REVIEW

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Skin-induced tolerance and its reversal by Toll-like receptor ligands

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Abstract

In 1970, Gershon was the first to propose that T cells, in addition to their helper activity, can also play a role as regulatory cells capable of suppressing immune responses. However, the initial strong interest in T cell-mediated suppression was followed by a period of doubt and skepticism. Since the late 1990s the "S" word started to be used in immunology again and interest in T suppressor cells has grown, ushering in a new renaissance for the field. In this article the author presents the current knowledge about a new subject called "skin-induced tolerance". Suppression is induced via epicutaneous immunization and is described in both Th1- and Tc1-mediated contact sensitivity reactions. The subject of skin-induced tolerance is also considered in the regulation of experimental models of autoimmune diseases such as allergic autoimmune encephalomyelitis and collagen-induced arthritis and finally in an animal model of graft rejection. The last part of this presentation will introduce the very fresh subject of "contrasuppression", or the reversal of skin-induced suppression.

Key words: suppression, contrasuppression, Toll-like receptors, contact sensitivity, autoimmunity.

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INTRODUCTION

Our bodies are constantly exposed to different microorganisms present in the environment. However, contact with pathogenic microorganisms rarely results in infection. This is because our bodies are protected by both innate and adaptive immune mechanisms. There are two major classes of adaptive immune responses. The first class is mediated by antigen (Ag)-specific T cell receptor (TCR)$\alpha\beta^+$ $CD4^+$ (delayed type hypersensitivity – DTH) or $CD8^+$ (T cell-mediated cytotoxicity) lymphocytes and is induced by intracellular pathogens [31]. The second type is the humoral immune response mediated by antibodies produced by B lymphocytes [31]. The main function of the humoral response is to destroy extracellular microorganisms and prevent the spread of infection. For complete health, every living being must be continuously protected from infection and tumors by their immune system. At the same time there must be mechanisms to protect the organism from the development of inappropriate immune responses that are harmful to one's own body (allergy, autoimmunity) as well as to silence inflammatory responses and allow their resolution. This demonstrates the importance of strict control of immune responses by regulatory mechanisms to balance the helpful and harmful aspects of inflammation.

In 1970, Gershon was the first to propose that T cells, in addition to their helper activity, can also play a role of regulatory cells capable of suppressing immune responses [18]. Many scientists then focused their attention on T suppressor (Ts) cells, trying to characterize both their phenotype and mechanism of action. After a time of huge interest in suppression, the world of cellular immunology started to doubt if Ts really existed and many findings were considered to be over-interpretations of inconclusive data. For nearly 30 years, scientists who had not abandoned their belief in Ts cells had to mask their findings under less controversial names such as "down-regulation", "infectious tolerance", or "active unresponsiveness". Since late 1990s the "S" word started to be used in public again and interest in Ts cells was reborn [2]. Since that time it has been shown that Ts cells not only play an important role in down-regulating immune response, but that they also keep autoreactive T and B cells under control [35]. It is commonly accepted that self tolerance is based on two major mechanisms: clonal deletion in the thymus and anergy in the periphery [20, 53]. At present it is well accepted that a number of different T cell subsets contribute to T cell-

-mediated suppression. The first population of Treg cells are among the naturally occurring $CD4^+CD25^+$ lymphocytes. These develop directly from $CD4^+$ T cell precursors during positive selection as a result of their interaction with thymic epithelial cells [34]. The second group of $CD4^+$ Treg cells, known as "induced Tregs", is comprised of the Tr1 and Th3 cell populations [15, 34]. Two other groups of T cells with suppressor activity belong to the $CD8^+$ and NKT cell populations [32]. These different subsets are induced through different mechanisms and perhaps play different roles in the regulation of the immune response. The mechanisms by which these different subsets mediate suppression are currently a very important topic of research in the field of T cell-mediated suppression.

In this article the author reviews the current knowledge about a hot topic called "skin-induced tolerance" in three types of experimental models: Th1- and Tc1--mediated contact sensitivity (CS), experimental models of autoimmune diseases such as allergic autoimmune encephalomyelitis (EAE) and collagen induced arthritis (CIA), and finally rejection of allogeneic skin grafts. In the second part of this presentation the role of Toll-like receptors (TLRs) in the reversal of skin-induced suppression (or "contrasuppression") will be discussed.

SKIN-INDUCED TOLERANCE

Both the skin and mucosa are constantly exposed to many antigens and play a crucial role in protecting the body from different pathogens present in the external world. While the development of an 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 Ag via the digestive tract or nasal mucosa leads to both a local immune response and a state of profound immunosuppression in the periphery [11, 74, 77]. This suppression seems to play an important role in avoiding the development of immune responses to non-pathogenic antigens. In his review, Weiner pointed out that certain types of regulatory T cells are preferentially induced in mucosa to maintain tolerance [14, 73]. He also suggested that a special mucosal milieu may create tolerogenic dendritic cells (DCs) that induce different types of suppressor cells.

Although the skin is considered an organ where immune responses are easily induced [3, 64, 67], little attention has been given to skin-induced tolerance [54]. Because skin and mucosa have a similar function in our bodies (i.e. as a barrier to external pathogens), it is possible that epicutaneous (e.c.) application of Ag, apart from inducing a strong immune response, may also induce peripheral tolerance. Wang et al. [71, 72] showed that e.c. application of the protein Ag ovalbumin (OVA) resulted in allergic dermatitis accompanied by the appearance of interleukin (IL)-4-secreting T lymphocytes. Additionally, Herric et al. [25] showed that e.c. immunization with protein Ag induced a Th2-mediated model of asthma. These data suggested to us that, similar to mucosal immunization, deposition of protein antigens on the skin may also induce T cells producing anti-inflammatory cytokines that would suppress Th1--mediated responses causing their suppression. This subject will be discussed in the next section.

REGULATION OF CS BY Ts CELLS INDUCED VIA e.c. IMMUNIZATION

As discussed above, e.c. immunization with protein Ag induces the appearance of IL-4-secreting T cells, which may suggest that immunization by this route may suppress a subsequent Th1 response to the same Ag. To verify our hypothesis we developed the experimental model described in Fig. 1. TNP is a hapten, and previous research has shown that CS responses to TNP-protein conjugates are Th1 dependent. To potentially induce a Th2 response to TNP, mice were subject to prolonged exposure to TNP conjugated to Ig protein by applying gauze patches soaked with TNP-Ig. The patches were applied on days 0 and +4. Then on day +7 the patches were removed and the animals were actively sensitized with Th1-dependent hapten and tested for CS four days later [60]. We found skin patch immunization with

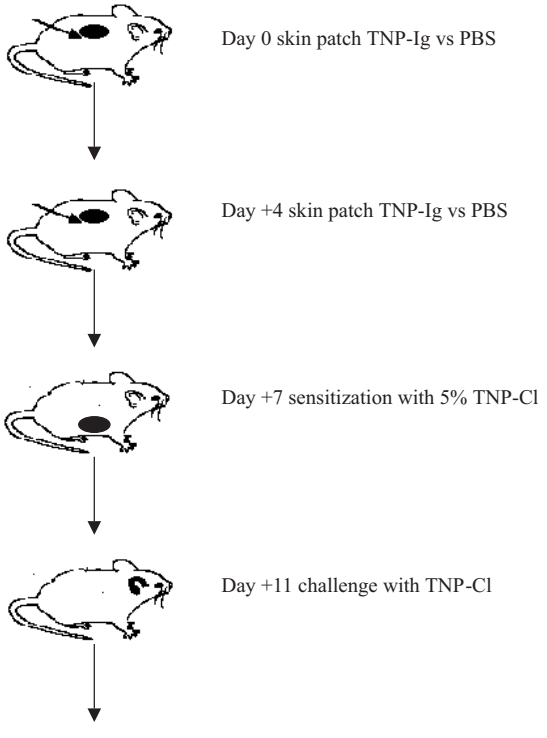

Fig. 1. Skin-induced tolerance via epicutaneous immunization with TNP-Ig. Mice were exposed to TNP-Ig or PBS on day "0" and "+4" spread over the gauze patch. Then, on day "+7" patches were removed and mice were actively immunized e.c. with 5% TNP-Cl and tested for CS four days later.

TNP-Ig before active sensitization with TNP-Cl caused a reduction of CS by 90% compared with a positive control (animals patched with phosphate-buffered saline (PBS) and then sensitized with hapten). We made similar observations in CD8 Tc1-mediated CS to dinitroflurobenzene, another hapten (Szczepanik et al., in preparation). Further experiments on the TNP-Cl model showed that the optimal dose of Ag that induces this phenomenon is between 30 and 100 μg/animal. Additionally we found that e.c.-induced suppression lasts for about four weeks.

It is noteworthy that immune cells isolated from the lymph nodes and spleens of skin-patched mice could transfer tolerance to recipients. In these experiments, immune cells from tolerant mice were mixed with immune cells from sensitized mice prior to transfer to naïve recipients ("transfer-out" protocol). The tolerant cells prevented the induction of CS in the recipients. We considered the possibility that the tolerance was the result of *in vitro* cell manipulation or of overloading the immune system with too many cells, some of which could be undergoing apoptosis and therefore be tolerogenic. To exclude these possibilities we used another experimental system, a "transfer-in" protocol, where CS-effector cells from sensitized mice were transferred into naïve recipients (positive control) or into mice that were previously tolerized by skin patch. We found that endogenous suppression was similar to that observed with the "transfer-out" protocol described above, indicating that inhibition of the Th1-mediated response was not an artifact of *in vitro* cell manipulation. In additional transfer-out experiments we found that skin-induced suppressor cells were very potent in that they were able to significantly inhibit CS effector cells when transferred into naïve recipients at a ratio 1 to 10.

We performed negative selection experiments in order to define the phenotype of the skin-induced suppressor cells. These demonstrated that they belong to the population of $TCR\alpha\beta^+$ lymphocytes. There are some reports that invariant $V\alpha14^+$ NKT cells can have immunoregulatory function in many experimental systems [63]. However, experiments performed with NKT cell-deficient $J\alpha18^{-/-}$ mice showed that these are not involved in skin-induced tolerance.

In order to further characterize the T cells that mediate suppression, we used anti-CD4 or anti-CD8 antibodies to deplete these leukocyte subsets from the suppressor population isolated from skin-patched mice. As either antibody eliminated suppressor activity, we conclude that both $CD4^+$ and $CD8^+$ lymphocytes contribute to suppression. However, reconstitution of $CD4^-$ cells with the CD8-depleted cell population did not restore the suppressor activity of skin tolerance-induced suppressor cells, suggesting that these cells may co-express both CD4 and CD8 co-receptors. Dual expression of CD4 and CD8 on the Ts was suggested by showing that skin patching causes the appearance of about 2–3% $TCR\alpha\beta$ $CD4^+CD8^+$ double-positive T cells in subcutaneous lymph nodes and in the spleen, compared with the normal range of between 0 and 1% [8]. Increased numbers of $CD4^+CD8^+$ double-positive T cells began on day +7 (day of removal of the second patch) and remained elevated for a week. The proportion of $CD4^+CD8^+$ cells then declined to that observed in control mice e.c. treated with PBS alone.

The role of this $CD4^+CD8^+$ peripheral population of leukocytes in *in vivo* immune responses is still unclear. It has been reported that in both mice and humans, peripheral $CD4^+CD8^+$ T lymphocytes increase during the neonatal period as well as in certain infections, autoimmune diseases, tumors, transplantation, and also with age [33, 39]. To confirm our hypothesis that e.c. immunization induces $CD4^+CD8^+$ double-positive Ts cells, we mixed four-day TNP-Cl immune CS--effector cells from sensitized mice with FACS-sorted $CD4^+CD8^+$ T cells from tolerized e.c.-treated mice. These were then transferred into naïve recipients that were then tested for CS to hapten. This cell mixing experiment showed that $CD4^+CD8^+$ T cells isolated from e.c.-immunized mice are indeed responsible for suppression.

The cells responsible for skin-induced tolerance may be related to the double-positive $CD4^+CD8^+$ intestinal intraepithelial lymphocytes that may be responsible for oral tolerogenic mechanisms in the intestinal environment, a process also thought to be dependent on the production of Th2 cytokines [16]. Other studies show that intraepithelial lymphocytes are $CD4^+CD8^+$ $TCR\alpha\beta^+$ cells that can release immunoregulatory cytokines such as transforming growth factor (TGF)-β and IL-10, suggesting suppressor activity. As noted above, $CD4^+CD8^+$ T cells in naïve mice are less than 1% of peripheral lymphocytes and their origin is unclear. They could 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 lymphokines such as IL-4 or are present due to prior "natural" suppressive events. In our case we speculate that $CD4^+CD8^+$ T cells with suppressor activity originate from prematurely escaped $CD4^+$ lymphocytes that acquire a CD8 co-receptor. Our hypothesis is based on the observed increase in $CD4^+CD8^+$ T cells in lymph nodes 7 days after e.c. 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 cells.

There are many reports showing that Ts cells belong to the population of $CD4^+CD25^+$ lymphocytes. In a FACS sorting experiment employing skin-induced suppressor cells we showed that one third of the $CD4^+CD8^+$ T cells expressed CD25 and two thirds were $CD25^-$. However, both $CD4^+CD8^+CD25^+$ and $CD4^+CD8^+CD25^-$ T cells were able to inhibit adoptive transfer of CS. Therefore, in this system both $CD25^+$ and $CD25^-$ $CD8^+CD4^+$ cells have suppressive activity, but we cannot rule out that the cells express CD25 below the levels detected by FACS or that they become

$CD25^+$ after transfer *in vivo,* and thus also become responsive to IL-2.

Skin-induced Ts cells are very potent suppressors of CS responses. For instance, only very low numbers of Ts cells were required to observe suppression; as few as 2×10^3 per mouse were able to inhibit the effector function of 7×10^7 effector cells *in vivo.* We previously also found that small numbers of $TCR\gamma\delta^+$ suppressor cells could inhibit Th1 TNP-Cl-induced CS effector cells, since only 2.5×10^3 of these cells efficiently suppressed 7×10^7 CS-effector immune cells [59]. The mechanism by which skin-induced Ts cells inhibit CS responses is not known. It is possible that these cells interfere with either sensitization to the Ag or with the response following challenge. To determine if Ts cells inhibit either or both of these processes, we transferred skin-induced Ts cells on the day of immunization or challenge. The observed suppression of CS in both cases might suggest that Ts cells induced by e.c. immunization operate both at the afferent and efferent phase of Th1-mediated CS. However, this experiment does not exclude the possibility that Ts cells transferred at the time of immunization do not affect the induction of Th1 effector cells and can persist in the body of the recipient to suppress Th1 cells only in the efferent phase, but also at the time of challenge.

Further study of the Ag specificity of skin-induced tolerance showed suppression of both CS and DTH responses in mice immunized epicutaneously with four different non-cross-reacting Ags, such as TNP-Cl, oxazolone (OX), OVA, and keyhole limpet haemocyanin (KLH), prior to contact sensitization or intradermal immunization with the protein Ag KLH. These results show that the final mediation of suppressor T cell activity is Ag nonspecific. The data of the *in vivo* experiments were confirmed *in vitro* and showed that skin-induced tolerance with two non-cross-reacting Ags, TNP-Ig and OVA, resulted in inhibition of proliferation of TNP-specific immune cells.

To avoid the cumbersome and inconvenient patch method of generating skin tolerance, we developed another technique based on daily application of neutral cosmetic Nivea™ cream containing different proteins as additives to the shaved skin [51]. This set of experiments confirmed our previous findings using the patch method in that e.c. deposition of Ag emulsified in cream also leads to systemic Ag-nonspecific unresponsiveness. Using this method of immunization, we also showed that proteins such as collagen, elastin, and keratin, which are normal additives of many cosmetics, induce strong Ag-nonspecific suppression of Th1-mediated CS. Additionally, we found that commercially available cream containing both collagen and elastin strongly inhibited CS when applied e.c. before TNP-Cl sensitization.

The above findings raise the possibility that protein-enriched cosmetic creams may influence the immune status of their human users as they do experimentally in mice. At present we are not aware of any information on this subject. The amounts of antigens we used to immunize the mice were proportionally not much different from the amounts used by humans during normal skin care on a daily basis. Although creams are used by humans for a much more extended period of time (many months and even years vs. one week), the anatomy and physiology of mouse skin differs from its human counterpart in several respects. These differences include skin thickness, which in the mouse would favor easier Ag penetration, although the thick and hydrophobic horny layer of human skin is now frequently removed by peeling, thus making the skin more permeable to subsequently applied cosmetics. Moreover, mouse and human skin have different cellular composition, e.g. the presence of TCRγδ cells in mouse skin and their absence in human [56]. Thus, further studies will be required to determine if commercial skin creams really are immunosuppressive in humans.

As mentioned above, e.c. immunization with protein Ag induces Ag-nonspecific suppression of cell-mediated immune response. In classic T cell-mediated suppression, both suppressor and effector cells are often specific to the same Ag, i.e. the suppression is Ag-specific and limited to that Ag. The nonspecific tolerance observed in our experiments is possibly akin to the phenomenon known as "bystander suppression" or determinant spreading [10, 28], in which suppressor cells elicited by a particular Ag secrete Ag-nonspecific cytokines that down-regulate the immune responses of other Ag-specific T cells. In the well-studied model of oral tolerance, orally administrated Ag appears to interact with gut-associated lymphoid tissue (GALT) to generate Ts cells that produce TGF-β, IL-4, and IL-10 [56] and also mediate bystander suppression [28]. It was shown both *in vivo* and *in vitro* that orally induced suppression is dependent on Ag-specific triggering of Ts cells. Once Ag-specific triggering occurs, suppression is mediated by nonspecific suppressive cytokines produced by the Ts cells [10]. In the case of our skin-induced tolerance model, CS to one Ag could be inhibited by all the tested Ags used to induce skin tolerance. Thus the presence of the Ag responsible for inducing Ts cells is not required during elicitation of the Ts effect [58]. This might suggest that skin-induced suppressor cells work either via different mechanisms from those that are active in bystander suppression in mucosa oral tolerance, or an original Ag persists for a long period in peripheral lymph organs, or alternatively, anti-inflammatory cytokines released by Ts cells interfere with the induction and/or effector phase of the T effector cell-mediated immune response.

The association of non-Ag-specific suppression with inhibitory cytokines led us to determine if a specific cytokine milieu is required for the induction of Ts cells via e.c. immunization, as observed in some systems [49]. In one system, both IL-10 and TGF-β play an important role in the differentiation of naïve $CD4^+$ cells into Ts [28]. The mechanism by which a special cytokine environment drives the induction of regulatory cells is not fully understood. It has been reported that pulmonary DCs that were isolated after nasal mucosal exposure to

an Ag secreted IL-10 and were able to induce $CD4^+$ T regulatory type I (Tr1) cells [1]. Another study showed that DCs isolated from mesenteric lymph nodes of mice tolerized by feeding with an Ag showed increased production of TGF-β which facilitated the induction of another down-regulatory cell, the Th3 regulatory cell [73]. There is also some evidence that IL-4 prevents the maturation of DCs, and subsequent interaction of these immature DCs with T cells results in T cell tolerization [1].

These data do not fully explain how anti-inflammatory cytokines are involved in the induction of Ts cells. However, it appears that a unique suppressive immunological milieu made up, at least in part, by cytokines plays a crucial role in the induction of immunological tolerance. As noted before, exposure to an Ag via skin induces $CD4^+CD8^+$ regulatory cells, similarly to mucosal immunization. We speculate that these two organs, exposed to similar and innocuous Ag at the surface of an organism in contact with the environment, are similarly capable of inducing suppressor cells to prevent needless reactivity.

To answer the question as to whether a specific cytokine milieu is required to induce suppressor cells via skin immunization, we tolerized mice with TNP-Ig alone or with TNP-Ig plus the anti-cytokine antibodies anti--IL-4, anti-IL-10, or anti-TGF-β or control antibody on the skin before induction of CS. All of the anti-cytokine monoclonal antibodies significantly diminished the suppression induced by skin immunization with TNP-Ig, suggesting that anti-inflammatory cytokines are involved in the induction of Ts cells. At present it is unclear how these cytokines regulate the differentiation of T lymphocytes into Ts cells. Further experiments are in progress.

Finally we tried to determine how e.c.-induced Ts cells inhibit Th1 effector cells. To determine the mechanism of skin-induced tolerance, mitomycin C-treated tolerized lymph node cells were co-cultured with 4-day TNP-Cl immune cells with or without the anti-cytokine monoclonal antibodies anti-IL-4, anti-IL-10, or anti--TGF-β. Anti-TGF-β but not anti-IL-4 or anti-IL-10, resulted in a significant inhibition of suppression as demonstrated by increased effector-cell proliferation in response to TNP-Ig. Therefore, TGF-β likely plays a crucial role in the effector stage of suppression. Indeed, analysis of cytokine production by lymph node and spleen cells isolated from TNP-Ig-tolerized mice showed increased production of TGF-β compared with cells isolated from control mice (animals patched with PBS alone). There was no significant difference in IL-4 and IL-10 production by lymph node and spleen cells isolated both from TNP-Ig-tolerized and control mice [60]. Therefore, the *in vivo* mechanism of skin-induced, non-Ag-specific suppression is most likely through the production of TGF-β by Ts cells (Fig. 2).

As discussed above, orally administrated Ag appears to interact with GALTs and generate T regulatory cells that produce TGF-β, IL-4, and IL-10 [49] and mediate bystander suppression [28]. Our data thus suggest that e.c. exposure to an Ag also results in the induction of Ts cells that act through similar mechanisms. This emphasizes the similarity of the immune tolerance mechanisms operating on these two body surfaces. The ease of e.c. generation of non-Ag-specific Ts cells may have important implications for designing therapeutic schemes aimed at modulating immune responses to self antigens involved in autoimmune diseases. This subject will be discussed in the following section.

EPICUTANEOUS IMMUNIZATION WITH PROTEIN Ag CAN PROTECT FROM THE DEVELOPMENT OF Th1-DEPENDENT AUTOIMMUNE DISEASES

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) characterized by the presence of cellular infiltrates comprised primarily of lymphocytes and macrophages and localized areas of demyelination in the CNS. MS is thought to be initiated by self reactive $CD4^+$ Th1 T cells with specificity for a variety of antigens contained within the myelin sheath [23]. EAE is a well-characterized animal model that mimics the clinical disease of MS, including the presence of cellular infiltrates and demyelination in the CNS. Treatment modalities for MS are limited, the most common being steroids or antimitotic drugs such as cyclophosphamide acting nonspecifically on the immune system, resulting

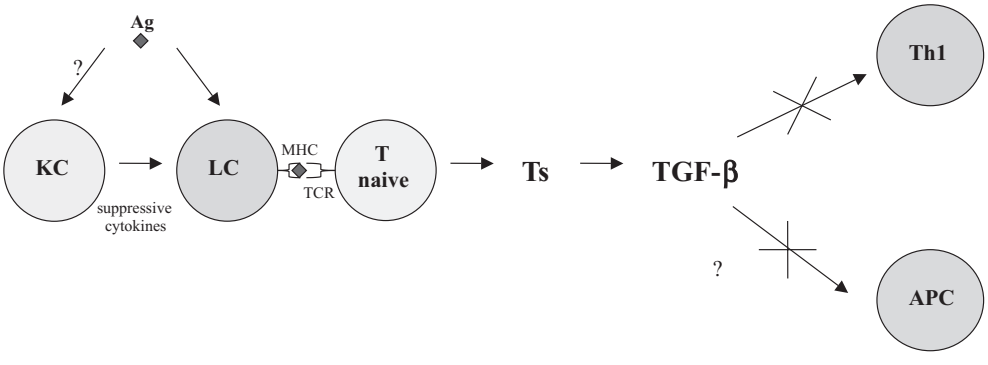

Fig. 2. The mechanism of skin-induced suppression. KC – keratynocyte, LC – Langerhans cell, Ts – T suppressor cell, APC – antigen presenting cell, Th1 – T helper cell type1.

in a general immunosuppression accompanied by many severe side effects [57]. Thus numerous efforts have been undertaken to develop a treatment able to control the autoimmune response specifically. One of these methods relied on mucosal deposition of an Ag. It is worth mentioning that oral tolerance has been studied in many experimental models, including EAE, where it was found that animals fed with myelin basic protein (MBP) were protected from disease [14, 29]. Other studies showed that orally induced Ts cells secreted anti-inflammatory cytokines, such as TGF-β, IL-4, and IL-10 [11]. 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. MBP- and proteolipid protein-specific TGF-β secreting Th3-type cells have been observed in the peripheral blood of MS patients treated orally with bovine myelin preparation and not in patients who received placebo [17]. Despite these promising observations *in vitro*, a clinical trial failed to show any therapeutic benefit of bovine MBP feeding beyond the placebo effect [74, 76].

As discussed extensively in the previous section, our own research on the CS model has shown that Ag applied on the skin induces profound peripheral suppression of the Th1-mediated immune responses [51, 60]. These findings raised the possibility that e.c. immunization with the proper antigens could induce Ts cells that would protect from EAE, similar to their protection from CS. Indeed, we have found that e.c. immunization with MBP prior to the induction of EAE resulted in protection from developing EAE, as the incidence was reduced by 50%. In addition, disease severity was also significantly reduced in those tolerized mice that did develop disease [61]. The lowest dose of MBP that significantly suppressed disease was 100 μg/animal. Further experiments showed that e.c. immunization induced a population of Ts cells that function in an Ag-nonspecific manner. This was shown by suppression of EAE when mice were patched with the non-relevant antigens OVA or TNP-Ig. The Ag-nonspecific suppression along with the ability to transfer the suppression to a naïve animal are consistent with a mechanism of bystander suppression [74]. Bystander suppression was first described *in vitro* by Weiner's laboratory in a model of oral administration of Ag and was characterized as the ability of cells from an MBP-fed animal to inhibit the proliferation of an OVA-specific cell line across a trans-well [43]. In addition, bystander suppression is characterized by the ability to transfer suppression via the adoptive transfer of lymphocytes from a tolerized animal to a naïve recipient [74]. In the same laboratory, the mechanism of bystander suppression was found to be the production of TGF-β by both $CD4^+$ and $CD8^+$ T cells [12].

We found that Ts cells induced via e.c. immunization mediated inhibition both *in vitro* and *in vivo* by the production of TGF-β , similarly to oral tolerance. *In vitro* we showed that MBP-specific T cell proliferation was significantly lower in mice e.c. immunized with a variety of protein antigens, including MBP and OVA. Furthermore, using blocking monoclonal antibody, the suppression of proliferation was shown to be due to TGF-β. Blocking of the other Th2 cytokines IL-4, IL-10, and IL-13 had no effect on T cell proliferation. A role for TGF-β in the suppression of EAE was confirmed *in vivo* by injecting mice with anti-TGF-β-neutralizing monoclonal antibody; the mice subsequently exhibited a normal EAE disease course. TGF-β is a pleiotropic, multifunctional cytokine that acts on a wide variety of cell types with functions ranging from development to carcinogenesis to the regulation of immune responses [40]. In our *in vitro* studies, the mechanism of suppression is possibly through the inhibition of IL-2-dependent T cell proliferation [36]. However, *in vivo* suppression may be due to multiple functions of TGF-β including inhibition of T cell proliferation, inhibiting IL-12--induced Th1 differentiation and MBP-specific T cell priming or skewing of the immune response to a Th2 response [6, 52]. All these mechanisms are possible and each would likely result in the prevention and suppression of EAE. In our studies it is not clear whether TGF-β exerts its suppressive effect in the CNS or in the periphery. It is likely active on primed MBP-specific T cells in both locations, since TGF-β receptors are upregulated following T cell activation [36]. Although daily injection of TGF-β had suppressive effects on both EAE and CIA [38], other studies have shown an association with unresolved inflammation and fibrotic events as well as a predisposition for recurrent infections [69]. Thus the delivery of TGF-β to a particular location by Ts cells may overcome the problems of systemic delivery.

The presence of regulatory T cells with the ability to suppress a variety of immune functions has been shown in a number of autoimmune models [68]. At present there are reports that more than one type of Ts cell exists, which include $CD4^+$, $CD8^+$, TCRγδ and NKT cells [15, 32, 34, 68]. Many Ts cells exert their inhibitory activity by the production of cytokines, including IL-10, IL-4, and TGF-β [68]. Ts cells which secrete TGF-β include both $CD4^+$ and $CD8^+$ T cells [12].

In our experiments we have found that the suppression induced via e.c. immunization was transferable to naïve recipients by the adoptive transfer of either lymph node cells or spleenocytes or $CD4^+CD8^+$ splenocytes from tolerized mice. These data are in line with our previous findings in the CS system. Finally we showed that e.c. treatment of mice with MBP after the first signs of disease significantly ameliorated the ongoing disease.

Rheumatoid arthritis (RA) is another example of an autoimmune disease mediated by chronic inflammation. While this disease is not an uncommon condition, with a prevalence of 1% worldwide, its pathogenesis is not yet clear [21]. At present it is thought that RA is mediated by $CD4^+$ Th1 cells that recognize unidentified antigens present in joints. Upon recognition of Ag, these auto-reactive Th1 cells release pro-inflammatory cytokines that initiate inflammatory processes in the

joints. This results in polymorphonuclear cell and macrophage accumulation within the joint and subsequent cartilage destruction and bone erosion with extra-articular manifestations. However, it is important to keep in mind that the pathogenic mechanism of RA is complex, as in addition to T cells, IgG and IgM immunoglobulins and complement are also involved [21, 31]. CIA in genetically prone strains of mice, rats, or rhesus monkeys has been used as an experimental model of RA, as it shares many of the histological and immunological characteristics found in RA [4, 5, 22].

There are many different drugs that are used in the therapy of autoimmune diseases, including RA. It is noteworthy that apart from drugs such as gold salts, sulfasalazine, cortocosteroids or methotrexate, there are also medicines that interfere with the immune processes involved in the pathogenesis of RA. To the group of drugs that affect immune response and are used to treat autoimmune diseases belong cyclosporin A, antibodies that neutralize tumor necrosis factor (TNF)-β or IL-1R antagonists. However, many of those drugs inhibit the immune response non-specifically and cause many side effects [48]. Various attempts have been made to develop a vaccine that would protect from autoimmune diseases such as RA and MS. One that made use of oral tolerance using collagen showed protection from CIA in experimental animals. Unfortunately, there was no significant benefit in RA patients fed collagen [77].

In our experiments we tried to find if, similarly to CS and EAE, e.c. immunization with protein Ag could protect from CIA. In the first set of experiments we found that e.c. immunization with collagen prior to the induction of CIA delayed the development of the disease. In addition, the inflammatory response in the joints was reduced by 50% compared with controls. We found that optimal protection occurred within a range of 30 and 100 μg of collagen per animal. Similarly to the protection from CS and EAE, skin-induced protection from CIA was Ag nonspecific, 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 the skin-induced suppression. Further experiments revealed that depletion of both CD4 and CD8 cells abrogates e.c.-induced tolerance [41]. Further experiments are required to determine if, similarly to CS and EAE, suppression of CIA is mediated by $CD4^+CD8^+$ double-positive cells.

To summarize, our data suggest that e.c. exposure to an Ag, similarly to mucosal immunization, results in the induction of Ts cells that have much the same mechanism of action and therefore emphasize the similarity of immune tolerance mechanisms operating on these two body surfaces. The ease with which Ts cells can be generated through e.c. immunization may have important implications for designing therapeutic schemes aimed at modulating immune responses to self antigens involved in autoimmune diseases.

SKIN-INDUCED TOLERANCE AND GRAFT REJECTION

Tissue transplantation is an important treatment technique. 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 towards 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 beings 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 [78]. The male-specific Ag H-Y provides a well-characterized system for the study of graft rejection. Female mice on an $H\text{-}2^b$ background (e.g. C57BL/6) are able to generate a strong cellular response against H-Y-disparate grafts [70].

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 present highly developed and important protection against invading pathogens. This may explain why it is so difficult to circumvent these mechanisms to prolong the survival of a skin graft [45]. There are some differences between the process of rejection of skin grafts in major, complete minor, and H-Y minor histocompatibility-mismatched models [44]. In the first two models, cytotoxic T cells play a major role in skin graft rejection, whereas in the latter case T helper cells are primarily involved. Many of the mechanisms involved in graft rejection are the same mechanisms responsible for DTH and CS [78]. Indeed, Wang et al. [70] reported 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 the 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 Ag. Host-versus-graft and graft-versus-host responses to the male-specific Y-H Ag can be alleviated by treatment with immunosuppressive drugs. However, these drugs are usually hepatotoxic and increase the risk of both infection and tumor development. 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 if e.c. immunization would be effective in inducing tolerance and prolonging graft survival.

We evaluated the effect of pretreatment of female C57 BL/6 mice with e.c OVA on the survival of syngeneic male skin grafts. To pre-immunize the female mice, a 1-cm^2 gauze patch soaked with a solution containing 100 μg OVA in PBS was applied to the shaved back. The patch was secured by adhesive tape wrapped around the midsection. Control mice were patched with gauze soaked in PBS alone. The patches were applied on day 0, replaced on days 4, 7, and 11, and removed on day 14. Skin grafts from the ear of syngenic male mice were then implanted on the recipient's thorax on day 22 or day 42. The bandages were removed 7 d later and the grafts were inspected daily. 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 e.c. pretreatment of female C57 BL/6 mice with OVA prolonged the survival of the Y-H-miss-matched male skin grafts. Epicutaneously induced suppression of transplantation reactivity declines with time [42]. Further experiments are required to determine what mechanisms are involved in protection against skin graft rejection.

REVERSAL OF SKIN-INDUCED SUPPRESSION BY TLR LIGANDS

As we discussed in previous sections, e.c. immunization with protein Ag induces profound suppression of both Th1 and Tc1 immune responses. The Ag-nonspecific nature of the suppression raises question as to whether e.c. exposure to antigens would prevent the development of immune responses to new antigens, for example pathogen-associated antigens. This is clearly an unacceptable possibility. However, in contrast to the experimental models discussed here, pathogen antigens are also affiliated with pathogen-associated molecules that are recognized by innate immune receptors (TLRs), which may help to overcome tolerance. We therefore hypothesized that immunization with protein Ag through the skin in the presence of TLR ligands could reverse the suppression of CS reactivity observed after e.c. immunization with protein Ag alone.

Among the currently identified TLRs, TLR2 binds the largest number of microbial products [30], including peptidoglycan, lipoteichoic acid [26], zymosan [66], and lipopolysaccharide (LPS) from certain species of bacteria [13, 75]. TLR4 also binds LPS from a variety of Gram-negative organisms [27]. TLR3 is involved in the recognition of double-stranded RNA (dsRNA), which is the most representative viral component [62], whereas TLR5 uniquely recognizes a protein molecule, bacterial flagellin [55]. Unmethylated CpG sequences, characteristic of bacterial DNA, are the specific ligands for TLR9 [24]. We examined the effect of the addition of TLR ligands to the e.c. immunization protocol on the subsequent development of suppression in a model of CS (Fig. 3). Gauze soaked with a solution containing 100 μg TNP-Ig was applied to the shaved skin. In some groups of mice, crude bacterial lysates were included in the TNP-Ig solution. In other groups, incomplete Fruend's adjuvant or complete Fruend's adjuvant were homogenized with TNP-Ig (1:1). The patch was left in place from day 0 until day 4, when it was replaced by a fresh patch. On day 7 the mice were actively sensitized with TNP-Cl or OX. We found that crude bacterial lysates and specific TLR ligands (zymosan, peptidoglycan, and lipoteichoic acid for TLR2; dsRNA for TLR3; LPS for TLR4; and CpG for TLR9) applied to the skin together with an Ag (TNP-Ig) were able to overcome skin-induced suppression [50]. The TLR4 ligand LPS was able to reverse suppression completely, while TLR2 ligands (peptidoglycan and lipoteichoic acid) were less effective. LPS-induced reversal of suppression was dependent on intact TLR4, as demonstrated by the failure of LPS to reverse suppression in C3H/HeJ mice, which are naturally deficient in TLR4. In dose-response experiments, all TLR ligands tested were capable of

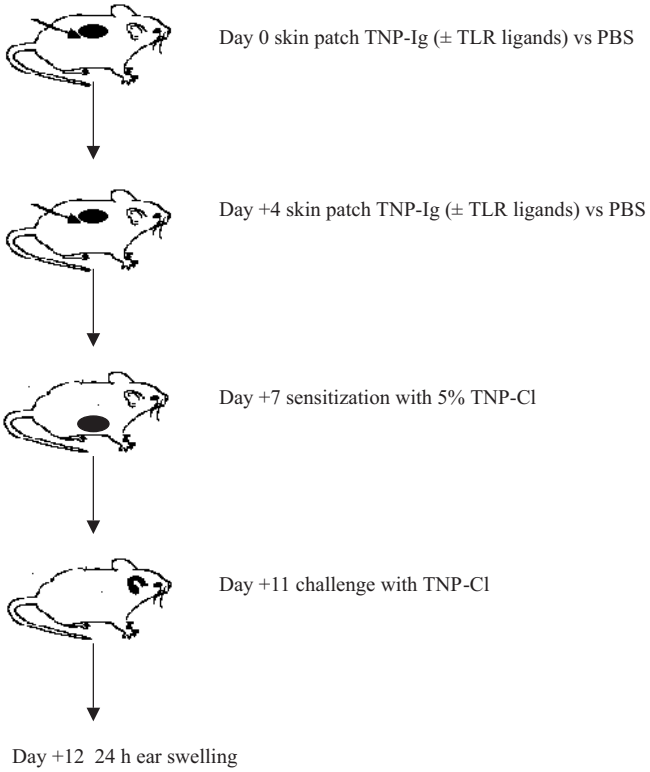

Fig. 3. Epicutaneous immunization with TNP-Ig and TLR ligands to reverse skin-induced tolerance. Epicutaneous was performed by patch application of TNP-Ig with or without TLR ligands or with PBS vehicle alone on day "0" and "+4". TNP-Cl sensitization was performed by topical application of 5% TNP-Cl to the shaved abdomen on day "+7". After 4 days mice were challenged on the ear with TNP-Cl, and ear swelling was measured 24 h later.

reversing suppression to some degree given a high enough dose, suggesting that all operate by the common downstream pathways employed by TLR ligands. Unlike the Ag-nonspecific suppressive effect of e.c. immunization with Ag alone, the reversal of suppression by addition of the TLR ligand LPS is specific to the Ag used in e.c. immunization.

Several possible mechanisms responsible for the reversal of suppression should be considered. The concept that hapten-substituted (TNP) Ag applied e.c. in the presence of TLR ligands induces specific effector cells that mediate the CS reaction to homologous hapten can be ruled out, since such treatment does not lead in itself to active sensitization. Two other scenarios are possible. One is that this procedure prevents the development of the suppressor cell population during e.c. immunization. However, as shown in our crisscross experiment with TNP-Cl and OX, this was not the case [50]. Abrogation of suppression to one Ag by presenting it e.c. together with TLR ligand did not prevent the development of Ag-nonspecific suppression to the other non-cross-reacting Ag. Our *in vivo* findings are fully supported by *in vitro* TGF-β measurement, where we did not find any significant difference in the level of this suppressive cytokine [60] between TNP-Ig- and TNP--Ig/LPS-treated mice We consider this to be the most likely explanation that Ag in the presence of TLR ligands induces a particular subpopulation of lymphocytes which make effector cells that mediate the CS response resistant in an Ag-specific manner to suppressor-cell signals. This putative cell behaves like the $CD4^+$ contrasuppressor (Tcs) cell, previously described by us, induced by intravenous injection of TNP-immune complexes. This cell also screened effector cells of CS against suppression and had no capability to disable suppressor cells directly [19]. Our further experiments showed that $CD4^+$ $TCR\alpha\beta^+$ Tcs cells from mice that underwent e.c. immunization with protein Ag in the presence of TLR ligands can reverse suppression in recipients that have undergone e.c. immunization with protein Ag alone [41]. The terminology of T cells with such mode of action is still unsettled and they are termed interchangeably contrasuppressor or up-regulatory cells. We tentatively propose that skin Langerhans cells (LCs; DCs found in the skin), which are immature under normal conditions, preferentially drive toward the development of Ts cells. Under the influence of TLR ligands, which leads to the local production of a potpourri of cytokines by both LCs themselves and keratinocytes, the LCs mature and acquire the ability to trigger contrasuppressor cells. The ability of LCs to deliver either tolerogenic or immunogenic signals, dependent on their maturation stage, has been demonstrated previously [37]. A similar mechanism, where a TLR ligand was able to convert tolerogenic macrophages into immunogenic ones, was demonstrated by us [9]. Previous studies have indicated that TLR ligands are capable of overcoming the suppressive effect of $CD4^+CD25^+$ regulatory T cells [47]. In an *in vitro* model, the ability of pathogen-specific T cells to overcome suppression by regulatory T cells was shown to be dependent on TLR-induced production of IL-6 by DCs *in vitro*. Although the suppressor cells induced by e.c. immunization with protein Ag alone are both $CD25^+$ and $CD25^-$ [60], it is possible that DC-derived IL-6 is required to reverse CS suppression by e.c. immunization with Ag and TLR ligands. In our study we found that lymph node cells isolated from mice e.c. immunized with TNP-Ig/LPS produced significantly increased levels of IL-6 and IL-12 compared with PBS- or TNP-Ig-treated mice. There was also a significant difference in TNF-α production between TNP-Ig- and TNP-Ig/LPS-treated animals. This may suggest that reversal of e.c. immunization-induced tolerance via TLR stimulation can be mediated by pro-inflammatory cytokines such as IL-6, IL-12, and TNF-α. The hypothetical mechanism of e.c.--induced contrasuppression is illustrated in Fig. 4. However, we believe that the mechanism of regulatory action is more complex and also likely requires the participation of other, less conventional cell products (such as free cell receptors) [46]. We hypothesize that the ability to reverse suppressed T cell responses may have important therapeutic implications. Suppressive $CD4^+CD25^+$ T regulatory cells prevent immune responses to certain poorly immunogenic melanomas [65], and even immunogenic tumors have been described to induce $CD4^+$ T cell populations that suppress concomitant immunity against distal tumor inoculation [7]. If e.c. immunization with Ag in the presence of TLR ligands is capable of actively reversing suppression, and is not limited to particular species of laboratory animals, then it may provide a means of enhancing immunity in situations when it is required.

Finally, we want to stress that the experimental demonstration that TLR ligands can prevent the induc-

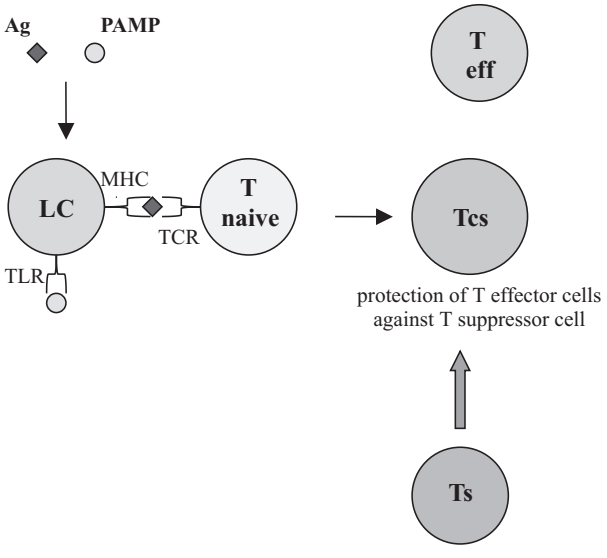

Fig. 4. The mechanism of skin-induced contrasuppression. LC – Langerhans cell, PAMP – pathogen-associated molecular patterns, TLR – Toll-like receptor, Teff – T effector cell, Ts – T suppressor cell, Tcs – T contrasuppressor cell.

tion of tolerance by skin-deposited protein antigens is not merely a laboratory artifact, as it has an equivalent in "normal" situations. Skin is permanently exposed to environmental antigens, including different proteins, which potentially can induce suppressor cells. The presence of skin microbial flora as a source of TLR ligands can thus be regarded as a safety valve to prevent suppression and thus safeguard the homeostasis of the immune system.

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