coupled cells [40]. Of these, mucosal tolerance has received most attention. This protocol relies on mucosal deposition of an antigen. Oral tolerance has been studied in many experimental disease models, including EAE, where it was found that animals fed with MBP were protected from disease [16]. Other studies showed that orally induced Treg cells secreted anti-inflammatory cytokines, such as TGF-β, IL-4 and IL-10 [6]. Promising achievements in the field of mucosal tolerance in EAE encouraged clinicians to treat MS patients by feeding them bovine MBP daily to suppress disease. In MS patients, MBP- and PLP-specific TGF-β secreting Th3-type cells have been observed in the peripheral blood of patients treated orally with bovine myelin preparation and not in patients who received placebo [12]. Despite these promising observations, clinical trials failed to show any therapeutic benefit of bovine MBP feeding beyond the placebo effect [48,50].

Our findings in T cell mediated CHS model raised the possibility that EC immunization with the proper antigens could induce regulatory cells that would protect from animal model of multiple sclerosis-EAE. Indeed, we have found that EC immunization with myelin basic protein (MBP) prior to induction of EAE resulted in protection from developing EAE, as incidence was reduced by 50%. In addition, disease severity was also significantly reduced in those tolerized mice that did develop disease [39]. In support of our findings showing that EC immunization is an effective strategy to protect from EAE is a study from the Janeway laboratory, in which a similar level of protection was induced in TCR transgenic mice bearing a TCR specific for MBP [5]. Further, our study showed that protection from the disease correlated with decreased number of mononuclear leukocytes isolated from the CNS [21]. Additionally, histological examination showed only a slight mononuclear cell infiltration in the spinal cords of mice EC immunized with MBP prior to disease induction when compared to positive control group [34]. Further experiments showed that EC induced protection from EAE is transferable with lymph node and spleen cells [39]. Employing $TCR\delta^{-/-}$ and $CD1d^{-/-}$ mice, we showed that EC induced suppressor cells do not belong either to the population of

TCRγδ cells or CD1d restricted NKT cells [41]. Then, using $\beta_2m^{-/-}$ mice, negative selection and positive selection of skin-induced suppressor cells, we demonstrated that Ts cells protecting from EAE belong to the population of TCRαβ$^+$ CD4$^+$ CD8$^+$ double positive T lymphocytes [41]. Both *in vitro* and *in vivo* experiments with blocking antibodies showed that mechanism of skin-induced suppression is most likely through the production of TGF-β by Ts cells [39]. In addition, we found that EC immunization induced a population of regulatory cells that function in an antigen nonspecific manner. This was shown by suppression of EAE *in vivo* and lymph node cell proliferation *in vitro via* EC immunization with foreign antigens OVA or TNP-Ig.

Finally, we showed that EC treatment of EAE mice with MBP after first signs of disease significantly ameliorated ongoing disease [39].

Maneuver of EC immunization seems to bear fruit as we found that myelin peptides applied EC to MS patients activated dendritic Langerhans cells in the skin at the site of immunization and induced a unique population of granular dendritic cells in local lymph nodes. In the periphery, EC immunization with myelin peptides resulted in the generation of type 1, IL-10 producing regulatory T cells, suppression of specific autoreactive proliferative responses and inhibition of IFN-γ and TGF-β production [17].

Our the most current study demonstrates that induction of immune tolerance *via* EC immunization with myelin peptides translates into attenuation of disease activity as determined by MRI and clinical measures [42]. In this study we conducted a double-blind, placebo controlled, trial with three myelin peptides, MBP85–99, PLP139–151 and MOG35–55, administered EC in a form of gauze patch in remitting relapsing MS (RRMS) patients for one year. Additionally, our study showed that this method of therapy is safe and well tolerated by patients [42].

Inflammatory bowel disease

Inflammatory bowel disease (IBD) in humans, such as ulcerative colitis (UC) and Crohn's disease (CD), is a complex chronic inflammatory disease of largely unknown cause that affects 3.6 million people in Europe and the USA, mainly of Caucasian descent [13]. The contribution of the host immune system and genetic factors that predispose to IBD have been extensively investigated. At present it is believed that IBD relates to genetic predisposition, an environmental trigger and an aberrant immune reaction [27].

The immune response reflects defects in both innate and adaptive immunity. Defects of the innate immune response lead to inappropriate responses to commensal gut flora including the production of various cytokines such as IL-12 and IL-23 or IL-13 resulting in either Th1/Th17 or Th2 responses [27].

Pathogenesis of IBD is not fully understood but it is well accepted that CD is Th1/Th17-T cell-driven process whereas UC is Th2-like T-cell-driven process [36].

To study pathogenesis of human diseases and to test new therapeutics animal models are used. One of the models that mimic UC can be induced in susceptible mouse strains by intrarectal instillation of the haptens, *e.g.* TNBS or oxazolone dissolved in ethanol with or without skin preimmunization [49].

Our findings in CHS and EAE let us to speculate that maneuver of EC immunization with a proper antigen might become a universal method to induce tolerance that could control unwanted immune responses. To verify this hypothesis we tested efficacy of skin-induced tolerance in animal model of colitis ulcerosa. We found that indeed EC application of the protein antigen TNP-Ig prior to colitis induction alleviates disease severity what was determined by the body weight, the length and the weight of the colon, the histological activity index (HAI) and myeloperoxidase activity (MPO) in tissue homogenates [22].

It is commonly accepted that CD is Th1/Th17-T cell-driven process whereas UC is Th2-like T-cell-driven process [36]. On the other hand, it was already shown that TNBS-colitis exhibits heighten Th1–Th17 response [1]. To verify whether observed amelioration of colitis in mice patched with TNP-Ig is related to the suppression of Th1 and Th17 responses we measured production of proinflammatory cytokines in culture supernatants. This experiment showed that EC immunization with TNP-Ig prior to colitis induction significantly inhibits IFN-γ and IL-17A production when compared to mice EC treated with PBS alone prior to disease induction [22]. Finally, our *in vitro* experiments showed that EC immunization with TNP-Ig prior to disease induction results in increased production of IL-10 by mesenteric lymph nodes when compared with animals that were EC treated with PBS before colitis induction [22]. IL-10 itself functions as an anti-inflammatory cytokine, and limits excessive tissue disruption caused by inflammation. Thus, it is possible that observed amelioration of the disease in our system after skin immunization with TNP-Ig is partly mediated by IL-10. Our finding is in line with reports from other laboratories showing that indeed IL-10 plays an important role in controlling inflammatory response during TNBS-colitis [8]. Further experiments are required to determine the source of anti-inflammatory cytokines in our system.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is another example of an autoimmune disease mediated by chronic inflammation. This chronic inflammatory disease affects about 1% of the adult population and occurs twice as frequently among women than men [14]. The onset may appear at any age, but the peak of incidence comes in the 25–55 age range. At present it is considered that pathogenesis of RA is based on inflammatory response mediated by CD4$^+$ Th1 and Th17 lymphocytes [2]. Studies on animals are a valuable source of information on the role of the immune system in the RA pathogenesis. The most commonly applied model imitating RA is the collagen-induced arthritis (CIA).

Our study showed that EC immunization with collagen prior to induction of CIA delayed development of the disease. In addition, the inflammatory response in the joints was reduced by 50% when compared to control group. Protection from the disease was dose dependent and maximal reduction of CIA occurred with a range of 30 and 100 μg of collagen per animal [19]. Similarly to findings in CHS and EAE, skin-induced protection from CIA was antigen non-specific as we found that the non-cross reacting antigens MBP, OVA and TNP-Ig were also protective. Suppression was transferable with lymph node and spleen cells. Employing negative selection techniques we found that TCRαβ but not TCRγδ cells are involved in skin induced suppression. Further experiments revealed that depletion of both CD4 and CD8 cells abrogates EC induced tolerance [19]. Further experiments are carried out to determine the mechanism of skin-induced suppression in CIA model.

Skin-induced tolerance and skin graft rejection

Organ transplantation is an important treatment strategy. In many cases, an adaptive immune response to the grafted organ is responsible for failure of the graft. Transplantation barriers can be attributed to genetic disparity between the donor and the recipient. The antigens that are present on genetically disparate tissues are known as histocompatibility antigens. Anti-graft immune responses directed toward the major histocompatibility complex (MHC) are particularly strong, and for this reason, recipients are routinely paired with MHC matched donors. However, minor histocompatibility (H) antigens, which cannot

be easily matched, can also induce anti-graft immunity resulting in graft rejection.

Minor histocompatibility (H) antigens remain a barrier to the transplantation of organs and tissues between individuals matched for MHC antigens in human being and by H-2 in mice. Minor H antigens are composed of peptides derived from polymorphic intracellular proteins. They function to stabilize MHC or H-2 molecules during biosynthesis for expression at the cell surface. These antigens are encoded by both autosomal and Y chromosome genes [51]. The male-specific antigen H–Y provides a well characterized system to study graft rejection. Female mice on H-2^b background (*e.g.* C57BL/6) are able to generate a strong cellular response to H-Y disparate grafts [44].

Following a skin graft, the immune events that occur in the area surrounding the graft and in the draining lymph nodes likely mimic those that occur during an infection with bacteria, virus or fungi. These are highly developed and important protection from invading pathogens. This may explain why it is so difficult to circumvent these mechanisms to prolong the survival of the skin graft [30]. There are some differences between the process of rejection of skin grafts in major, complete minor and H–Y minor histocompatibility mismatched models [28]. In two first models, cytotoxic T lymphocytes play a crucial role in skin graft rejection whereas in the latter cases, T helper cells are primarily involved. Many of the mechanisms involved in graft rejection are the same mechanisms responsible for DTH and CHS [51]. Indeed, Wang et al. [44] demonstrated that mutant female C57BL/6 mice, which are unable to generate anti-H–Y cytotoxic T-lymphocytes, but are able to mount an H–Y specific DTH reaction, reject H–Y-disparate skin grafts. Class II-restricted Th1 $CD4^+$ cells are also involved in the induction of graft-*versus*-host reaction (GVHR). Consequently, bone marrow grafts between HLA-matched siblings are often complicated by GVHR directed at minor H disparities, including the male-specific H–Y antigen.

Host *versus* graft and graft *versus* host responses to the male-specific Y–H antigen can be alleviated by treatment with immunosuppressive drugs. However, these drugs are usually hepatotoxic and increase the risk of both infection and tumor development [9]. Clearly, the ability to induce tolerance to minor H–Y antigens would be clinically advantageous and there is an urgent need to provide pre-clinical data with experimental models. We therefore endeavored to determine whether EC immunization with a protein antigen would be effective to induce tolerance and prolong graft survival.

We evaluated the effect of pretreatment of female C57BL/6 mice with EC OVA on the survival of syngeneic male skin grafts. The female mice were treated with gauze patch soaked with a solution containing 100 μg OVA in PBS. The control mice were patched with gauze soaked in PBS alone. Patches were applied on day 0, replaced on days 4, 7 and 11 and removed on day 14. Skin grafts from the ear of syngeneic male mice were then implanted on the recipient's thorax on day 22 or day 42. The bandages were removed 7 days later and the grafts were checked daily [20]. Scab formation on 10% of the skin transplant surface was considered to be the beginning of skin rejection, whereas full-scab formation represented complete rejection of the graft. We found that EC pretreatment of female C57 BL/6 mice with OVA prolonged the survival of the Y–H-mismatched male skin grafts. Epicutaneously induced suppression of transplantation reactivity declines with time [20]. Further experiments are required to determine mechanisms involved in protection from skin graft rejection.

Coda

Both skin and mucosa constantly exposed to many antigens play 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. So, in both skin and mucosa discrimination between pathogens and innocuous antigens in order to induce either immune response or tolerance is of utmost significance.

Our work shows that EC exposure to an antigen, similar to mucosal immunization, results in the induction of regulatory cells. The inhibitory mechanisms are also much the same, emphasizing the similarities between these two tissues. 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.

Conflict of interest

There are no known conflicts of interest associated with this publication.

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