Regulatory T cells in cutaneous immune responses

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Abstract

Regulatory T cells (Treg) are a subset of T cells with strong immunosuppressive activity. In the skin, it has recently been revealed that Treg play important roles not only in the maintenance of skin homeostasis but also in the regulation of the immune responses, such as contact hypersensitivity and atopic dermatitis. Furthermore, the skin plays important roles in the induction of Treg in the periphery. In this review, we will provide an overview of the mechanism of Treg-mediated immunosuppression and discuss the role of Treg in the skin.

(88 words)

Contents

Introduction

Regulatory T cells (Treg) are a subset of T cells with strong immunosuppressive activity. Treg were originally identified as $CD4^+CD25^+$ T cells [1] [2]. When mice were depleted of $CD4^+CD25^+$ cells, they spontaneously developed autoimmune diseases and allergies, indicating that $CD4^+CD25^+$ T cells are essential for the maintenance of self-tolerance. Later on, the forkhead box p3 (Foxp3) gene was identified as the master transcriptional factor of Treg [3].

There are at least two kinds of $Foxp3^+$ Treg: naturally occurring Treg (nTreg) and inducible Treg (iTreg) [4]. nTreg develop in the thymus, and play an important role in the maintenance of self-tolerance and immune homeostasis. Scurfy mice, which possess a defective *Foxp3* gene, exhibit hyperactivation of $CD4^+$ T cells and overproduction of proinflammatory cytokines, and typically die within a month after birth [5]. Patients with IPEX syndrome (immune dysregulation polyendocrinopathy, enteropathy, X-linked syndrome) have a mutation in the human *FOXP3* gene, and are therefore regarded as the human counterpart of scurfy mice [6]. iTreg, on the other hand, are induced from naïve T cells in the presence of transforming growth factor (TGF)-β, and develop in the periphery. Retinoic acid facilitates the differentiation of naïve T cells to $Foxp3^+$ Treg [7] [8] and may be related to the establishment of oral tolerance, although it remains to be determined whether iTreg are functionally stable and to what extent they contribute under physiological conditions.

In addition to $Foxp3^+$ Treg, there are other types of Treg, such as Tr1 and Th3 cells; these are induced in the periphery [4] [9] [10]. Tr1 cells can be induced through the antigenic stimulation of naïve T cells in the presence of IL-10 *in vitro*, and exert a suppressive effect *in vitro* by inducing large amounts of IL-10 and TGF-β. Th3 cells

produce TGF-β in an antigen-specific manner, and exert a suppressive effect. Intriguingly, however, both are Foxp3 and CD25 negative. No further details of this population are discussed in this manuscript.

Evidence has accumulated regarding the regulatory roles of Treg not only in self-tolerance, but also in a variety of pathophysiological immune responses, such as gastritis [11], arthritis, encephalomyelitis [12], inflammatory bowel disease (IBD) [13], insulin-dependent diabetes [14] and various allergic skin diseases such as contact hypersensitivity or atopic dermatitis.

In this review, we will provide an overview of the mechanism of Treg-mediated immunosuppression, mainly focusing on $Foxp3^+$ Treg, and discuss the role of Treg in the skin immune responses, focusing on contact hypersensitivity and atopic dermatitis.

1. Mechanism of suppression by Treg

Treg potently suppress the proliferation of T cells when Treg are co-cultured with responder cells that have been stimulated with a specific antigen or a polyclonal T cell receptor stimulator *in vitro*. Multiple suppression mechanisms have been proposed based on *in vitro* assays; for example, IL-10 [13], TGF-β [15], and IL-35 [16] have been considered as possible soluble suppressive factors of T cell proliferation. Absorption of IL-2 by Treg may also be involved in inhibiting T cell proliferation [17]. It has also been reported that Treg exert their regulatory functions by cell-cell contact-dependent factors, such as CD39/CD73 [18] and granzyme/perforin [19]. In addition to these direct suppressive effects, Treg indirectly suppress T cell proliferation by affecting the function of APCs. It has been reported that Treg inhibited the T cell

stimulatory capacity of APCs by down-regulating CD80 and CD86 expression through cytotoxic T-lymphocyte antigen (CTLA)-4 and lymphocyte function-associated antigen (LFA)-1 [20]. Using two-photon microscopic analysis, Tadokoro et al [21] and Tang et al [14] have revealed that Treg inhibit stable contact and interaction between APCs and effector T cells. Treg also stimulate DCs to express the enzyme indoleamine 2,3-dioxygenase (IDO), which catabolizes the conversion of tryptophan to kynurenine, a toxic factor to T cells [22]. In addition to their effect on APCs, it has also been reported that Treg down-regulate mast cell function by suppressing mast cell degranulation and anaphylactic response through OX40-OX40L interaction [23]. The mechanisms by which suppression is achieved may vary depending on context, however, and it has not yet been determined how these *in vitro* findings correlate with *in vivo* suppression.

2. Characterization of Treg in the skin

Treg exist in all non-lymphoid tissues; the skin has a particularly high proportion of Treg in the steady state [24, 25] [26]. Treg in the skin are $CD44^{+}$ and $CD103^{high}$ [24, 25] [26], and express the chemokine receptors CCR4, CCR5, CCR6 and CCR7. $CCR5^{+}$ Treg preferentially migrate to cutaneous lesions of *Leishmania major* infection [27]. Mice with a complete loss of CCR4 on Treg develop spontaneous lymphocytic infiltration and severe inflammation in the skin and lungs, accompanied by peripheral lymphadenopathy and increased differentiation of skin tropic $CD4^{+}Foxp3^{-}$ T cells. Using α-1,3-fucosyltransferase VII (Fut7) deficient mice, Dudda et al [26] have reported the importance of E- and P-selectin ligand for Treg migration to the skin. Loss of these selectin bindings caused skin-specific inflammation, indicating the essential role of skin-resident Treg for maintaining immune homeostasis locally.

3. Treg induction and expansion in the skin

Ultraviolet (UV) radiation to the skin is well known to cause immunosuppression, and is accordingly applied as a treatment for a wide variety of skin diseases. Recently, it has been revealed that one of the immunosuppressive mechanisms involved in this effect is mediated by Treg, which are induced by UV irradiation [28]. It has been proposed that the cells responsible for this induction of Treg are epidermal Langerhans cells (LCs), an important group of skin-resident dendritic cells. Loser et al. [29] have reported that the receptor activator of NF-kappaB ligand (RANKL) was induced in keratinocytes by UV exposure, and RANKL-activated LCs were responsible for the development of UV-induced Treg. It has also been reported that the induction of Treg by UV irradiation was completely abolished by the depletion of LCs using Langerin-DTR mice or steroid mometasone [30] [31]. In addition, it has recently been reported that IL-10-producing and OX40 ligand-expressing mature LCs are responsible for the induction of Treg upon UV exposure [31], suggesting the importance of LCs for Treg induction. In addition to UV-induced immunosuppression, similar findings were observed concerning the mechanisms involved in immunosuppression during skin grafting. Yoshiki et al. [32] have reported that the development of contact hypersensitivity (CHS) was suppressed when mice were sensitized with a hapten through full-thickness grafted skin. In this model, $CD4^+CD25^+$ but not $CD4^+CD25^-$ T cells in draining lymph nodes (LNs) were responsible for this suppression. In addition, a high expression of RANKL was observed in the grafted skin, and recombinant RANKL stimulated LCs to produce IL-10. These findings suggest that the LCs play important roles in the peripheral induction of Treg. Recently, it has been reported that glucocorticoids modify LCs to produce TGF-β

and expand regulatory T cells in humans [33], implying that glucocorticosteroids may exert their anti-inflammatory functions by inducing Treg.

The phenotypes and suppression mechanisms of UV-induced Treg are different from those of nTreg. Schwartz et al. [34] [35] have reported that the administration of $CD4^+CD25^+$ cells from UV-irradiated DNFB-sensitized mice impaired sensitization of CHS. These UV-induced Treg did not suppress the CHS response when administered before elicitation, though natural $CD4^+CD25^+$ Treg did. Direct injection of UV-induced Treg into the elicitation sites did suppress the CHS response, however. They accordingly concluded that UV-induced Treg did not express skin-homing receptors for E- and P-selectins, and so failed to suppress elicitation. In addition, they reported that UV-induced Treg changed APCs in LNs from a stimulatory to a regulatory phenotype by modulating the co-stimulatory molecules on APCs, which, in turn, further induce Treg [36].

Although the importance of LCs has been suggested as mentioned above, other groups have reported the importance of dermal DCs in UV-induced immunosuppression and peripheral Treg induction. Wang et al. [37] reported the UV-induced immunosuppression was abolished by selective depletion of Langerin-positive dermal DCs, suggesting the importance of Langerin-positive dermal DCs in Treg induction. It has also been reported that retinoic-acid producing CD103-negative dermal dendritic cells have the ability to induce Treg in draining LNs [38], in contrast to the equivalent phenomenon in the gut, where CD103-positive DCs are responsible for the induction of Treg [39]

4. Treg in CHS

CHS, a frequently used mouse model of contact dermatitis, is a prototype of skin immune response, and the role of Treg in CHS has been gradually revealed.

The development of CHS is divided into two phases: sensitization and elicitation [40]. In the sensitization phase, low molecular weight compounds called haptens are cross-linked to epidermal proteins and taken up by resident DCs such as LCs and dermal DCs. Subsequently, these cells are matured by proinflammatory cytokines such as TNF-α, IL-1β, and prostaglandin E_2, and migrate to the draining LNs to present antigens in a CCR7 and CXCR4-dependent manner [41, 42]. After antigen presentation, naive T cells are activated and differentiated into antigen-specific Th1 and Tc1 cells under the influence of polarizing signals such as IL-12 and other chemical mediators [43]. Th17 cells are also involved in the pathogenesis of CHS [44]. When the skin is re-exposed to the same hapten after establishment of the sensitization, an antigen-specific T cell-mediated inflammation that is known as elicitation phase is provoked. Upon re-exposure to the same hapten, keratinocytes and mast cells produce chemokines and pro-inflammatory cytokines such as TNF-α and IL-1β, which activate endothelial cells and induce the expression of E- or P-selectins [45-47]. Then, neutrophils and antigen-specific T cells enter the dermis and release IFN-γ, which further stimulates keratinocytes to induce massive leukocyte infiltration [48].

a. Treg in the CHS response - elicitation phase

The effect of Treg on CHS has mainly been investigated in the elicitation phase. Ring et al. have purified $CD4^+CD25^+$ Treg from naïve mice and administered them into TNCB-sensitized recipient mice intravenously one day before elicitation [49].

Administration of Treg significantly suppressed the ear swelling response and inflammatory cell infiltration into the skin compared to those of vehicle-treated mice. Ring et al. have reported that these suppressive effects are mediated by soluble factors, especially IL-10. Administration of a culture supernatant of Treg suppressed the CHS response, and this suppression was reversed by an anti-IL-10 Ab. Furthermore, Treg from IL-10-deficient mice failed to suppress the CHS response by inhibiting the leukocyte influx into the inflamed skin.

The same group has recently reported that the adenosine produced by Treg is involved in blocking the influx of leukocytes into the skin by downregulating E- and P-selectins on endothelial cells [50]. Adenosine triphosphate (ATP) is first degraded by CD39 to adenosine diphosphate (ADP) and then to adenosine monophosphate (AMP). The AMP is serially dephosphorylated by CD73 to adenosine. Treg are strongly positive for both CD39 and CD73 expression; therefore, Treg convert ATP to adenosine and suppress the CHS response. On the other hand, conventional T cells exhibit only a low basal expression level of CD39. Accordingly, injection of adenosine or Treg abrogated the ear-swelling response in CHS, which was not seen using Treg from CD39-deficient mice [50]. Moreover, Treg further upregulate CD39 expression after activation; this activation is a prerequisite for Treg to acquire their suppressive capacity.

b. Treg in the CHS response - sensitization phase

While reports on the role of Treg in the sensitization phase have been rather limited compared to those discussing the elicitation phase, some interesting reports have recently been published. Dubois et al. [51], for example, have reported the involvement of Treg in the induction of oral tolerance and inhibition of DNFB-induced CHS. Oral tolerance was induced by feeding DNFB orally prior to DNFB sensitization. Although

no such tolerance induction was seen in $CD4^+$ T cell-deficient mice, transfer of naïve $CD4^+CD25^+$ T cells restores oral tolerance in those mice, in a manner independent of IL-10 [51]. The same authors also showed that administration of neutralizing anti-CD25 monoclonal antibody (mAb) impairs oral tolerance in WT mice. Intriguingly, administration of anti-CD25 mAb before sensitization had no significant affect on the ear swelling response, suggesting that $CD4^+CD25^+$ T cells are responsible for oral tolerance induction, while the role of Treg in the sensitization phase remained unclear. Ring et al. have recently reported that the administration of Treg suppressed the extent of sensitization in CHS by inhibiting DCs and CD8 T cells in the draining LNs [52]. In their report, Treg and DCs established a gap junctions, which caused a reduction in the capacity of DCs to stimulate CD8 T cells. In their next report, the same authors stated that Treg activation in draining LNs was mediated by ATP, because Treg acquired an activated phenotype upon ATP treatment *in vitro*, while blockage of ATP receptors on Treg abrogated ATP-mediated activation and suppressive function of Treg *in vivo* [53].

c. The role of endogenous Treg in CHS

As described above, exogenous administration of Treg suppresses CHS both in the sensitization phase and in the elicitation phase. It remains unclear, however, whether endogenous Treg play the same suppressive role under physiological conditions. To this end, specific depletion of Treg *in vivo* is required. Although $CD4^+CD25^+$ has been used as a marker for Treg, CD25 is expressed in activated CD4 cells as well as in Treg. Therefore, Foxp3 is a more definitive marker of Treg, but because Foxp3 is a transcriptional factor that exists intracellularly, the purification of live Treg or depletion by means of neutralizing mAb has been technically difficult.

To solve these problems, Foxp3 reporter mice expressing human CD2 and human CD52 chimeric protein have been generated and designated as Foxp3$^{hCD2/hCD52}$ mice. Since Foxp3^{+} cells co-express hCD2 on the cellular surface, live Foxp3^{+} Treg are sorted with anti-hCD2 mAb and depleted with neutralizing anti-hCD52 Ab [25]. The mice have been used in the investgations into the role of endogenous Treg in CHS. Depletion of Treg in the elicitation phase caused the ear swelling response to be enhanced and prolonged compared with that seen in the control, indicating that Treg is responsible for terminating skin inflammation in CHS [25].

In addition, the role and mobility of Treg in the skin during CHS was investigated. Kaede-transgenic mice are genetically engineered to ubiquitously express Kaede protein, a photoconvertible protein that changes its fluorescence from green to red under exposure to violet light. Therefore, mobility of cells from the skin under physiological conditions can be analyzed. Treg were found to localize abundantly in the inflamed skin seen in CHS, and these skin Treg were found to migrate further back to draining LNs. Treg from the skin showed significantly higher mRNA expression of T cell suppression-associated molecules such as IL-10, TGF-β and CTLA4. Consistently, Treg from the skin exhibited significantly stronger suppressive activity both *in vivo* and *in vitro* (**Figure 1**). These results suggest that Treg in the skin also play important roles in the termination of dermatitis and possibly in the control of systemic immune responses.

It has been suggested that Treg in the skin contribute to its homeostasis, since chronic depletion of skin Treg leads to the development of spontaneous dermatitis [24] [26]. Schneider et al. have reported that CCR7-deficient mice showed a reduced number of Treg in draining LNs and an enhanced inflammatory response in CHS after repeated hapten application [54], which suggests the homing of Treg to draining LNs through

CCR7 plays an important role in eliciting the function of Treg.

Endogenous Treg regulate the extent of sensitization as well as that of challenge in CHS. Depletion of Treg during the sensitization phase leads to enhanced skin inflammation [55]. Mice depleted with Treg population showed increased numbers of memory T cells and higher expression levels of costimulatory molecules in DCs in draining LNs compared with control mice, suggesting that endogenous Treg modulate DC function and thus regulate the extent of sensitization [55]. Recent findings on the role of Treg in CHS are summarized in Table 1, and schematic views of those findings are illustrated in Figures 3 and 4

5. Atopic dermatitis (AD) and Treg

Atopic dermatitis is one of the most common skin inflammatory disorders. New insights point to an important role of structural abnormalities in the epidermis combined with immune dysregulation [56]. Although studies on the role of Th2 cells have focused on the pathophysiology of AD, recent reports have indicated the importance of other T cell subsets such as Th17 [57] and Treg.

Ou et al. [58] have compared the numbers and functionality of peripheral blood mononuclear cells (PBMC) between healthy controls and AD patients, and reported that AD patients have higher numbers of Treg, each with a suppressive activity comparable to that of Treg in healthy controls, in the peripheral blood. Others have also reported that increased numbers of Treg in the PBMC of AD patients [59] and expansion of Treg were positively associated with disease activity in AD [60]. On the other hand, it has also been reported that the numbers of Treg among the PBMC are similar between AD and healthy controls [61]. In AD skin lesions, it was initially reported that Treg were

absent, while Tr1 were detected [62]. Later on, however, several groups reported the existence of Treg in AD skin lesions [63, 64]. Because AD is a chronic inflammatory disease with multiple disease stages and multiple factors, and because some treatments for AD such as cyclosporine [59, 61, 65], glucocoriticoids [33] and UV radiation [28], can alter the number of Treg in the PBMC, the interpretation and comparison of these studies will require careful attention.

Based on observations of IPEX syndrome patients, who show atopic-like dermatitis and high IgE levels, however, it seems probable that the number of Treg is related to the development of AD lesions [6]. As for the function of Treg in AD, it has been reported that their suppressive activity is similar to that of Treg in healthy controls [58]. Reefer et al., however, have reported that a new subtype of Treg with Th2-promoting ability has been observed in AD and that its functions depend on the expression of CCR6 [66]. In this report, CCR6-negative CD25-high positive Treg produced Th2 cytokines, and co-culture with effector T cells selectively enhanced IL-5 production, suggesting the heterogeneity of Treg in AD.

Recently, dysfunction of Treg has been reported in psoriasis [67], another chronic inflammatory skin disease. Treg in both lesional skin and blood from psoriasis patients showed reduced suppressive activity [67], and such dysfunction was dependent on the signaling from IL-6, which was abundantly produced in psoriasis lesion [68]. Local cytokine milieu in AD may also alter the function of Treg in AD skin.

Conclusion

We have reviewed the roles of Treg in cutaneous immune responses. A considerable

amount of knowledge on Treg has been accumulated, and multiple mechanisms and various molecules are reported to be involved in Treg-mediated immunosuppression. It is likely that the suppressive mechanisms of Treg may differ depending on disease stage and the skin immune response type. Analysis using Foxp3-diphtheria toxin receptor knockin mice or Foxp3$^{hCD2/hCD52}$ mice, which enable us to deplete Treg conditionally and specifically, will further reveal the molecular mechanisms and physiological functions of Treg in cutaneous immune responses.

It is crucially important to clarify how and to what extent those molecules are involved in Treg function in humans. From a clinical perspective, the precise mechanism by which Treg function in the elicitation phase is an important issue to be addressed, since most patients with cutaneous immune disease have already been sensitized. We expect that further effort in the investigation of Treg will give us important clues supporting the development of innovative therapeutic approaches for various skin diseases.

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Figure legends

Figure1. Possible mechanisms involved in suppression by Treg

(a) Soluble factor-dependent mechanisms. Treg produce large amounts of IL-10, IL-35, and TGF-beta, all of which suppress naive/effector T cell activation. Treg also absorb IL-2, which causes cytokine deprivation-induced apoptosis among effector T cells. (b) Contact-dependent mechanisms. CTLA-4 on Treg deliver negative signals to T cells. CD39/CD73 on Treg catalyze ATP and generate pericellular adenosine, exerting an anti-inflammatory effect. Treg also may kill responder T cells by a granzyme or perforin-dependent mechanisms. (c) Indirect mechanisms. Treg inhibit the interaction between DCs and effector T cells. Treg also downregulate DC activation and thus cause immunosuppression.

Figure 2. Proposed mechanism of Treg induction by skin DCs

UV exposure or skin grafting induces RANKL expression on keratinocytes, which stimulate LCs. RANKL-stimulated LCs then induce Treg in draining LNs. Under conditions of UV exposure, it has also been proposed that the UV-induced Treg affect DCs and modify their functions from a stimulatory phenotype to a regulatory phenotype, which further induces Treg. In addition to LCs, CD103-negative dermal DCs can induce Treg in draining LNs.

Figure 3. Possible mechanism of suppression by Treg in sensitization phase of CHS

Treg are activated in draining LNs by ATP. They down-regulate DC activation through gap junction formation and subsequent T cell proliferation, which controls the extent of sensitization.

Figure 4. Possible mechanism of suppression by Treg in elicitation phase of CHS

Treg suppress effector T cells in the LNs and inhibit leukocyte influx into the periphery through IL-10 or CD39-dependent mechanisms. In addition, Treg migrating into the skin could suppress the effector T cell functions in the skin. Furthermore, a fraction of Treg in the skin migrate back to the draining LNs through afferent lymphatic vessels, and can return from there to the skin. These skin-derived Treg possess higher suppression activity than LN-resident Treg, and contribute to the termination of skin inflammation.

Table 1. An overview of recently published papers about Treg and CHS

	Major findings	Reference
Sensitization	Attenuated sensitization by Treg induced by RANKL-activated LC in a UV-immunosuppression model	[29]
	Attenuated sensitization by Treg induced by IL-10 from RANKL-activated LC in a skin graft immunosuppression model	[32]
	Attenuated sensitization by Treg induced by orally administered antigen in an oral tolerance model	[51]
	Treg attenuate sensitization by modifying DC function through gap junction formation	[52]
	Treg acquire an activated phenotype by means of ATP in draining LNs	[53]
	Enhanced ear swelling response resulting from the depletion of endogenous Treg	[55]
Elicitation	Reduced ear swelling response resulting from the inhibition of the leukocyte influx through IL-10 from Treg	[49]
	Reduced ear swelling response resulting from the inhibition of the leukocyte influx through adenosine from Treg via CD39/CD73 (inhibition of E- and P-selectin expression in endothelial cells)	[50]
	Treg acquire activated phenotype by means of ATP in blood.	[53]
	Enhanced and prolonged ear swelling response resulting from depletion of endogenous Treg	[25]

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Figure 1 Possible mechanisms involved in suppression by Treg

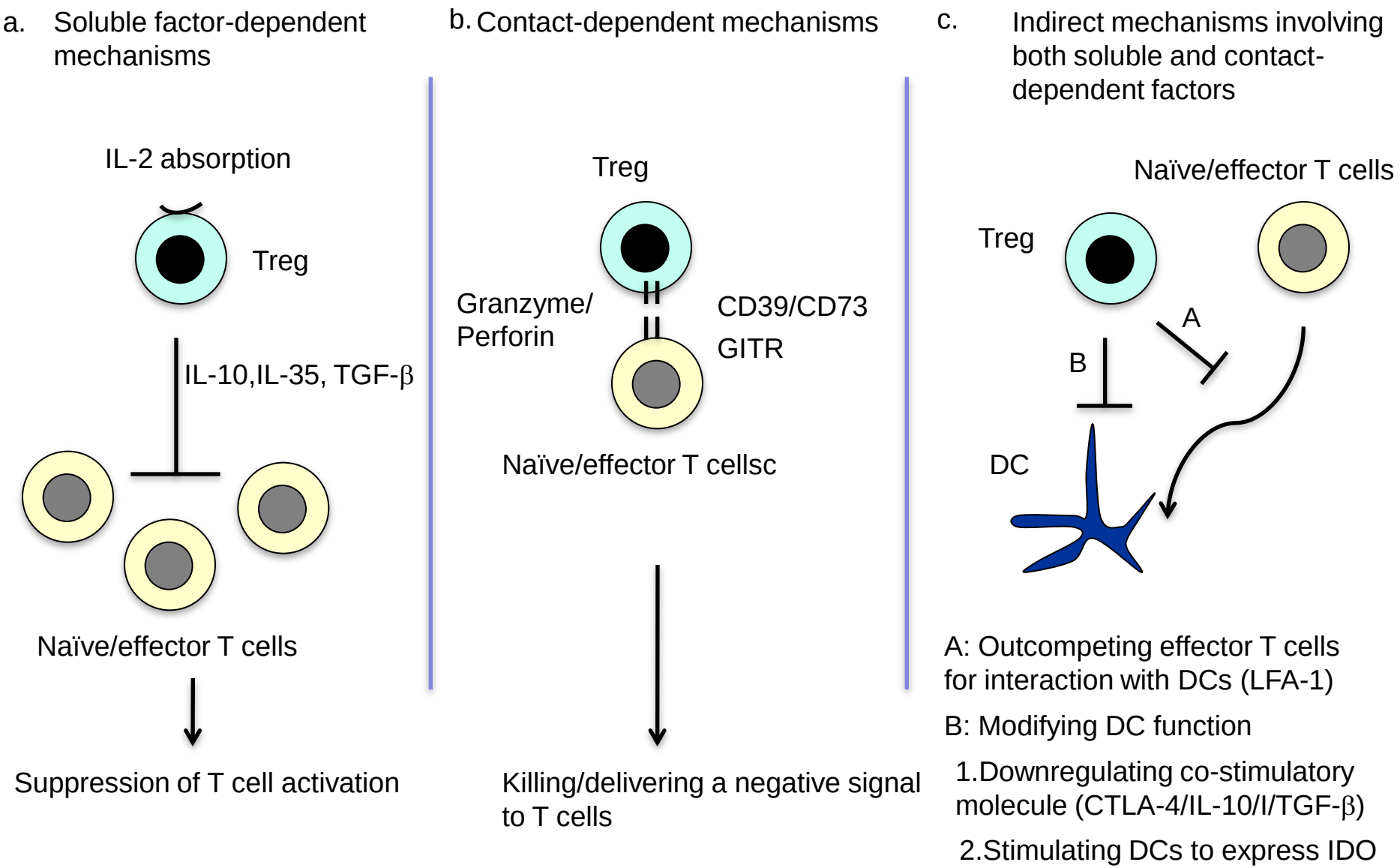

Figure 2

Proposed mechanism of Treg Induction by skin DCs

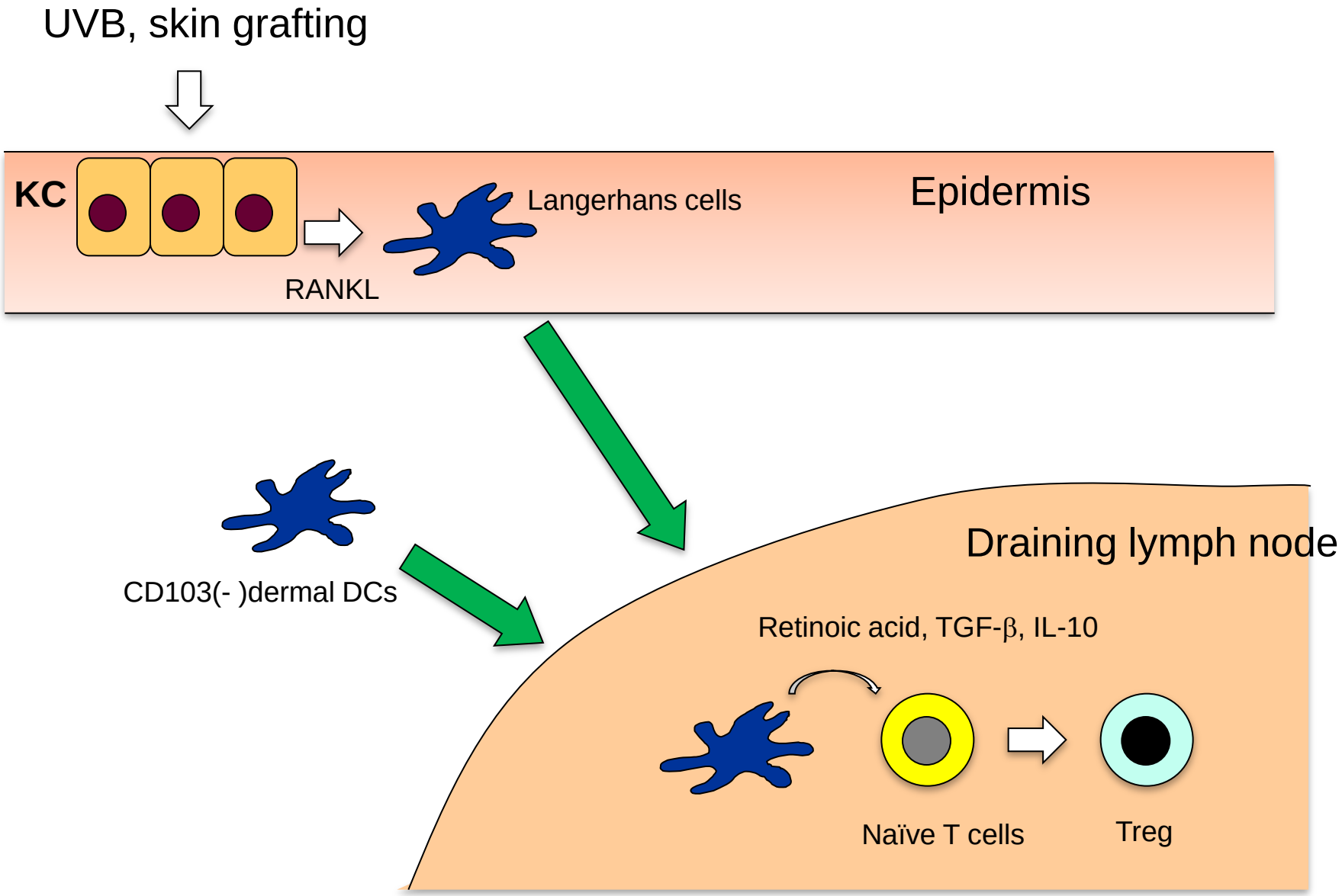

Figure 3 CHS: Sensitization phase

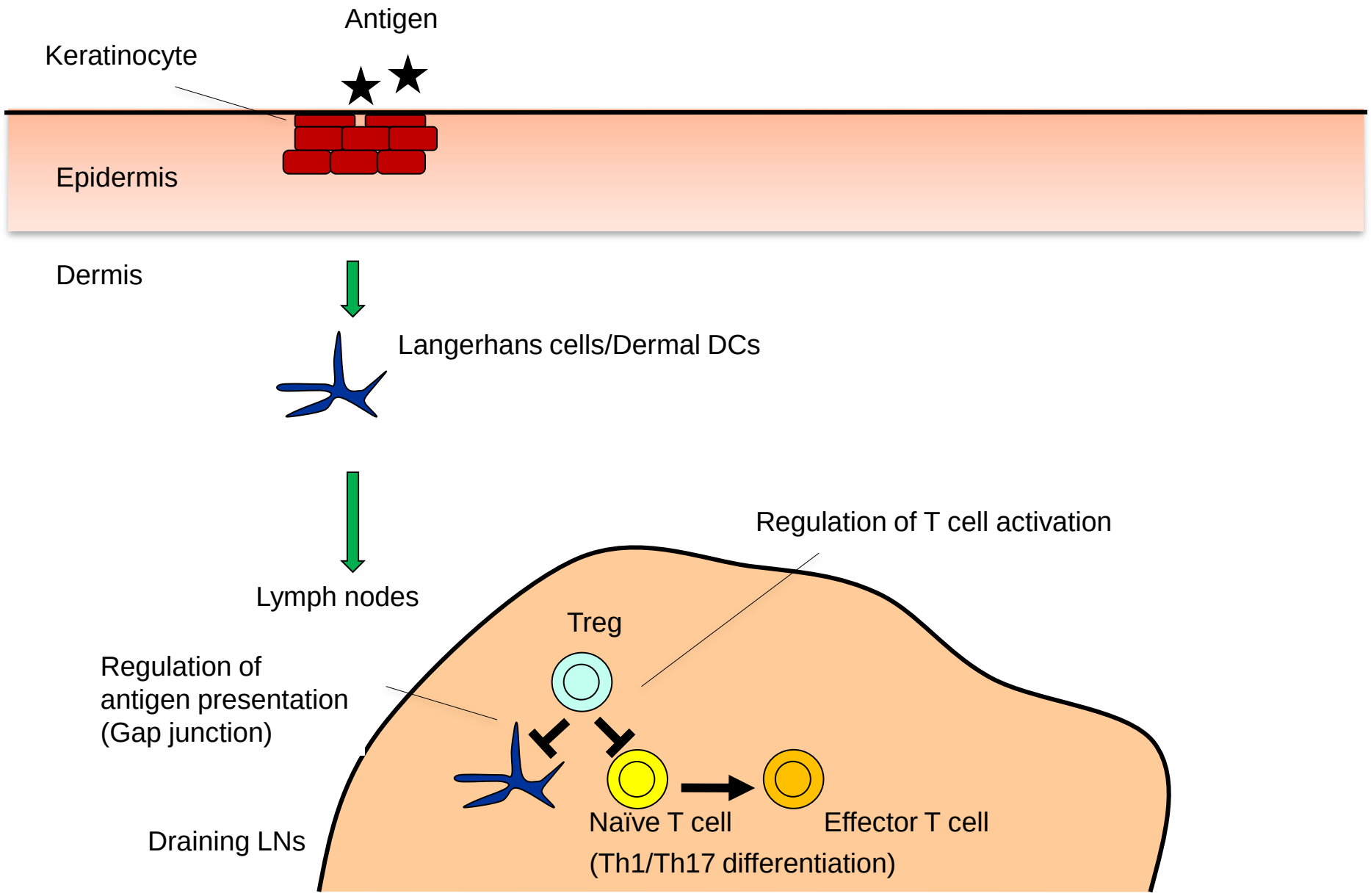

Figure 4 CHS: Elicitation phase