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Letter to the Editor

Possible involvement of CCR4⁺CD8⁺ T cells and elevated plasma CCL22 and CCL17 in patients with Rhododendrol-induced leukoderma



Keywords:

Rhododendrol-induced leukoderma; CCR4; CCL22; Immune response

Rhododendrol (4-(4-hydroxyphenyl)-2-butanol), brand name: Rhododenol) is a naturally occurring phenol used in skin-whitening cosmetics and has been reported to cause a hypopigmentary disorder called Rhododendrol-induced leukoderma (RIL) which developed in approximately 16,000 (2%) consumers [1]. Recent pathological analyses of RIL have revealed a direct cytotoxic effect of rhododendrol on melanocytes, which may explain the loss of melanocytes in the lesional skin [1]. In addition, we speculated that local infiltration of various immune cells may be involved in the pathophysiology of RIL [2]. A skin homing receptor C-C chemokine receptor type 4 (CCR4) is reported to be expressed in a greater proportion of CD8⁺ T cells in the blood of nonsegmental vitiligo patients than that observed in healthy individuals [3]. This receptor is required for skin homing of T cells via its ligands, C-C chemokine ligand (CCL) 17 [4] and CCL22 [5]. Although CCR4 is primarily expressed on CD4⁺ T cells and regarded as a marker of type 2 helper T cells [6], CD8⁺ T cells also express CCR4. These CCR4⁺CD8⁺ T cells function to secrete multiple cytokines rather than act as cytotoxic cells [7]. We herein investigated the frequencies of CCR4⁺ T cells and its ligands in the blood and lesional skin of RIL patients.

Nineteen patients who gave written informed consent were informed in Table S1. Percentage of CCR4-expressing cells among CD3⁺ T cells was 27.40% in the RIL patients and 28.20% in the nonsegmental vitiligo patients in contrast to 18.55% in healthy

controls (RIL, $P = 0.0282$; nonsegmental vitiligo, $P = 0.0500$; Fig. 1A) while CCR4⁺CD4⁺ T cells was not increased in the patients. Interestingly, CCR4⁺CD8⁺ T cells were significantly increased in the patients (RIL, $P = 0.0109$; nonsegmental vitiligo, $P = 0.0104$; Fig. 1B). The percentage of CCR4⁺CD8⁺ T cells correlated to the duration after withdrawal of rhododendrol before sampling (Fig. 1C). Therefore, we focused on CCR4⁺CD8⁺ T cells in the RIL patients for further experiment. To investigate whether the CCR4⁺CD8⁺ T cells infiltrated into the RIL skins, a double immunohistochemical analysis of CCR4 and CD8 was performed (Fig. 1D), resulting in increased number of CCR4⁺CD8⁺ T cells in the RIL tissue samples (24.25/field for the RIL lesions compared to 6.50/field for healthy control skins; $P = 0.0569$) Fig. 1E). In addition, we found significantly increased ratio of CCR4⁺CD8⁺ T cells to whole CD8⁺ T cells in the RIL samples (39.43% for the RIL lesions compared to 8.576% for the nonsegmental vitiligo lesions and 6.280% for healthy skin samples; nonsegmental vitiligo, $P = 0.0498$; normal control, $P = 0.0144$; Fig. 1F). Peripheral blood T cells from ten patients analyzed twice. Most of them showed reduced CCR4 expression on CD8⁺ T cells over time (Fig. 1G). Regarding analysis for CCR4 ligands, CCL22 was expressed by infiltrating mononuclear cells in the lesional skin of RIL patients (Fig. 2A–C), although no CCL17 expression was observed (data not shown). Number of CCL22⁺ cells was increased in vitiligo and RIL lesions compared to healthy skins (Fig. 2D). The cells expressing CCL22 were positive for CD1a or CD68, suggesting dermal dendritic cells or macrophage, respectively (data not shown). The plasma CCL17 and CCL22 concentrations were elevated in the RIL patients compared to those in vitiligo and healthy individuals (Fig. 2E and F depicted CCL22 and CCL17, respectively).

As increased frequencies of CCR4⁺CD8⁺ T cells in the peripheral blood have been reported in patients with cutaneous diseases, in which immunity is regarded to be involved [3,8], we investigated the frequency of CCR4⁺ T cells in RIL patients in order to explore the possible involvement of immunity and potential therapeutic targets for this disease. Consequently, we found that the frequency of CCR4⁺CD8⁺ T cells was increased in the peripheral blood of patients with RIL as well as those with nonsegmental

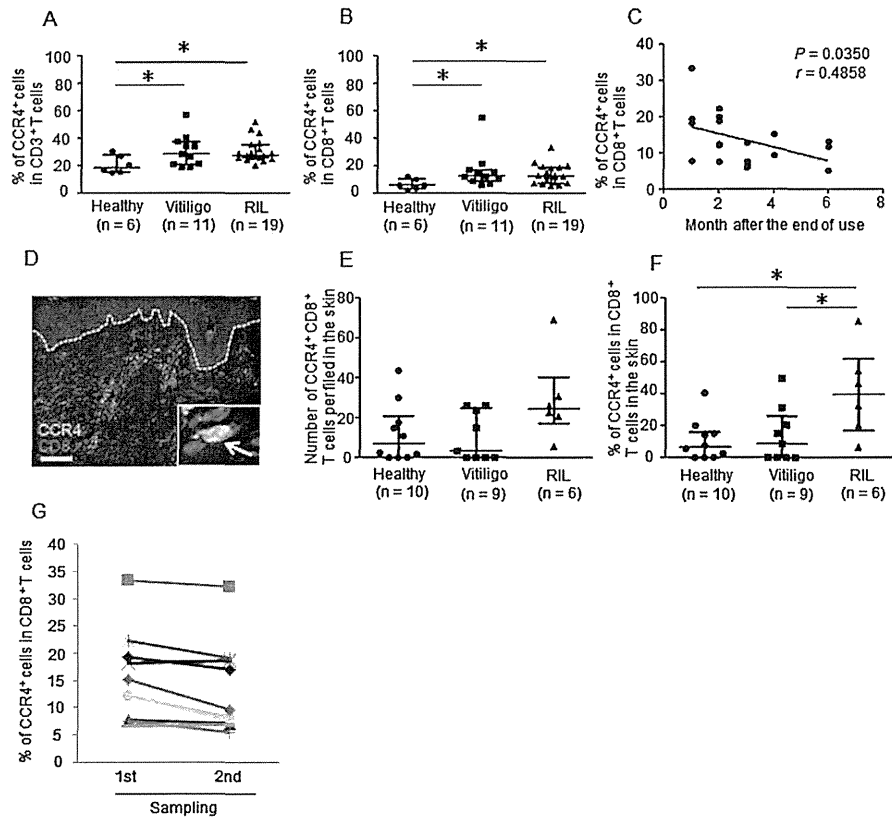


Fig. 1. Increased percentage of CCR4⁺ cells among CD8⁺ T cells in the peripheral blood and skin of Rhododendol-induced leukoderma patients. The percentages of CCR4⁺ T cells among (A) CD3⁺ T cells, (B) CD8⁺ T cells in the PBMCS of healthy control, nonsegmental vitiligo and RIL samples are shown. (C) Correlation between percentage of CCR4⁺ cells in CD8⁺ cells and month after the withdrawal of Rhododendol-containing cosmetics. (D) Representative double immunostaining for CCR4 (green) and CD8 (red) in the RIL skin. Inset shows double positive cell with higher magnification. Bar indicates 100 μ m. (E, F) Infiltration of CCR4⁺CD8⁺ T cells into the skin of the healthy control, nonsegmental vitiligo and RIL samples. Bars represent the median \pm interquartile range. * $P \leq 0.05$. (G) Percentage of CCR4⁺ cells in CD8⁺ cells were compared in ten RIL patients.

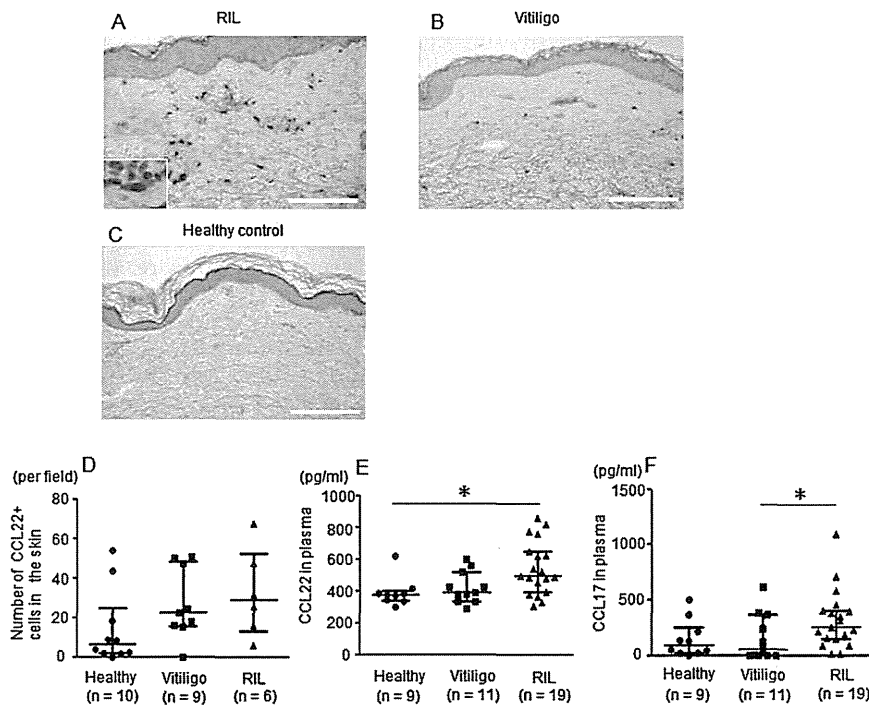


Fig. 2. Increase in CCL17 and CCL22 in the plasma and skin of the Rhododendol-induced leukoderma patients. Representative immunostaining for CCL22 (red, higher magnification view shown in box) in the lesional skin of RIL patient (A), nonsegmental vitiligo patient (B), and healthy control (C). Bar indicates 200 μ m. (D) Comparison of numbers of CCL22⁺ cells in the skin sample among RIL, nonsegmental vitiligo, and healthy control. Comparison of concentration of plasma CCL22 (E) and CCL17 (F) among RIL, nonsegmental vitiligo, and healthy control. Bars represent the median \pm interquartile range. * $P \leq 0.05$.

vitiligo, as reported previously by Zhang et al. [3]. Although both the RIL and vitiligo patients in this study showed increased frequencies of CCR4⁺CD8⁺ T cells, these cells are expected to target different antigens in each disorder. Pathogenic CD8⁺ T cells in nonsegmental vitiligo, which may express CCR4 in order to migrate into the skin, are thought to be specific for melanocyte antigens [9]. In fact, melanocyte antigen-specific CD8⁺ T cells are reported to express another skin homing marker, cutaneous leukocyte-associated antigen (CLA) [9]. On the other hand, in patients with RIL, considering that symptoms usually develop only at Rhododendrol-applied sites [2], CCR4⁺CD8⁺ T cells may target endogenous molecules modified by rhododendrol. In addition to the specificity of skin homing T cells, further studies focusing on the functions of these CCR4⁺CD8⁺ T cells, such as cytokine secretion, are needed to elucidate what role CCR4⁺CD8⁺ T cells play in the pathogenesis of these diseases. Considering that CCL22 is a dominant ligand in inducing receptor desensitization and internalization [5], the reduction of CCR4 expression on CD8⁺ T cells over time may be explained by the dominant expression of CCL22 in the lesional skin and plasma. In cases of atopic dermatitis, CCL17/TARC is considerably important with respect to the inflammatory process [10]; although both CCL17 and CCL22 in plasma were increased in the RIL patients, CCL22-associated chemotaxis contributing to skin inflammation may be the more characteristic of RIL. It is limitation of this study that specimens from 98% of the consumers without leukoderma were not analyzed due to ethical considerations.

In conclusion, the frequency of CCR4⁺CD8⁺ T cells and CCL17 and CCL22 concentrations were increased in the blood of RIL patients. In addition, CCR4⁺CD8⁺ T cells and CCL22⁺ not CCL17⁺ monocytes were preferentially found to infiltrate into the lesional skin in the affected patients, providing the possibility of their involvement in skin inflammation and hypopigmentation in RIL by chemo-attractive fashion. We believe that these results help to clarify the pathogenesis of cosmetics-induced leukoderma, which may lead to the development of novel therapeutic agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jdermsci.2015.02.014>.

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Letter to the Editor

Melanocyte-specific cytotoxic T lymphocytes in patients with rhododendrol-induced leukoderma



To the Editor,

Various chemicals have been reported to cause leukoderma or occupational vitiligo. Historically, mono-bensyl hydroquinone was a typical causative agent for depigmentation of the skin

of tannery workers wearing rubber gloves [1]. Since then, it has become apparent that some substituted phenols cytolyse melanocytes. Vitiligo-like lesions may appear not only at the contact sites of chemicals but also subsequently at non-contact areas [2]. Despite their leukoderma-evoking potential, substituted phenols are still used as whitening cosmetic agents because of their efficacies. Recently around 2013, in Japan and some Asian countries, women who used whitening cosmetic agents containing rhododendrol (4-(4-hydroxyphenyl)-2-butanol) have pre-

We have shown that transcriptional noise is well predicted by molecularly detailed models for the two most common promoter architectures in *E. coli* as the various genetic knobs are tuned. This agreement is not the result of fitting theory curves to data, because the predicted curves are generated using physical parameter values reported elsewhere in the literature and in that sense are zero-parameter predictions. Earlier reports of “bursty” transcription (5, 21) are based on the observation that the Fano factor is greater than 1 for constitutive mRNA production (as well as direct kinetic measurements). Various explanatory hypotheses have been proposed, including transcriptional silencing via DNA condensation by nucleoid proteins (22), negative supercoiling induced by transcription, or the formation of long-lived “dead-end” initiation complexes (23). Although our data do not rule out these hypotheses, we find that extrinsic noise is sufficient to explain the deviation from Fano = 1 in our constitutive expression data (Fig. 2B). Thus, we find no need to invoke alternative hypotheses to explain the observed “burstiness” of constitutive transcription.

Many interesting earlier experiments make it difficult to interpret differences between promoters and induction conditions in terms of distinct physical parameters because of the wide variety of promoter architectures in play as well as the diverse mechanisms of induction. We have instead taken a “synthetic biology” approach of building promoters from the ground up. By directly controlling aspects of the promoter architecture, our goal has been to directly relate changes in promoter architecture to changes in observed gene expression variability. We believe that this work has demonstrated that mutations in regulatory DNA can alter gene expression noise. This suggests that gene expression noise may be a tunable property subject to evolutionary selection pressure, as mutations in regulatory DNA could provide greater fitness by increasing (or decreasing) variability. Demonstrating the relevance of this hypothesis in natural environments remains an ongoing challenge.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/346/6216/1533/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S11
Tables S1 to S3
References (24–32)

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IMMUNE TOLERANCE

Detection of self-reactive CD8⁺ T cells with an anergic phenotype in healthy individuals

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Immunological tolerance to self requires naturally occurring regulatory T (T_{reg}) cells. Yet how they stably control autoimmune T cells remains obscure. Here, we show that T_{reg} cells can render self-reactive human CD8⁺ T cells anergic (i.e., hypoproliferative and cytokine hypoproducing upon antigen restimulation) in vitro, likely by controlling the costimulatory function of antigen-presenting cells. Anergic T cells were naïve in phenotype, lower than activated T cells in T cell receptor affinity for cognate antigen, and expressed several coinhibitory molecules, including cytotoxic T lymphocyte–associated antigen-4 (CTLA-4). Using these criteria, we detected in healthy individuals anergic T cells reactive with a skin antigen targeted in the autoimmune disease vitiligo. Collectively, our results suggest that T_{reg} cell–mediated induction of anergy in autoimmune T cells is important for maintaining self-tolerance.

Naturally occurring CD25⁺CD4⁺ regulatory T (T_{reg}) cells, which specifically express the transcription factor FoxP3, actively maintain immunological self-tolerance and homeostasis (1). Developmental or functional anomalies of natural T_{reg} cells can cause autoimmune diseases (such as type I diabetes), allergy, and immunopathological diseases (such as inflammatory bowel disease) (2). How T_{reg} cells effectively control potentially hazardous self-reactive T cells in humans remains an open question. In particular, it is unknown whether T_{reg} cell–mediated suppression for a limited period has a critical long-lasting effect on cell fate and antigen reactivity of autoimmune T cells.

To address this issue, we examined proliferation, cytokine production, and cell fate of antigen-

specific CD8⁺ T cells in peripheral blood mononuclear cells (PBMCs) from healthy individuals stimulated in vitro with self-antigen peptide in the presence or absence of natural FoxP3⁺CD25⁺CD4⁺ T_{reg} cells. Melan-A (also known as MART-1) used in the experiments is a self-antigen expressed by normal melanocytes and some melanoma cells and targeted in vitiligo vulgaris, an autoimmune disease of the skin (2–5). In the absence of T_{reg} cells, Melan-A–specific CD8⁺ T cells [detectable by major histocompatibility complex (MHC) tetramers and peptide tetramers] expanded over 10 days from very few cells to a sizable fraction when cultured with peptide-pulsed autologous antigen-presenting cells (APCs) (Fig. 1A) (6). Natural T_{reg} cells, which appeared to be activated by endogenous self-peptides and class II MHC on autologous APCs (7–9), suppressed the expansion of Melan-A tetramer–positive (Tet⁺) CD8⁺ T cells in a dose-dependent manner. Similar stimulation with irrelevant peptide NY-ESO-1, another self- and tumor antigen, failed to induce Melan-A Tet⁺ CD8⁺ T cells. In cultures containing T_{reg} cells,

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we noted an accumulation of Tet⁺CD8⁺ T cells that had divided once and then stopped further proliferation. This proliferation-aborted population increased in ratio, whereas the population under multiple cell divisions reciprocally decreased, in proportion to the number of added T_{reg} cells. The proliferation-aborted cells had significantly lower tetramer staining intensity than the cells that had vigorously proliferated in the absence of T_{reg} cells (peak a versus b in Fig. 1A, Fig. 1B, and fig. S1). The staining intensity of T cell receptor- $\alpha\beta$ (TCR- $\alpha\beta$) chains was equivalent in both populations, which indicated that the lower tetramer staining intensity was not due to down-modulation of TCR but to lower TCR affinity for the Melan-A peptide, as supported by significantly lower ratios of tetramer versus TCR- $\alpha\beta$ staining

intensities (Fig. 1C). Functionally, they produced reduced levels of cytokines such as interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), and interleukin 2 (IL-2) (Fig. 1, D and E), despite the addition of exogenous IL-2 to maintain cultured T cells. Furthermore, upon secondary stimulation, they remained hypoproliferative and produced very low amounts of cytokines (fig. S2). Thus, antigenic stimulation under T_{reg} cell-mediated suppression allows responder T cells with relatively low affinity TCRs for a self-antigen to divide once but prevents their further proliferation, which drives them into a profoundly and stably hypoproliferative and cytokine-hypoproducing state, which can be immunologically defined as “energy” (10–13).

In contrast with anti-Melan-A responses, CD8⁺ T cells from the same donor, who had detectable

serum anticytomegalovirus (CMV) immunoglobulin G (IgG) antibody, had CMV peptide-specific T cells with a memory phenotype (fig. S3, A to D). CMV-specific CD8⁺ T cells, whether they were in a naïve or memory cell fraction, vigorously proliferated and produced inflammatory cytokines even at a high T_{reg}-to-responder T cell ratio, with no significant differences in CMV tetramer staining intensity among CD8⁺ T cells proliferating in the presence or absence of T_{reg} cells (fig. S3, E to H). However, high numbers of T_{reg} cells completely inhibited the proliferation of polyclonally activated naïve CD8⁺ T cells without allowing a single cell division (fig. S4). The nondividing CD8⁺ T cells proliferated as actively as nonsuppressed cells upon restimulation after removal of T_{reg} cells.

Collectively, T_{reg}-cell dosage, the immunological states of responder T cells (e.g., in a naïve or memory state), and their TCR affinity for cognate antigen contribute to T_{reg} cell-mediated induction of energy. This is an active process and differs from a mere naïve nonproliferative state.

Microarray gene expression analysis revealed that activated or anergic Tet⁺CD8⁺ T cells or Tet⁺CD8⁺ T cells obtained from T_{reg}-absent or -present cell cultures were substantially different in gene expression profiles (Fig. 2A). As the most striking differences, the transcription of *CTLA4*, encoding the coinhibitory molecule CTLA-4 (14), was significantly up-regulated, whereas *BCL2*, encoding the apoptosis-inhibiting molecule B cell lymphoma-2 (BCL-2) (15), was down-regulated in anergic CD8⁺ T cells, as confirmed by quantitative reverse transcription polymerase chain reaction (RT-PCR) (Fig. 2B). There were no significant differences in the expression of *PDCDI* encoding the coinhibitory molecule PD-1; the genes encoding the energy-related molecules *GRAIL*, *CBL-B*, and *EGR-2* (16–19); *BAT3*, *TBX21*, and *EOMES*, putative markers for exhausted CD8⁺ T cells (20, 21); and *p27KIP1*, a cyclin-dependent kinase inhibitor. Anergic CD8⁺ T cells did not express *FoxP3* (Fig. 2B and fig. S5A). The majority (>90%) of anergic CD8⁺ T cells expressed both *CTLA-4* and the chemokine receptor *CCR7*, which differed from the phenotype of activated or naïve CD8⁺ T cells (Fig. 2, C and D, and fig. S5, B to D) (22, 23). Functionally, during secondary stimulation of anergic Tet⁺CD8⁺ T cells with Melan-A peptide-pulsed APCs after removal of T_{reg} cells, antibody blockade of *CTLA-4* and *PD-1* at doses enhancing cytokine production by activated conventional T cells failed to rescue proliferation resistance or cytokine hypoproduction of anergic CD8⁺ T cells (fig. S5E) (24). Addition of a high dose of IL-2 induced apoptosis in restimulated Tet⁺CD8⁺ T cells rather than abrogating their hyporesponsiveness. Nevertheless, anergic CD8⁺ T cells were not in the process of immediate apoptosis (fig. S6), despite their lower *BCL2* expression than activated T cells (Fig. 2B). Thus, anergic CD8⁺ T cells induced by T_{reg} cell-mediated suppression are distinct from activated or naïve T cells in gene and protein expression profiles. They also appear to be different from “exhausted” CD8⁺ T cells, which develop as PD-1⁺ hypoproliferative and cytokine-hypoproducing cells

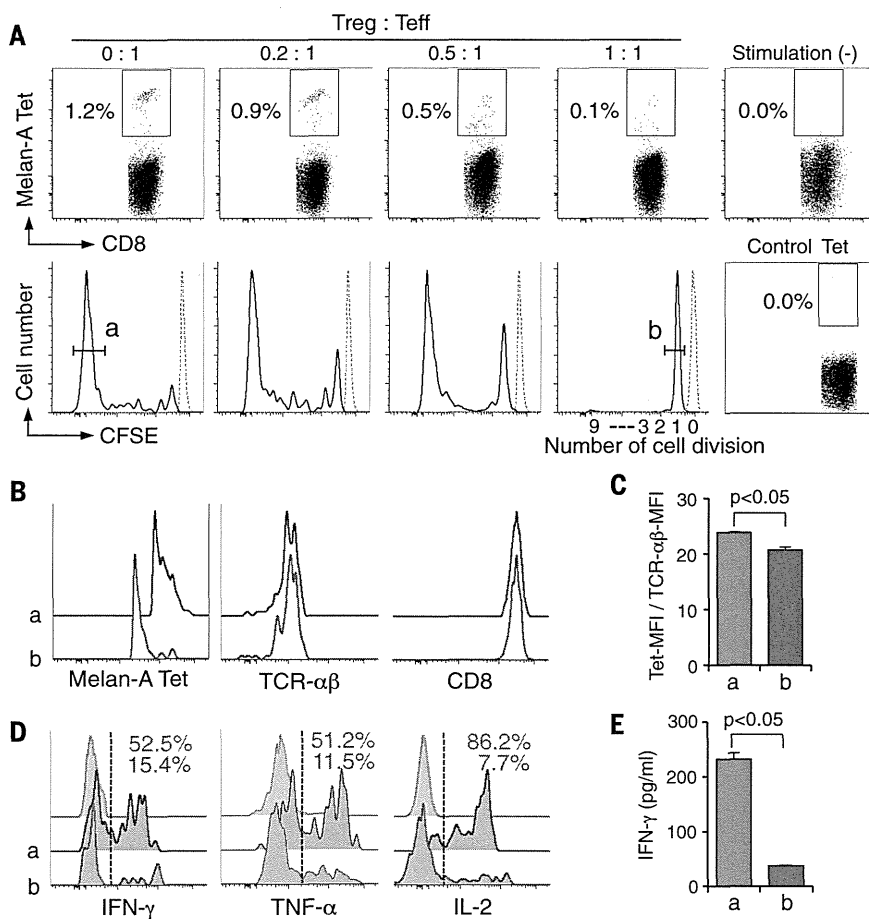


Fig. 1. Natural T_{reg} cells render low-affinity self-reactive CD8⁺ T cells anergic upon antigen stimulation. (A) Melan-A-specific CD8⁺ T cell induction. CFSE-labeled CD8⁺ T cells of HLA-A*0201⁺ healthy individuals were stimulated by T cell-depleted, γ -irradiated, and Melan-A₂₆₋₃₅ peptide-pulsed APCs with graded numbers of CD25^{high}CD4⁺ T_{reg} cells for 10 days (6). Dotted lines mean Tet⁺CD8⁺ cells showing no CFSE dilution. Control tet: NY-ESO-1₁₅₇₋₁₆₅/HLA-A*0201 tetramer. T_{eff} refers to CD8⁺ effector T cells. (B) Tet, TCR- $\alpha\beta$, and CD8 staining of Tet⁺CD8⁺ T cells. Results in (A) and (B) are representative of 10 independent experiments. (C) Relative tetramer staining intensities, calculated as mean fluorescence intensity (MFI) of Tet/MFI of TCR- $\alpha\beta$ staining of Tet⁺CD8⁺ T cells ($n = 5$). (D and E) Cytokine production of Tet⁺CD8⁺ T cells by intracellular staining (D) and enzyme-linked immunosorbent assay (E) (6). Representative result of three independent experiments. The labels a and b in (B) to (E) mean the cell accumulations like a or b in (A). Error bars indicate means \pm SEM. The significance was assessed by Student's two-tailed paired *t* test.

in chronic viral infections and in tumor tissues, because exhausted CD8⁺ T cells are reportedly CCR7⁻, CD45RA⁻, and BAT3⁺, and their exhaustion can be rescued by a PD-1-blocking antibody (21, 24–26).

T_{reg} cells suppress the activation and/or proliferation of responder T cells (27), at least in part, by down-regulating the expression of the costimulatory molecules CD80 and CD86 on APCs (fig. S7A) (28, 29). To determine whether low expression or down-modulation of CD80 and CD86 on dendritic cells (DCs) was responsible for the induction of antigen-specific anergic CD8⁺ T cells, we stimulated carboxyfluorescein succinimidyl ester (CFSE)-labeled CD8⁺ T cells with autologous immature or mature DCs pulsed with Melan-A

peptide in the presence of graded amounts of CTLA-4-immunoglobulin (CTLA-4Ig), which blocked CD80 and CD86 (fig. S7B) (30). In contrast to the vigorous proliferation of Tet⁺CD8⁺ T cells cultured with mature DCs, the majority of Tet⁺CD8⁺ T cells generated with immature DCs, and some with mature DCs with a high dose (100 μg/ml) of CTLA-4Ig, were proliferation-aborted after one cell division (Fig. 3A). The proliferation-aborted T cells (peaks c and d in Fig. 3A) were lower than proliferating T cells in Melan-A tetramer staining intensity (Fig. 3B), and highly expressed CTLA-4 and CCR7 (fig. S7, C and D); they formed a discrete CTLA-4/CCR7 double-positive population (Fig. 3C and fig. S7E). They produced significantly lower amounts of IFN-γ,

TNF-α, and IL-2 compared with Tet⁺CD8⁺ cells, having proliferated in culture with mature DCs (fig. S7F). Similar to peptide stimulation, polyclonal antibody against CD3-specific monoclonal antibody (mAb) stimulation of CTLA4⁻ naïve CD8⁺ T cells in the presence of CTLA-4Ig produced cells that were proliferation-aborted after one cell division (fig. S8A). Notably, increasing CTLA-4Ig dose proportionally intensified CTLA-4 expression by the aborted cells, while stably maintaining their high CCR7 expression (fig. S8, A and B). Taken together, antigen presentation with low CD80 and CD86 costimulation is able to drive CD8⁺ T cells to differentiate into CTLA-4⁺CCR7⁺ anergic cells. DCs with moderate CD80 and CD86 reduction can concurrently generate both

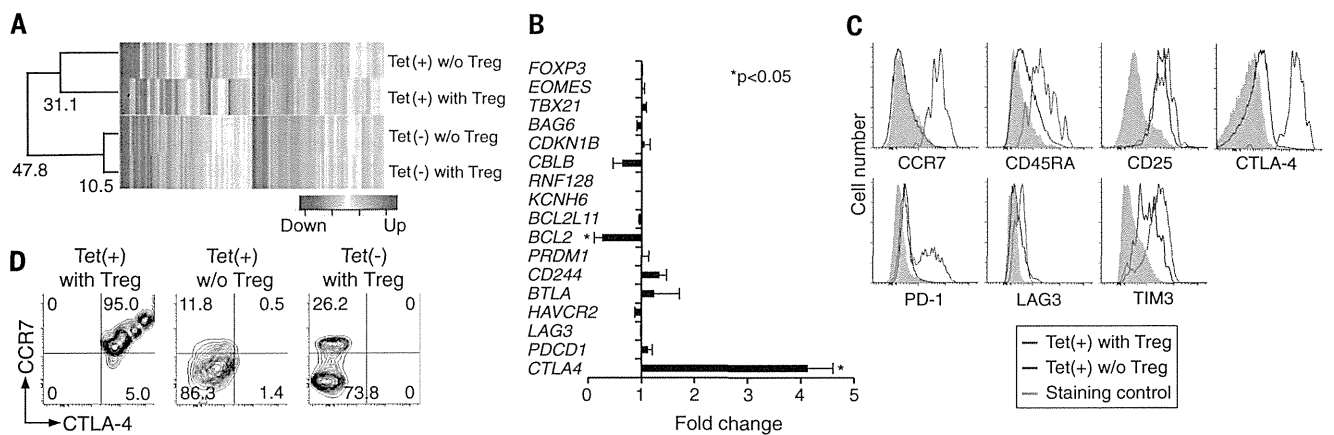


Fig. 2. Distinct phenotype and function of anergic CD8⁺ T cells produced by T_{reg} cell suppression. (A) Global mRNA expression profile. Tet⁺CD8⁺ T cells induced at CD8⁺ T cells: T_{reg} cell ratios of 1:0.5 and 1:0 were subjected to microarray analyses. Gene expression reportedly associated with CD8⁺ T cell function was compared among the indicated four groups and expressed as a heat map. Correlation distances shown were calculated by h-clust (6). Representative of two independent experiments. (B) mRNA expression measured by

quantitative real-time PCR. Fold changes of mRNA level as [Tet(+) with T_{reg}] versus [Tet(+) without T_{reg}] in five independent experiments are shown. Error bars indicate means ± SEM. (C and D) Expression of cell surface molecules by Tet⁺CD8⁺ T cells induced at CD8⁺ T cells: T_{reg} cell ratios, 1:1 and 1:0. Representative histogram staining pattern (C) and contour plot staining pattern of CTLA-4 and CCR7 (D). Data are representative of five independent experiments (n = 10). The significance was assessed by Student's two-tailed paired t test.

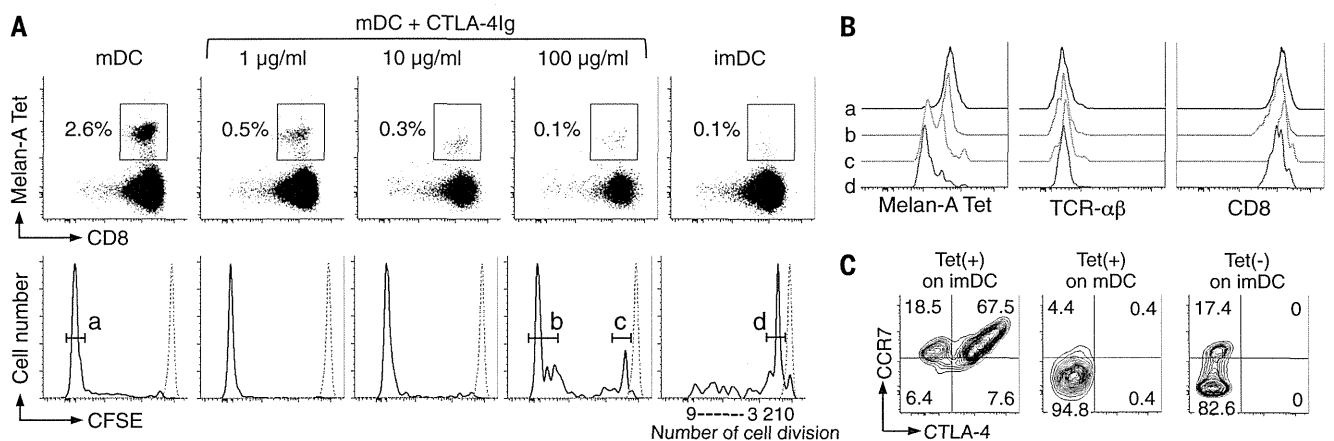


Fig. 3. DC expression of CD80 and CD86 controls the generation of CTLA-4⁺CCR7⁺ low-affinity anergic self-reactive T cells. (A) Melan-A-specific CD8⁺ T cell induction. CFSE-labeled CD8⁺ T cells of HLA-A*0201⁺ healthy individuals were stimulated with γ-irradiated, Melan-A₂₆₋₃₅ peptide-pulsed monocyte-derived immature or mature DCs. CTLA-4Ig was added into mature DCs cultures at indicated concentrations (6). (B) Tet, TCR-αβ, and CD8 staining intensity of Tet⁺CD8⁺ T cells shown in (A). (C) Representative contour plot staining pattern of Tet⁺ or Tet⁻CD8⁺ T cells shown in (A) for CTLA-4 and CCR7. Data in (A) to (C) are representative of five independent experiments.

activated T cells and anergic T cells, in part, depending on TCR affinity.

The above *in vitro* findings prompted us to ask whether healthy individuals harbored such anergic self-reactive CD8⁺ T cells. Direct *ex vivo* staining of CD8⁺ T cells in PBMCs of healthy donors ($n = 10$) for Melan-A peptide and MHC tetramer, with CD8⁺ T cells from vitiligo patients ($n = 10$) as a positive control, revealed that a small number of Tet⁺CD8⁺ T cells were indeed present in healthy individuals and constituted ~0.03% of CD8⁺ T cells in PBMCs, which contrasted with high percentages (~0.1%) in vitiligo patients (Fig. 4, A and B) (6). Two-thirds of the former had a naïve

(CCR7⁺CD45RA⁺) phenotype, whereas the majority of the latter had an effector or memory phenotype (Fig. 4C and fig. S9A) (4, 5). The Tet⁺CD8⁺ T cells from healthy individuals had significantly lower tetramer staining intensity than those from vitiligo patients (Fig. 4, D and E). They expressed CTLA-4 at higher levels than Tet⁺CD8⁺ T cells from vitiligo patients or Tet⁻CD8⁺ T cells from healthy individuals, or activated CD8⁺ T or natural T_{reg} cells (Fig. 4F and fig. S9, B to D), and ~90% of the Tet⁺CD45RA⁺CD8⁺ cells were double positive for CTLA-4 and CCR7 (Fig. 4G and fig. S9B). Functionally, Tet⁺CD8⁺ T cells directly prepared from healthy donors scarcely

produced IFN- γ , TNF- α , or IL-2, contrasting with active cytokine production by naïve Tet⁻CD8⁺ T cells (Fig. 4H) or Melan-A-specific CD8⁺ T cells from vitiligo patients (4, 3I).

To determine further the function of these anergic T cells, we cocultured CTLA-4⁺ and CTLA-4⁻ fractions of naïve (CCR7⁺CD45RA⁺) CD8⁺ T cells from healthy individuals and assessed the proliferative activity of Tet⁺CD8⁺ T cells present in each fraction (Fig. 4I). The CTLA-4⁻ fraction, which constituted less than 10% of naïve CD8⁺ T cells in healthy donors, contained the majority (~95%) of Melan-A Tet⁺CD8⁺ T cells before stimulation (Fig. 4, I to K). These CTLA-4⁻Tet⁺ cells were

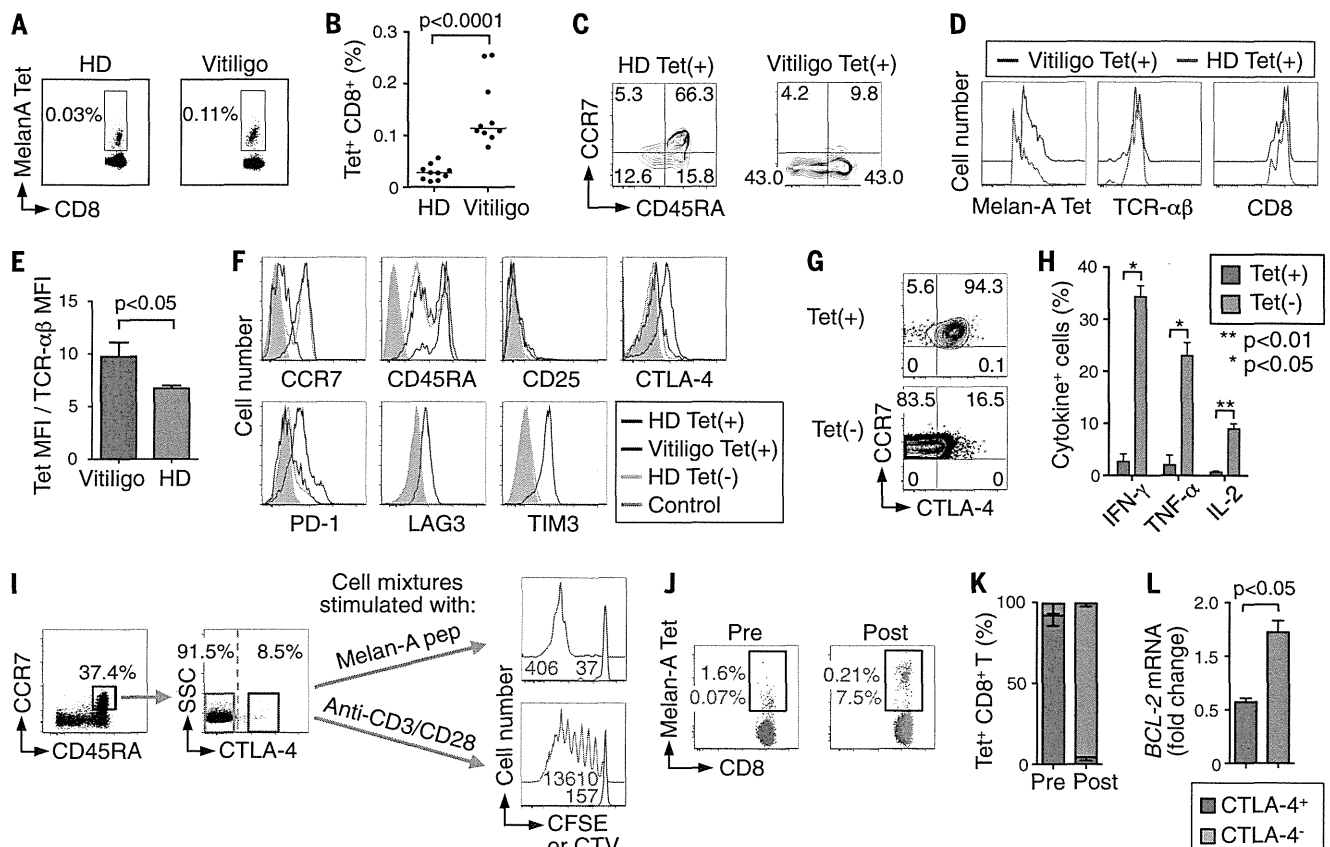


Fig. 4. Detection of low-affinity anergic self-reactive CTLA-4⁺CCR7⁺CD8⁺ T cells in healthy individuals.

(A) Melan-A Tet⁺CD8⁺ T cells in PBMCs of a healthy donor (HD) and a vitiligo patient. (B) Percentages of Tet⁺CD8⁺ T cells in HDs and vitiligo patients ($n = 10$). (C) CCR7 and CD45RA expression by Tet⁺CD8⁺ T cells in an HD and a vitiligo patient. (D) Tet, TCR- $\alpha\beta$, and CD8 staining intensity of Tet⁺CD8⁺ T cells in an HD and a vitiligo patient. (E) Ratios of MFI of tetramer staining to MFI of TCR- $\alpha\beta$ staining in Tet⁺CD8⁺ T cells in HDs and vitiligo patients ($n = 4$ each). (F) Expression of cell surface molecules by Tet⁺ or Tet⁻CD8⁺ T cells in a representative HD and a vitiligo patient. (G) Representative staining for CTLA-4 and CCR7 of Tet⁺ or Tet⁻ cells in CD45RA⁺CD8⁺ T cells of an HD. Data shown in (A), (C), (D), (F), and (G) are representative of four independent experiments. (H) Cytokine production by Tet⁺CD8⁺ T cells in HDs assessed by intracellular staining with CCR7⁺CD45RA⁺Tet⁺CD8⁺ T cells as control. Data summarize four independent experiments. (I) Proliferation and cytokine production of CTLA-4⁺ or CTLA-4⁻ naïve CD8⁺ T cells in HDs. CCR7⁺CD45RA⁺CD8⁺ T cells

from HD PBMCs were further separated into CTLA-4⁺ and CTLA-4⁻ cells, labeled with Cell Trace Violet (CTV) or CFSE, respectively, mixed at a 1:1 ratio, stimulated with Melan-A₂₆₋₃₅ peptide-pulsed APCs for 10 days (top) or CD3/CD28-specific mAb for 5 days (bottom), and assessed for proliferation by CTV or CFSE dilution (red and blue, respectively) (6). Numbers in right two figures represent the numbers of cells in each cell fraction. SSC, side scatter. (J) Representative tetramer staining of the cell mixtures before (Pre) and after (Post) Melan-A₂₆₋₃₅ peptide stimulation for 10 days. Numbers represent percentages of Tet⁺CD8⁺ cells in the CTLA-4⁺ or CTLA-4⁻ fraction (red and blue, respectively). (K) Percentages of Tet⁺CD8⁺ T cells in the CTLA-4⁺ (red) or CTLA-4⁻ (blue) fraction in the cell mixtures before (Pre) and after (Post) cell culture as shown in (I) and (J). (L) *BCL2* mRNA expression of CTLA-4⁺CCR7⁺CD45RA⁺Tet⁺CD8⁺ and CTLA-4⁻CCR7⁺CD45RA⁺Tet⁺CD8⁺ T cells measured by quantitative real-time PCR. Data in (I) to (L) are representative of at least three independent experiments. Error bars indicate means \pm SEM. The significance was assessed by Student's two-tailed paired *t* test.

hypoproliferative, low in *BCL2* expression, and prone to die upon Melan-A stimulation (Fig. 4, I to L). In contrast, the CTLA-4⁻ fraction, which initially contained fewer than 5% of total Tet⁺CD8⁺ T cells, gave rise to proliferating Tet⁺CD8⁺ T cells, which made up ~95% of total Tet⁺CD8⁺ T cells after stimulation (Fig. 4, I to K). In addition, polyclonal stimulation of the cell mixtures with CD3-specific and CD28-specific mAb revealed that the CTLA-4⁺ fraction as a whole was hypoproliferative (Fig. 4I) and cytokine hypoproducing (fig. S9E), in contrast with active proliferation and cytokine production of the CTLA-4⁻ fraction.

These results collectively indicate that healthy individuals harbor at least two distinct populations of self-reactive CD8⁺ T cells: one that is functionally anergic and expresses CTLA-4 and CCR7 and another that is CTLA-4⁻ and naïve in function and phenotype. The latter, especially those with high-affinity TCRs, may become activated and expand upon self-antigen stimulation in the absence or reduction of natural T_{reg} cells, as shown in Fig. 1A.

Thus, anergic self-reactive T cells, which are phenotypically distinct from other T cells, are physiologically present in the immune system. They appear to be generated, at least in part, as a result of T_{reg}-mediated suppression, which can determine cell fate of responder T cells (i.e., activated, anergic, or ignorant) upon antigenic stimulation, depending on the number and suppressive activity of T_{reg} cells, the TCR affinity and differentiation states of responder T cells, and the condition of APCs. This T_{reg}-dependent switching of re-

sponder T cell fate can be a key target in controlling autoimmunity and tumor immunity, as illustrated by our analysis of Melan-A-specific immune responses, as well as a variety of other physiological and pathological immune responses.

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SUPPLEMENTARY MATERIALS

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Supplemental Text
Figs. S1 to S9
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The role of cytokines and chemokines in the T-cell-mediated autoimmune process in alopecia areata

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Abstract: The aetiology of alopecia areata (AA) is still not fully understood. However, recent clinical and experimental studies have provided insights into the pathomechanisms of AA and revealed that it is an organ-specific and cell-mediated autoimmune disease. Some triggers, such as viral infections, trauma, hormones and emotional/physical stressors, may cause activation of autoreactive T cells that target hair follicle (HF) autoantigens. In these immunological responses, cytokines and chemokines are regarded as key players that mediate the autoimmune inflammation. This results in the collapse of HF

immune privilege, which is central to the pathogenesis of AA. This essay will focus on how cytokines and chemokines contribute to the immunological aspects of AA. The management of AA often remains difficult in a number of cases. Our review suggests that novel therapies for AA may involve targeting cytokines and chemokines.

Key words: alopecia areata – chemokine – cytokine – immune privilege – swarm of bees

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Why are we focused on chemokines in the pathogenesis of alopecia areata?

A 'swarm of bees' is a characteristic histopathological feature in the lesions of alopecia areata (AA) (1). However, the molecular mechanisms underlying this cell accumulation have not been uncovered. To attract immune cells around the hair bulb, some chemokines are upregulated in the hair bulbs. So, if we can hinder the surge in immune cells, the damage to the hair bulb can be decreased, which results in improvement of hair loss in AA. To find new treatments for AA, we focused on chemotactic activity in the pathogenesis of AA.

Key observations in the pathogenesis of alopecia areata

Actually, the detailed pathomechanisms of AA have not been fully elucidated. However, the following points represent key findings in AA (Fig. 1).

1. Genetic susceptibility is involved in AA development.
2. Physiological stresses, emotional stresses, virus infections and trauma are triggers of AA.
3. Collapse of hair follicle (HF) immune privilege (IP).
4. T-helper (Th)1 cytokines/chemokines are involved in AA.
5. Th1/Tc1 cells accumulate around the hair bulbs.
6. Th17 and AA.
7. HF autoantigens, such as melanin-associated protein, may be recognised by cytotoxic T cells.

These are causally related to AA. The points related to cytokine/chemokines will be discussed in detail in the following subheadings.

Virus infections and AA

Virus infection as an important trigger of hair loss in AA has been debated for a decade. Cytomegalovirus (CMV) has been refuted the association between CMV and AA (2–4). Hepatitis B virus (HBV), hepatitis C virus (HCV) and Epstein–Barr virus infections

have also been implicated as triggers for AA. Vaccinations to prevent these infections may also be a trigger of AA (5–7). We reported seven cases of AA, including both new onset and recurrent AA, following swine flu viral infection (8). After viral infection, Th1-mediated immune responses are generally induced by an overproduction of interferons (IFNs) (9), which may induce autoimmune AA (10). Among IFNs, type I IFNs (IFN- α , - β , - ϵ , - κ and - ω) and IFN-inducible myxovirus protein A play a principal role in the antiviral response (11–13). Indeed, a case of reversible alopecia universalis was treated with PEG-IFN- α -2b and ribavirin combination therapy for HCV infection (14). Therefore, IFN- α may play a critical role in the induction of hair loss preceded by a viral infection.

Th1 cytokines and AA

In the AA model mouse, the Th1 cytokine IFN- γ is a key molecule in the pathogenesis of AA (15). Murine IFN- γ -injected C3H/HeJ mice showed AA-like hair loss with infiltration of CD4+ and CD8+ T cells and upregulation of major histocompatibility complex (MHC) class I/II and IP-10, although another group failed to induce hair loss after injection with murine IFN- γ subcutaneously in C3H/HeJ mice (16). Furthermore, IFN- γ ^{-/-} mice are resistant to the development of AA (17). IFN- γ mRNA expression was significantly increased in the lesions of AA in C3H/HeJ mice (18). In another study, C3H/HeJ mice showed higher expression of TNF- α / β , IL-12 and IFN- γ mRNA (19). In humans, IFN- γ -producing cells were detected in the perifollicular infiltrate (20). Serum Th1 cytokines (IL-2, IL-12 and IFN- γ) and IL-1 receptor antagonist were increased in AA patients regardless of disease severity or coexistence of atopic disease (21). On the other hand, another study reported that IL-4, IFN- γ and TGF- β 1 mRNA levels were lower in peripheral blood mononuclear cells (PBMCs) from AA patients than in PBMCs from healthy individuals (22). IL-10 mRNA levels in AA were comparable with those in healthy

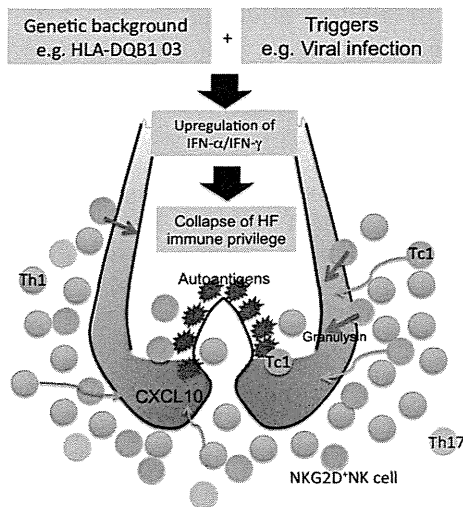


Figure 1. Summary of the pathogenesis of alopecia areata (AA). Unknown inducers such as viral infection upregulate IFNs that induce Th1 chemokines (CXCL10) and major histocompatibility complex (MHC) class I expression. Subsequently, Tc1 and Th1 cells accumulate around hair bulbs, and the hair follicle immune privilege (HF-IP) is collapsed, which results in the recognition of HF autoantigens by autoreactive CD8+ T cells.

subjects. Decreased levels of IFN- γ and TGF- β 1 were also seen in patients with atopic dermatitis (AD). This earlier study suggested that the cytokine profile of AD and AA is similar (i.e. decreased levels of IFN- γ and TGF- β 1) (22). Apart from this study, several studies have reported an increased level of IFN- γ (23–25). The reason for this discrepancy is unclear, but the source of the samples used for analysis was different between these studies (serum and PBMCs).

In addition, a recent study also reported that the levels of IL-2, IFN- γ , IL-13 and IL-17A were significantly increased, and the TGF- β 1 level was significantly decreased ($P < 0.05$) in AA patients compared with those in control subjects, using an enzyme-linked immunosorbent assay (ELISA) (26). The IL-2 levels positively correlated with disease duration and the number of AA patches on the scalp. We can also learn about the pathogenesis of AA from information on vitiligo. Harris stated that the comparison between AA and vitiligo is 'like comparing apples and oranges' (27).

Actually, the skin manifestations and the treatments are different (28,29). Although these two diseases show different clinical features, their pathogeneses are similar. Both diseases are regarded as tissue-specific autoimmune diseases, and the targeted autoantigens are melanocyte-specific proteins (30). In addition, both vitiligo and AA were considered to be Th1 diseases characterised by the consistent production of IFN- γ within lesional skin (31,32). Compared with the Th1/Tc1 theory in AA, a recent genome-wide association study (GWAS) of AA revealed an opposite result for cytokine balance (33). In this study, 23 SNPs from 17 loci were examined using a sample of 1702 Central European AA patients and 1723 controls. Results showed a significant association between rs20541 ($P_{\text{comb}} = 7.52 \times 10^{-10}$; odds ratio [OR] = 1.30 [1.23–1.38]) and AA, thus implicating IL-13 as a susceptibility locus for AA. AD is a commonly associated disorder in AA patients (34). In another study of IL-13 in AA, significant associations were found for rs20541 in both groups of AA patients with AD and AA patients without AD. However, no significant differences in the ORs were observed between the two groups. Therefore, there seems to be no effect on AD comorbidity. The variant rs20541 and variants in linkage disequilibrium with rs20541 are also associated with psoriasis, arthritis and asthma (35–38). IL-13 is produced by activated Th2 cells, basophils and mast cells (39). The Th2 cell is an essential effector in the recruitment of inflammatory cells *via* the secretion of IL-4, IL-5, IL-9 and IL-13 (37). Only IL-4 and IL-13 are able to induce an immunoglobulin isotype switch to IgE in B cells (40). Given that AA is generally accepted as a Th1/Tc1 disease, the association between IL-13 and AA remains unclear, and the situation may be very complicated. The summary of cytokine expression/production in AA is indicated in Table 1.

Th1 chemokines and AA

There have been several studies on the expression of chemokines and their receptors in AA lesions. In a mouse model, murine AA skin-grafted mice showed a marked increase of chemokine (C-X-C motif) ligands 9 and 10 (CXCL9 and CXCL10) as early as 5 weeks post-transplant before the development of AA (41). This increased expression before the development of AA indicated that these Th1 chemokines are involved in the early pathogenesis of AA (41). In a study by Gilhar et al., biopsied human AA samples were grafted onto SCID mice. Subsequently, the patients' lesional T cells, which

Table 1. Expression of cytokines and T-cell categories in alopecia areata

Cytokines	T-cell type	Animal	Skin mRNA	Protein	Serum	PBMCs mRNA	Infiltrating cells	References
IFN- γ	Th1/Tc1	Human			Increase			21,23,25
IFN- γ	Th1/Tc1	Human				Decrease		22
IFN- γ	Th1/Tc1	Human	Increase					23
IFN- γ	Th1/Tc1	Human					Increase	20
IFN- γ	Th1/Tc1	C3H/HeJ	Increase					18,19
IL-1 α		Human			Increase			25
IL-1 β		Human	Increase					23,24
IL-2	Th1/Tc1	Human			Increase			21,25
IL-2	Th1/Tc1	Human	Increase					23,24
IL-4	Th2/Tc2	Human				Decrease		22
IL-4	Th2/Tc2	Human			Increase			25
IL-10	Th2/Tc2	Human				Comparable		22
IL-12	Th1/Tc1	Human			Increase			21
IL-12	Th1/Tc1	C3H/HeJ	Increase					19
IL-13	Th2/Tc2	Human			Increase			23
IL-17A	Th17	Human			Increase			23
IL-17A	Th17	Human					Increase	50,51

had been cultured with follicular homogenate, melanocyte peptides or human melanoma cell homogenate, were injected into the grafted area (42). Prior to grafting, only four of 13 donor biopsies exhibited positive staining for CXCL10/IP-10, and follicular CXCL10 was present in only two of nine non-injected grafts. The difference in follicular CXCL10 staining between T-cell-injected (13/13) and non-injected (2/9) grafts was significant ($P < 0.002$, two-sided Fisher's exact test). This study provides supportive evidence for a Th1 immune response in AA.

Other studies have also shown Th1 chemokines, CXCL9/MIG and CXCL10/IP-10, in the lesions of AA patients (43,44). Benoit et al. (44) reported a strong expression of CXCL9, a moderate expression of MCP-1 and a weak expression of CXCL10 by *in situ* hybridisation. The authors suggested that the increased expression of CXCL9 and MCP-1 chemoattract effector cells.

Transcriptional profiling revealed upregulation of CXCL10 in AA lesions compared with non-lesional skin (45). Chemokines (CX3CL1, CXCL1, CCL5 and CXCL10) associated with cellular immunity were overexpressed in AA skin. CX3CL1 and CXCL10 are not only induced by IFN- γ , but also act as an amplifier of polarised Th1 responses (46). In addition to cytokines/chemokines, the study showed upregulation of ICAM-2 and ICAM-3 in AA skin, thus supporting a proposed mechanism that involves enhanced antigen-specific immune responses through increased dendritic cell (DC) migration and DC-T cell interaction in AA lesional skin (47).

A recent study (48) focused on TARC and AA. Mean serum TARC in AA totalis, universalis and diffuse AA was significantly higher than that in mild AA, suggesting a potential role of TARC in active progression of AA. Furthermore, serum TARC can be used as a biomarker of disease activity (48). This study provides new insight beyond the Th1/2 paradigm to clarify the immunological pathogenesis of AA.

Th17 and AA

Th17 cells have been characterised in mice as a novel subset of CD4⁺ T cells that produce IL-17A, IL-17F and IL-22, and these cells might be immune effectors in autoimmunity (49,50). Alli et al. (51) made an AA model mice, IMOG244.1 mice, that expressed dual T-cell receptor α (TCRA) chains, one of which, when combined with the single TCRB present, promotes the development of CD8⁺ T cells with specificity for HFs. Retroviral transgenic mice expressing this TCR develop spontaneous AA with an incidence of nearly 100%. In these mice, pathologic T cells primarily express IFN- γ and IL-17 early in the disease, with dramatic increases in cytokine production and IL-4 and IL-10 production with disease progression. In humans, Lew et al. (52) reported that one single nucleotide polymorphism (rs879577) of the *IL17RA* gene showed a significant difference between the AA patient and control groups ($P = 0.0288$). One single nucleotide polymorphism (rs4819554) of the *IL17RA* gene showed a significant difference between early and late onset AA ($P = 0.0421$). Moreover, Tojo et al. (53) found that IL-17-producing cells predominantly infiltrated around the HFs by TissueFAXS analysis. Tanemura et al. (54) also found CD4⁺ IL-17⁺ infiltrating cells around the hair bulbs in the lesions of AA by immunofluorescent staining. As described before, ELISA revealed that serum IL-17 is increased in AA patients (26). The association between AA and IL-17 is still uncertain, and further studies are required.

Collapse of immune privilege

Immune-privileged sites have been found at several important locations, including the anterior eye chamber, HFs and the nail unit (9,55,56). The HF-IP is maintained by several factors, including the lack of MHC class I in the proximal outer root sheath (ORS) and matrix cells (Table 2). The collapse of HF-IP may be the central pathogenic alteration in AA, although there is no clear evidence that loss of HF-IP leads to induction of AA. When HF-IP is collapsed by some inducers, HF autoantigens are exposed to autoreactive T cells *via* ectopic expression of MHC class I molecules (7). In AA lesions, MHC class I is highly expressed on hair matrix epithelium and subinfundibular epithelium (57). This ectopic MHC class I expression can be induced by IFN- γ . A dose-dependent enhancement in the expression of MHC class I was upregulated in all follicular compartments, including the ORS, dermal papilla and connective tissue sheath by a 7-day culture with IFN- γ (10–1000 U/ml) (58,59). Gilhar et al. have reported evidence of loss of HF-IP in AA model mice (C3H/HeJ) that involved injection of IFN- γ . Intravenous IFN- γ -injected C3H/HeJ mice showed upregulation of MHC class I expression in the outer and inner root sheath and infiltration of lymphocytes around the HFs (15).

The restoration or protection against HF-IP collapse can be a new treatment strategy for AA. As mentioned before, HF-IP can be reproduced by 75 IU/ml IFN- γ *in vitro*. To restore or protect the collapse of HF-IP, MHC class I expression will be used as an indicator, for example, calcitonin gene-related peptide (CGRP) may provide relative protection from interferon- γ -induced collapse of human HF-IP (60). Immunoreactivity for the CGRP receptor, CRLR, was seen in the ORS and in the venules and capillaries, including the capillaries inside the dermal papilla. When CGRP was added prior to administration of IFN- γ into the medium for HF culture, the IFN- γ -induced ectopic MHC class Ia overexpression was reduced, which indicated a protective effect of CGRP against the collapse of HF-IP (60).

Accumulation of Th1/Tc1 cells around the hair bulbs in AA

Almost all alopecia scientists know the histopathological appearance of AA, termed 'swarm of bees', which is characterised by peribulbar lymphocytic infiltration in the acute phase of AA (1). To guide the future direction of the targeted therapy with

Table 2. The mechanisms of HF immune privilege

Absent or barely detectable expression of MHC class I (8)
Melanocytes of the HF pigmentary unit of human anagen scalp HFs are MHC class I-negative (66,67)
Downregulation of the MHC class I pathway-related molecule β 2-microglobulin and transporter of antigen processing-2 (TAP-2) (59)
Downregulation of interferon regulatory factor-1 expression (59)
Upregulation of immunosuppressing factors such as TGF- β 1 and TGF- β 2, ACTH and α -MSH (68–73)
Absence of MHC class II ⁺ or NLDC-145 ⁺ Langerhans cells in the murine hair bulbs (68,74)
Very few CD1a ⁺ or ultrastructurally identified Langerhans cells can be detected in the human anagen hair bulb; these Langerhans cells must be functionally impaired because they do not express MHC II (66,67)
Sparse distribution of CD4 ⁺ T cells, CD8 ⁺ T cells and NK cells (66)
Absence of lymphatics (75)

HF, hair follicle; MHC, major histocompatibility complex.

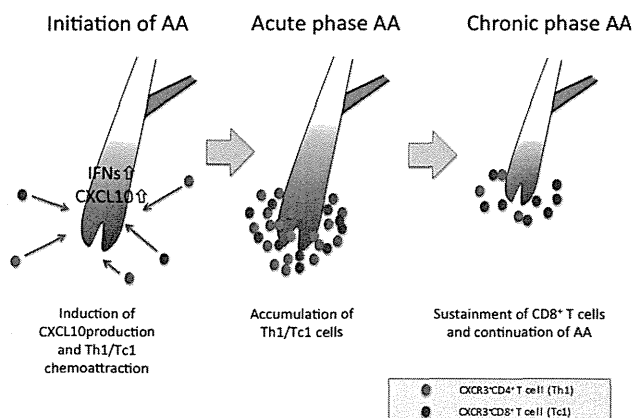


Figure 2. Summary of the immunological changes in alopecia areata (AA). Some stressors may initiate AA with upregulation of CXCL10 that results in the accumulation of CXCR3+ CD4+ and CXCR3+ CD8+ T cells in and around the hair bulbs. Persistence of CD8+ T cells around the hair bulbs may contribute to continuing hair loss in the chronic phase of AA.

inhibition of chemotaxis, we examined the numbers of CXCR3+ CD4+, CCR4+ CD4+, CXCR3+ CD8+ and CCR4+ CD8+ T-cell subsets, representing Th1, Th2, Tc1 and Tc2 cells, respectively, *in situ* and in PBMCs (61–63). This study revealed that both CD4+ and CD8+ T cells infiltrated around the HFs with invasion into the ORS. In addition, CXCR3+ T cells markedly infiltrated in and around the hair bulbs compared with CCR4+ T cells. In the chronic phase of AA, CD4+ T cells around the HFs were decreased in number, while the infiltration of CD8+ T cells remained constant. In this phase, the accumulation of CXCR3+ T cells was denser than CCR4+ T cells. The number of CXCR3+ CD4+ Th1 cells in PBMCs was significantly higher in acute phase AA patients than in chronic phase AA patients or healthy controls (63). Another study showed dominant infiltration of CCR5+ Th1 cells relative to CCR4+ Th2 cells in the C3H/HeJ mouse model (18). CXCL10 immunoreactivity was found in the outer and inner root sheath epithelial cells, dermal papilla cells

and juxtafollicular interstitial cells; this finding was supported by the results of real-time PCR analysis. These results suggest that CXCL10 contributes to the accumulation of CXCR3+ Th1 and Tc1 cells, leading to a dense perifollicular lymphocytic infiltrate (Fig. 2). Interestingly, real-time horizontal chemotaxis assay showed that the chemotactic velocities of circulating CD4+ and CD8+ T cell towards CXCL10 in acute phase AA were higher than those in chronic phase AA and in healthy controls, suggesting that Th1 and Tc1 are activated in the peripheral blood of acute phase AA patients (63).

A recent study reported interesting data showing that serum granulysin could be a possible key marker of the activity of AA (64). Accumulated Tc1 cells are key players in the cell-mediated cytotoxicity in the development of AA, which is mediated by granulysin.

Summary and future clinical perspectives

The pathogenesis of AA remains unclear, but current studies strongly suggest a T-cell-mediated autoimmune process. The hallmark of pathological change in AA is the accumulation of lymphocytes around hair bulbs. Overexpression of the Th1 chemokine, CXCL10, induces the infiltration of CXCR3+ Th1 cells around the hair bulbs in AA lesions. Therefore, the inhibition of chemotactic or cytokine activity could be novel therapies for AA. Our study has already shown that the antihistamine drug, olopatadine, downregulated T-cell chemotaxis towards CXCL10 by reducing CXCR3 expression, F-actin polymerisation and calcium influx in patients with AA (65).

A considerable number of questions regarding the aetiology of AA remain unresolved. However, Th1 chemokines and cytokines are targetable immune pathways that are important in solving the puzzle of the pathogenesis of AA.

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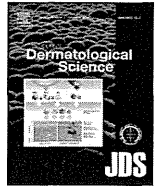
Conflict of interests

The authors have declared no conflicting interests.

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Invited Review Article

Attempts to accelerate wound healing



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ABSTRACT

Wound healing is a well-orchestrated process, where numerous factors are activated or inhibited in a sequence of steps. Immediately after the infliction of damage, the repair of wound starts. The initial step is an inflammatory change with activation of innate immunity, which is followed by proliferation phase, including fibroplasia, angiogenesis and re-epithelialization. Pathological impairment of wound healing process may lead to persistent ulceration as seen in diabetic patients. Various signaling pathways are involved in wound healing. TGF β /Smad pathway is a representative and well known to participate in fibroplasia, however, its comprehensive effect on wound healing is controversial. Experimental and clinical remedies have been being tried to promote wound healing. Advancement of cell engineering allows us to use stem cells and living skin equivalents.

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Contents

1. Introduction	169
2. Mechanisms underlying wound healing	169
2.1. Inflammation phase of wound healing	170
2.2. Proliferation phase of wound healing	170
2.3. Signaling involved in wound healing	170
2.4. Altered wound healing in diseases	170
2.5. Miscellaneous factors	171
3. Approaches to promote wound healing with mesenchymal stem cells or related materials	171
3.1. Introduction of stem cells into wound tissue	171
3.2. Introduction of living skin equivalents into wound tissue	171
3.3. Negative pressure to accelerate wound healing	171
4. Conclusion	171
Acknowledgements	171
References	171

1. Introduction

Wound healing is the restoration in injured skin tissue and one of the most important therapeutic targets. The process consists of inflammation, proliferation, and remodeling (Fig. 1). There have been reported multiple factors that accelerate and delay wound

healing. Recently, several perturbed signaling pathways were found in non-healing wounds. Various remedies have also been investigated to modulate the process of inflammation and proliferation. This review aims to highlight the recent approach for promotion of wound healing and new target for wound healing.

2. Mechanisms underlying wound healing

Inflammation is the common event to allergic diseases, autoimmune diseases, infectious diseases, and others. One cannot easily appreciate the key role of inflammation in wound healing;

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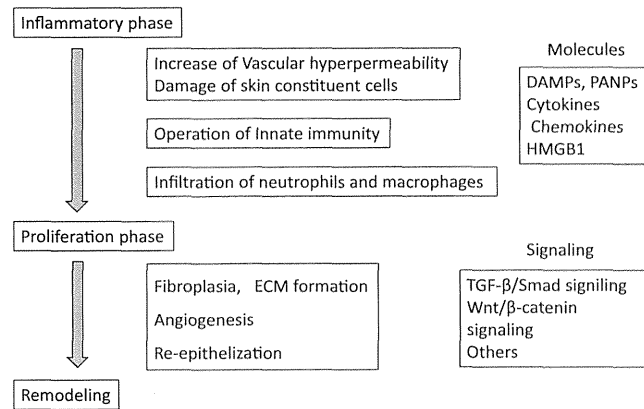


Fig. 1. Phases and factors involving wound healing. The process consists of inflammation, proliferation, and remodeling. Multiple factors accelerate and delay wound healing. Several signaling pathways work co-dependently.

however, inflammation is also essential for the healing process. Various signaling pathways, in particular transforming growth factor (TGF)- β /SMAD pathway, have been studied, but they remain to be elucidated. We focus on the inflammation and proliferation phases in wound healing.

2.1. Inflammation phase of wound healing

The inflammation phase starts with edema due to increased vascular hyperpermeability. Simultaneously, skin constituent cells are cytolysed. The damaged cells spread intracellular contents, including danger-associated molecular patterns (DAMPs), to the intercellular space of the epidermis and dermis. DAMPs activate inflammasome and elaborate caspase-1, which converts pro-interleukin (IL)-1 β and pro-IL-18 to IL-1 β and IL-18 [1]. In this scenario, pathogen-associated molecular patterns (PAMPs) serve as ligand for toll-like receptors (TLRs), leading to the production of pro-IL-1 β and pro-IL-18. The damaged keratinocytes also release IL-1 α , IL-33, and high-mobility group box 1 (HMGB1), which exaggerate inflammation or may regulate it [2]. Various proinflammatory cytokines and chemokines are thus produced in the wound tissue at the initial step.

Neutrophils and macrophages infiltrate at injured sites by virtue of inflammatory cytokines, chemokines, HMGB1, and fibronectin. These proinflammatory cells are necessary to remove debris and kill bacteria through phagocytosis and free radical production. Macrophages even stimulate angiogenesis and fibroplasia and extracellular matrix (ECM) production [3,4], thereby linking between the inflammatory and proliferation phases. However, it is notable that excess inflammation may delay the process of wound healing, because prolonged edema disturbs progression to the next step, and proinflammatory cytokines and toxic free radicals are harmful for skin constituents.

Studies using knockout mice of proinflammatory cytokines and its receptors suggested conflicting results on wound healing. The knockout mice of IL-6 [5] showed delayed healing, while the knockout of tumor necrosis factor (TNF) receptor p55 [6], interferon (IFN)- γ [7], and IL-12/23 [8] showed accelerated healing. The results of the knockout of anti-inflammatory cytokines are also conflicting. Knockout of IL-1 receptor antagonist (IL-1ra) delayed wound closure [9], while IL-10 knockout resulted in prompted healing [10].

Toll-like receptors (TLRs) are involved not only in innate immunity but also in non-infectious inflammation and tissue repair/regeneration. The knockout mice of TLR2 and TLR4 showed delayed healing with decreased mRNA expression of TGF- β and CCL5 mRNA expression in the wounded skin [11].

Reduction of chemokines or their receptor expression also may affect wound healing. Topical application of recombinant dermokin- β as well as its carboxy-terminal domain peptide inhibited mouse wound healing with reduced expression of CXCL1 and CXCL5, both of which are chemoattractant for neutrophils [12].

Therefore, we cannot make a sweeping generalization that acceleration of inflammation is beneficial or harmful. Probably, the waxing and waning of individual cytokines and chemokines might be different during wound healing, and the balance between them might determine the outcome.

2.2. Proliferation phase of wound healing

In the proliferation phase, angiogenesis, fibroplasia, and re-epithelialization occur [13]. Within this phase, fibroplasia and angiogenesis take place concurrently in a closely orchestrated manner to form ECM and granulation tissue. Factors involved in fibroplasia have been intensively studied in systemic sclerosis, keloids, and their rodent models [14–17]. Scleroderma is characterized by excess production and deposition of ECM proteins, at least partly because fibroblasts in scleroderma are resistant to Fas-mediated apoptosis and prone to survive. Thus, the dysregulation of fibroplasia leads to sclerotic disease and also provides an implication to understand wound healing.

2.3. Signaling involved in wound healing

Wound healing is a complex process in which a variety of transcription factors and related molecules participate, including TGF- β /Smad, E2F family, STAT3, homeobox genes, hormone receptors (androgen, estrogen, and glucocorticoid), peroxisome proliferator-activated receptors (PPARs), Wnt/ β -catenin signaling, AP-1, c-Myc, and Erg-1 [18]. These factors are not independent but mutually associated with each other. It is noted that Wnt/ β -catenin pathway forms a negative feedback loop for TGF- β pathway in fibroblast-to-myofibroblast transition [19].

The effect of TGF- β on wound healing is complicated. TGF- β is known to stimulate collagen production in dermal fibroblasts by fibroblast-to-myofibroblast transition [19]. Meanwhile, TGF- β inhibits proliferation of epidermal keratinocytes. An excess amount of TGF- β increases cell rigidity, resulting in keloid formation [20]. The gross effect of TGF- β on wound healing is controversial. On one hand, a small compound, HSc025, which antagonizes the TGF- β /Smad signal, accelerated dermal wound healing by modifying infiltration, proliferation and migration of distinct cellular components in mice [21]. Accordingly, increased level of TGF- β 1 was associated with retardation of wound healing in knockdown mice of Thy-1 (CD90), a glycosylphosphatidylinositol (GPI) linked cell surface glycoprotein [22]. On the other hand, decreased TGF- β was related to delayed wound healing. Wound healing was attenuated in TLR4 (-/-) mice with a decreased number of infiltrating TGF- β ⁺ macrophages and decreased TGF- β mRNA expression in the wounded skin [11]. Thus, it seems that TGF- β may function as a pleiotropic modulator in this process.

2.4. Altered wound healing in diseases

Impairment of wound healing in diabetic ulcer occurs as a consequence of ischemia, excessive reactive oxygen species (ROS), and inflammatory cytokine production. Whey protein (WP) successfully enhanced wound closure in diabetic rats by limiting prolonged inflammation, suppressing oxidative stress, and elevating the antioxidant defense system [23]. Supplementation of injury-related factors is being tried. In diabetic human and mouse skin, HMGB1 protein levels are decreased. HMGB1 protein is a multifunctional cytokine involved in inflammatory responses and

tissue repair. Topical application of HMGB1 prompted wound healing in diabetic mice [24]. Some peptides were also reported to be decreased in diabetic foot ulcer. Expression of antimicrobial peptides, LL-37, is decreased in diabetic ulcer [25]. Besides their antimicrobial effect, LL-37 supported the process of wound healing by increasing keratinocyte proliferation and migration and by promoting angiogenesis.

In contrast to diabetics, the efficacy of wound healing is increased in psoriatic patients, presumably due to accelerated turnover [26]. Immunohistochemistry showed strong immunostaining for subunits of the N-methyl-D-aspartate receptor (NMDAR), an ionotropic glutamate receptor, in the basal cell layer in psoriatic skin. L-Glutamate, the ligand for NMDAR, was also present at a high concentration in the epidermis. Therefore, glutamate could be a new target for wound healing.

2.5. Miscellaneous factors

N-(3-Oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) might be beneficial for wound healing. 3-Oxo-C12-HSL is synthesized by an autoinducer synthase in *Pseudomonas (P.) aeruginosa*, which regulates the production of virulence factors in this bacterium. Inoculation of *P. aeruginosa* strain significantly accelerated wound healing and inflammatory response in Sprague-Dawley rats [27]. These responses were reversed by inoculation with treatment with anti-3-oxo-C12-HSL antiserum.

Expressed in high metastatic cells (Ehm2) belongs to the FERM family of proteins, known to be involved in membrane-cytoskeletal interactions, and has been shown to promote cancer metastasis in melanoma. Ehm2 can act as a positive regulator of keratinocyte adhesion and motility. *In vitro*, Ehm2 knockdown reduced cellular adhesion, migration and motility, without affecting growth, cell cycle, or apoptosis [28].

Sensory neuropeptides from rat primary sensory neurons, such as vasoactive intestinal peptide, calcitonin gene-related peptide, and substance P, increased fibroblasts and keratinocytes proliferation [29]. This suggests that sensation upon wounding is related to the healing process.

3. Approaches to promote wound healing with mesenchymal stem cells or related materials

Mesenchymal stem cells enhance epidermal growth, angiogenesis, collagen deposition and wound contraction. Various methods using stem cells have been attempted to promote wound healing.

3.1. Introduction of stem cells into wound tissue

Install of stem cell is expected to exert a therapeutic effect on wound healing. To innovate more effective introduction, methods have been improved. A novel way of delivering stem cells was tried in a microsphere-based engineered skin [30]. It was effective even for the repairing of sweat glands.

Combination of low-level laser therapy (LLLT) with canine adipose-derived mesenchymal stem cells (ASCs) was attempted to obtain a sustainable effect in the recipient tissue [31]. LLLT increased the survival of ASCs with the decreased apoptosis of ASCs in the wound bed. Genetic modification of human ASCs was also investigated for the feasibility of long-term human ASC culture to enhance their therapeutic use [32]. Akt/v-myc human ASCs actively proliferated longer than control human ASCs. In this system, the authors found up-modulatory effects on the secretion of vascular endothelial growth factor (VEGF), migration of human ASCs, and vasculogenesis of co-cultured endothelial cells.

A certain stem cell population was decreased in chronic skin ulcers. The expression of p75 neurotrophin receptor (p75NTR), an

epidermal stem cell marker, was decreased in patients with chronic skin ulcers [33]. p75NTR could be a candidate for new therapeutic target for chronic skin ulcer patients.

3.2. Introduction of living skin equivalents into wound tissue

Administration of an artificial temporary matrix may be potentially beneficial to promote the migration of fibroblasts and keratinocytes. A new therapeutic option for this strategy is amelogenin, which aggregates under physiological condition to form larger hydrophobic structures like ECM [34]. The development of bioengineering has enabled us to use allograft-derived skin substitutes. Living dermal equivalent (LDE) supports the attachment, growth, and differentiation of keratinocytes. By using LDE, living skin equivalents with a monolayer epidermis and a stratified epidermis also can be formed [35]. The stratified epidermis significantly stimulated blood vessel formation and accelerated epidermal wound closure [35].

Living skin equivalents are now clinically used. Meta-analysis for the use of living skin equivalents suggested that the success rate of complete healing was equivalent to or better than that of standard therapy [36].

Epithelial-mesenchymal interactions suppressed α -smooth muscle actin expression in the fibroblast-rich dermal matrix [37]. This interaction might work to avoid the excessive dermal fibrosis.

3.3. Negative pressure to accelerate wound healing

Negative pressure wound therapy (NPWT) is an application of suction to wound. The vacuum, with negative pressure -75 to -125 mmHg, draws out fluid from the wound, increases blood flow to the area and promotes contracture of wound. It decreases duration to wound closure by promoting contraction of wound and promoting reepithelialization. NPWT is now clinically used for chronic ulcer and surgery site. Meta-analysis revealed that the application of NPWT on skin graft surgery site brought lower reoperation rates among skin graft patients in the NPWT group (7/65; 10.8%) compared to the standard dressing group (17/66; 25.8%) (risk ratio (RR) 0.42; 95% CI 0.19–0.92) [38].

The mechanism of NPWT has been investigated *in vitro*. The cell division control protein 42 (Cdc42) controls the direction of cell migration and regulates cell polarity in wound healing. Negative pressure accelerated monolayer keratinocyte healing involving Cdc42-mediated cell podia formation [39].

4. Conclusion

Research for factors affecting and accelerating wound healing is rapidly in progress. Since wound healing is a well-concerted process with numerous factors, a simple activation or inhibition of a single molecule might not result in favorable therapeutic efficacy. To better understand wound healing, comprehensive analysis of individual molecules/genes may be required.

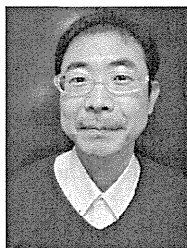
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IgG4-related skin disease

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Summary

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IgG4-related disease (IgG4-RD) is a recently established clinical entity characterized by high levels of circulating IgG4, and tissue infiltration of IgG4⁺ plasma cells. IgG4-RD exhibits a distinctive fibroinflammatory change involving multiple organs, such as the pancreas and salivary and lacrimal glands. The skin lesions of IgG4-RD have been poorly characterized and may stem not only from direct infiltration of plasma cells but also from IgG4-mediated inflammation. Based on the documented cases together with ours, we categorized the skin lesions into seven subtypes: (1) cutaneous plasmacytosis (multiple papulonodules or indurations on the trunk and proximal part of the limbs), (2) pseudolymphoma and angiolymphoid hyperplasia with eosinophilia (plaques and papulonodules mainly on the periauricular, cheek and mandible regions), (3) Mikulicz disease (palpebral swelling, sicca syndrome and exophthalmos), (4) psoriasis-like eruption (strikingly mimicking psoriasis vulgaris), (5) unspecified maculopapular or erythematous eruptions, (6) hypergammaglobulinaemic purpura (bilateral asymmetrical palpable purpuric lesions on the lower extremities) and urticarial vasculitis (prolonged urticarial lesions occasionally with purpura) and (7) ischaemic digit (Raynaud phenomenon and digital gangrene). It is considered that subtypes 1–3 are induced by direct infiltration of IgG4⁺ plasma cells, while the other types (4–7) are caused by secondary mechanisms. IgG4-related skin disease is defined as IgG4⁺ plasma-cell-infiltrating skin lesions that form plaques, nodules or tumours (types 1–3), but may manifest secondary lesions caused by IgG4⁺ plasma cells and/or IgG4 (types 4–7).

What is already known about this topic?

- IgG4-related disease (IgG4-RD) is a recently established clinical entity characterized by fibroinflammatory lesions, high levels of circulating IgG4 and tissue infiltration of IgG4⁺ plasma cells.

What does this study add?

- We comprehensively categorized the skin lesions of IgG4-RD into primary lesions with direct infiltration of IgG4⁺ plasma cells (three subtypes) and secondary non-specific inflammatory lesions where the role of IgG4 remains to be elucidated (four subtypes).
- Our study clarifies IgG4-related skin disease and its differential diagnoses.

IgG4-related disease (IgG4-RD) is a recently proposed clinical entity characterized by high levels of circulating IgG4 and tissue infiltration of IgG4⁺ plasma cells.^{1–4} IgG4-RD exhibits a distinctive fibroinflammatory change involving multiple organs,

including the pancreas,⁵ salivary glands,⁶ lacrimal glands,⁶ biliary tract,^{7,8} peritoneum,⁹ kidney,¹⁰ pituitary gland,¹¹ thyroid gland,¹² lung,¹³ prostate/testis¹⁴ and aorta^{15,16} (Table 1). Even lymph node lesions¹⁷ and orbital pseudotumours¹⁸ have been