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Mechanisms of tolerance and potential therapeutic interventions in Alopecia Areata

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Abstract

This review aims to address the mechanisms of compromised immune tolerance contributing to the development and maintenance of Alopecia Areata (AA). Our goal is to also highlight future treatment opportunities and therapeutics that will safely and efficiently restore hair growth and maintain patients in remission.

AA is a presumptive autoimmune disorder that coincides and genetically clusters to several other autoimmune diseases. In this review, we pay attention to the learnings from the mechanistic research and drug development in these other autoimmune conditions. Interestingly, most of these diseases have been linked to compromised central and peripheral tolerance, and increased intestinal inflammation with enhanced gut permeability. Break of tolerance and priming of the autoreactive T-cells to attack antigenic epitopes in the hair follicle most likely requires several steps which include escape from negative selection and compromised peripheral tolerance. Local skin-related changes are also of importance due to the patchy manifestation of the skin areas with loss of hair, particularly in the early disease. Here, we discuss the defective mechanisms of tolerance, both central and peripheral, and hypothesize that the disease is driven by areas of tolerance break, and that these could be targeted for successful therapeutic interventions.

Keywords: Alopecia areata, Tolerance, Autoimmunity, Tregs, Dysbiosis, Helminth infection

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Abbreviations

AA – Alopecia Areata
AID – Autoimmune disease
Aire – Autoimmune regulator
APC – Antigen-presenting cells
APS – Autoimmune polyendocrine syndrome
CeD – Celiac disease
CTLA – Cytotoxic T-lymphocyte-associated protein
CXCL – Chemokine (C-X-C motif) ligand
DC – Dendritic cell
EAE – Experimental autoimmune encephalomyelitis
ES – Excretory/Secretory
FEZF – Zink finger
FOXP3 – Forkhead box P3 (Treg)
HF – Hair follicle
HLA – Human leukocyte antigen
IFN – Inteferon
IL – Interleukin
ILC – Innate lymphoid cell
IP – Immune privilege
MHC – Major histocompatibility complex
MS – Multiple sclerosis
mTEC – Medullary thymic epithelial cell
NK – Natural Killer
NKG2D – Natural killer group 2D
RA – Rheumatoid arthritis
SCFA – Short-chain fatty acid
SLE – Systemic lupus erythematosus
TEC – Thymic epithelial cell
TGF – Transforming growth factor
TolDC – Tolerogenic Dendritic cell
TRA – Tissue restricted antigens
Treg – Regulatory T cell
T1D – Type 1 diabetes

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1 Introduction

Alopecia Areata (AA) is one of the most common autoimmune diseases (AID) with a reported lifetime risk of 2% and a prevalence of 0.1-0.2% (Gilhar et al 2012, Villasante Fricke & Miteva 2015). However, there are no recent updates on the prevalence or lifetime risk and one should keep in mind that these numbers are from 1975-1989. AA equally affects men and women and currently there are no approved medicines or effective treatment options for most AA patients, leaving a vast unmet medical need. Genetic profiling of AA has demonstrated deficiencies in the mechanisms of both peripheral and central tolerance (Tazi-Ahnini et al 2002, Petukhova et al 2010, Bellacchio et al 2014). AA risk genes are shared with many other AID, such as rheumatoid arthritis (RA), type I diabetes (T1D), coeliac disease (CeD), systemic lupus erythematosus (SLE), multiple sclerosis (MS) and psoriasis (Petukhova et al 2010). This supports the hypothesis of a common-cause genetic and mechanistic relation in the clustering of these diseases (Gregersen & Olsson 2009).

The resolution of the ongoing inflammation in and around the hair follicles is defective in AA patients as compared to healthy individuals (Ito et al 2013). Although genetic predisposition to a dysregulation of immune reponses is a crucial part of the pathogenesis, it seems that environmental factors which trigger reduced immunological tolerance also play a role. These factors are often linked with the modern ways of living; such as stress, sterile environment and altered western diet that affect epithelial barriers and functions of both the gut, lung and skin – organs that are in close communication with the immune system. The complexity implies a role for regulatory T-cells (Tregs), tolerogenic dendritic cells (DCs) and adequate barrier functions. The dysregulated tolerogenic mechanisms explain the development of autoimmunity in general, but the development of the autoimmune attack specifically against the hair follicle in AA remains unanswered. The hair follicle barrier seems to break and become leaky by the cytokine milieu, but it is not clear if this is a cause or a consequence of the immune cell attack (Gilhar 2010, Ito 2010). It is also not known if the priming of autoreactive T-cells, the $CD8^{+}NKG2D^{+}$ in the case of AA, is initiated locally or remotely (in e.g. the gut mucosa), or both. Moreover, the complexity of AA involves also triggers for anagen induction from telogen, or from placode, for normal hair growth and these are not yet fully understood (Schneider et al 2009). All genes involved in hair cycling have been mapped, at least in mice (Sennet et al 2015 www.hair-GEL.net), which may be an important future tool to understand anagen triggers in human disease.

Another interesting perspective is the increasing prevalence of many AIDs (Cooper & Stroehla 2003, Shoenfelt et al 2008, Elfström et al 2014, Lerner et al 2015) and the link to intestinal dysbiosis and increased gut permeability (Visser et al 2009). Although this link has not yet been proven for AA, the diseases that are associated with intestinal permeability closely resemble the list of diseases with genetic association to AA. Moreover, a negative correlation is seen with regards to a reduction of parasite infections in industrialized countries and the increased prevalence of autoimmunity (Shoenfelt et al 2008). Whilst there is limited data on the prevalence of AA in regions of high helminth infection, a paper by Villasante Fricke and Miteva (2015) reports intriguing data showing incidences of 0.57 and 0.7% in Mexico and India respectively, both countries where parasite infection is high in comparison to rates of 1.7 to 3.8% in the US, UK and Singapore. Less exposure to dirt, bacteria, viruses and parasites in our daily lives may also provide minimal training of the immune cells and a less tolerogenic immune system (the hygiene hypothesis), which would be especially detrimental in susceptible people.

In this review, we discuss the defective mechanisms of tolerance, central and peripheral (including the hair follicle itself), in AA. Understanding the underlying mechanisms may help to design and develop new and effective therapeutic interventions for AA.

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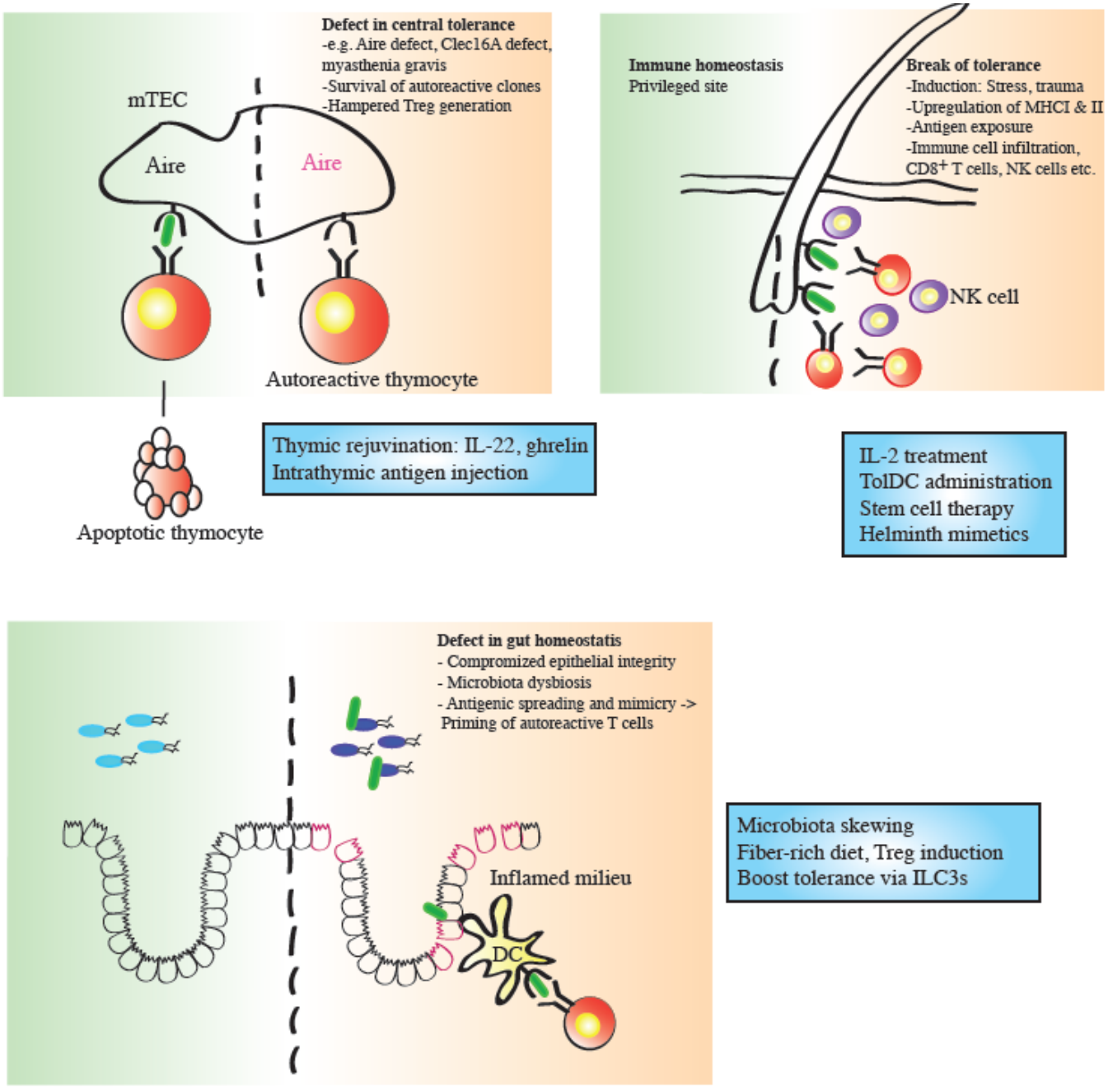

Figure 1: Schematic illustration of sites that contribute to maintenance and/or break of tolerance in AA and possible therapeutic approaches. **Upper Left:** Deficiencies in central tolerance have been described as a possible underlying mechanism in disease development. Here, a defect results in the escape of autoreactive T-cells into the periphery and an improperly educated pool of thymic Tregs. **Upper Right:** The peripheral tolerance function with regards to AA is often assigned to the immune privilege (IP) and its guardians (e.g. TGFβ, αMSH, IGF-1) where an infection or a stress reaction is likely to initiate breakage of the barrier. The collapse of the IP reveals MHC I epitopes and attracts cytotoxic immune cells that attack the sensitive and highly proliferative structures that provide normal hair growth. A defective regulatory function of the immune system in the skin, e.g. by dysfunctional Tregs, non-tolerogenic DCs and/or proinflammatory macrophages, may contribute to the defective resolution of inflammation and thus reduced ability to restore IP. **Lower Left:** The notion that AA

clusters with other autoimmune and inflammatory diseases that coincide with gut inflammation and an increased intestinal permeability, points towards the gut as a potential driver of disease. This may occur by priming of autoreactive T-cells by antigen presenting cells (e.g. DCs) either at site or in lymph nodes where cytotoxic $CD8^+$ T-cells are educated to destroy HF structures, and driven by the local production of chemokines such as CXCL10 (attracting $CD8^+NKG2D^+$ cells) and NKG2D ligands (attracting natural killer cells) in and around the hair follicles. By boosting underlying tolerance or the local ability to restore IP around hair follicles we postulate that AA can be managed, or even cured. Aire: Autoimmune regulator, DC: Dendritic cell, HDAC: Histone deacetylase, MHC I & II: Major histocompatibility complex I & II, mTEC: Medullary thymic epithelial cell, NK cell: Natural killer cell, TolDC: Tolerogenic dendritic cell.

2 Pathophysiology of AA

AA was originally considered an organ-restricted autoimmune disease in the early 20^{th} century when it was established that autoreactive T-lymphocytes were attacking hair follicle components (Kalish et al 1992, Bodemer et al 2000); however with increasing severity of disease, nail and eye involvement are also commonly seen (Gandhi et al 2003, Pandhi et al 2009). Recently, it was established that in a mouse model of AA the $CD8^+NKG2D^+$ cells are required for the hair follicle (HF) destruction and are solely responsible for the induction of disease (Xing et al 2014). These cells are also documented in humans but their function still needs to be unravelled. Much is now known about the disease (Gilhar et al 2012, Hordinsky 2013, Islam et al 2015, Villasante Fricke & Miteva 2015, Santos et al 2015, Sette et al 2015), with the exceptions of the exact etiology (McElwee et al 2013). Most importantly, several autoantigens of the hair follicle are suggested but are as yet unconfirmed (Tobin et al 1997, Gilhar & Kalish 2006, Leung et al 2010, Wang et al 2016).

Currently, there are no launched medicines for AA. In more than 60% of all cases, AA is established in early age and the early onset also correlates to increased disease severity (Gilhar et al 2012). The progression of AA upon diagnosis is unpredictable; it may remain relatively stable, develop into fast complete hair loss over a course of several nights or weeks, or spontaneously resolve itself. Ten percent of patients develop complete hair loss, a condition termed alopecia universalis, which is very rarely spontaneously resolving (Medscape, Alopecia Areata Clinical Presentation by Chantal Bolduc, MD). Comorbidities are common (Chu *et al* 2011, Huang *et al* 2013, Villasante Fricke & Miteva 2015, Suárez-Fariñas *et al* 2015), the most frequent ones being atopic dermatitis (in approximately 20-40% of all AA patients), thyroid dysfunction (e.g Grave's Disease or Hashimoto's, in approximately 8-10%), and vitiligo (in approximately 3-8%). The pathophysiology of AA is complex; T lymphocytes, cytokines, neuropeptides, normal hair cycling and genetic background all play important roles in the induction and maintenance of the disease. Since AA autoantibodies have not been confirmed to cause the hair follicle destruction (Ledesma & York 1982, Tobin et al 1994, Gilhar et al 2012) little research has been focussed on B-cells whereas $CD4^+$ Th1, Th2, Th17 and Treg signatures have been extensively studied and their respective role in driving the disease is still debated (Gilhar et al 2007, Freyschmidt-Paul et al 2006, Suárez-Fariñas et al 2015, Li et al 2015). It is, however, now well established that the cytotoxic $CD8^+$ T-cells, natural killer cells and cytokines/chemokines like IFNγ, CXCL10 and $NKG2D^+$ ligands have the strongest link to the disease (Xing et al 2014).

Shared genetic background to other autoimmune diseases like thyroid disease, vitiligo, RA, T1D, CeD, SLE, MS and psoriasis has been recognised (McDonagh & Tazi-Ahnini 2002, Hordinsky & Ericson 2004, Petukhova et al 2010, Betz et al 2015). Two different subsets of genetic deviations in addition to the HLA loci were highlighted (Petukhova et al 2010); the first with genes critical for regulatory T cell maintenance (e.g. CTLA4, IL-2/IL-21, IL-2Rα) and the second with significance to the NKG2D receptor (ULBP ligands as well as MICA/MICB). These loci are also essential in the pathogenesis of

RA, T1D and CeD – diseases that resemble AA with regards to the tissue destruction performed by unregulated cytotoxic lymphocytes.
Viral infections and vaccinations are often associated with the onset of AA, which is of note as this connects mechanisms of major histocompability complex (MHC) class I expression, molecular mimicry and neuropeptides (e.g. Substance P) released by neuronal synapses in the skin to the specific round patches seen in AA. We investigated epitope similarities between 5 postulated autoantigen(-s) of the hair follicle structures and common viruses and found many shared epitopes, especially with regards to herpes virus but also with the human immunodeficiency virus, HIV (unpublished data). Other viruses and bacteria (CMV, hepatitis B, hepatitis C, Epstein Barr, swine flu and helicobacter pylori) have also been associated with AA (Skinner et al 1995, Islam et al 2015, Campuzano-Maya 2011). It is intriguing to note that herpes virus has more epitope similarities and is a common infection in line with AA being one of the most prevalent autoimmune disorders, but this needs further investigation.
A recent hypothesis suggests that autoreactive T-cells are primed either in the gut mucosa or in peripheral lymphoid tissues by gut-primed DCs/APCs. For example the wheat gluten derived protein α-gliadin demonstrates similar epitopes to trichohyalin (Tobin et al 2015), which is a possible autoantigen in the hair follicle (Erb et al 2013, Tobin 1997, Wang et al 2016). Gluten antigens have also been associated with the hair follicle peptide peroxiredoxin 5 (PRDX5) (Jabbari et al 2013) which is one of the genes associated with AA (Petukhova et al 2010). Other dietary proteins with similarities to hair follicle epitopes, have to our knowledge not yet been investigated, e.g. cow milk proteins which are known irritants to the immune system (Docena et al 1996, Vojdani 2015), and it is interesting to note that dairy products are often excluded as part of an anti-inflammatory diet that may be recommended for AID. We propose that understanding the antigenic specificity, origin and site of priming of autoreactive T-cells might open up for new therapeutic angles to in a more preventive way treat AA and to maintain patients in remission.

3 Mechanisms of immune tolerance

3.1 Central tolerance

Thymus is the organ of T cell development and as such its tasks include establishing a T-cell repertoire which is both able to recognize antigenic epitopes in the context of antigen presenting MHC molecules, but to be ignorant towards self antigens. While recognition of MHC molecules is established in the process of positive selection mainly in the thymic cortex, negative selection (or clonal deletion) takes place in the thymic medulla micromilieu with the goal of purging the developing thymocyte population from autoreactive clones (Klein et al 2014). To achieve this comprehensively, medullary thymic epithelial cells (mTECs) are highly specialized to express an impressive set of tissue restricted antigens (TRAs) (Sansom et al 2014). A key transcription factor in mTECs is the autoimmune regulator (Aire) which controls the expression of TRAs (Anderson et al 2002) by releasing stalled RNA polymerases (Giraud et al 2012).

3.1.1 Central tolerance in autoimmunity and AA

Mutation in AIRE causes autoimmune polyendocrine syndrome type 1 (APS-1), which is characterized by the triad; chronic mucocutaneous candidiasis, Addison's disease and hypoparathyroidism (The Finnish-German APS-1 Consortium 1997). In addition, APS-1 patients often suffer from AA (Ahonen et al 1990). Genotyping of APS-1 patients has revealed that the AIRE mutant allele 961G is a risk factor for alopecia universalis and for early onset of disease (Tazi-Ahnini et al 2002). Another mutant allele of AIRE is the S250C variant that has also been reported together with AA (Bellacchio et al 2014). Further, APS-1 patients with alopecia totalis have autoantibodies against differentiating keratinocytes in the hair follicles (Hedstrand et al 1999). Interestingly, Down syndrome (trisomy 21) is another patient group

with increased frequency of AA from 2% to 30% (Du Vivier and Munro 1974). At the same time it has been shown that AIRE-expression (AIRE-gene is located at 21q22.3) is altered in the patients with Down syndrome (Skogberg et al 2014). Another genetic link between thymus and AA is the CLEC16A gene which has been associated to AA in a genome wide association study (Jagielska et al 2012). The molecular importance of Clec16A in known to be of importance in the thymus where it is involved in autophagy in TECs (Schuster et al 2015). A further link between thymic dysfunction and AA is the association between AA with myasthenia gravis and thymoma (Kubota et al 1997, Suzuki et al 2005). In addition, thymectomy and glucocorticoid treatment as therapy for a case of myasthenia gravis has been reported to markedly improve AA in the patient (Kamada et al 1997).
Recently, it was shown that AIRE is not the only transcription factor in the thymus that controls TRA-expression but instead accompanied by FEZF2 (also known as Zfp312 and Fezl) (Takaba et al 2015). The two transcription factors seem to control different sets of TRAs and hence work in tandem to make the thymic TRA-repertoire more comprehensive than either of them can establish alone (Takaba et al 2015). This is a major step forward in the understanding of central tolerance and sheds light on the discrepancy of autoimmune manifestations between APS-1 patients compared to Aire$^{-/-}$ mice. Interestingly, both Aire$^{-/-}$ mice and mice deficient in Fezf2, specifically in thymic epithelial cells, have autoantibodies in their sera against different structures of the skin (Takaba et al 2015). It is, however, not addressed yet whether FEZF2 defect is linked to autoimmunity (or with AA in particular) in humans. Finally, total lifetime and early childhood traumatic events have been associated with AA (Willemsen et al 2009). This is interesting together with the notion that elevated concentrations of glucocorticoid steroids as a consequence of prolonged stress affects thymic size and output (Dominguez-Gerpe and Rey-Mendez 2003, Doppman 1986, Hanson et al 2009). Moreover, perinatal generation of Tregs generated in the thymus early in life plays a specific role in the maintenance of self-tolerance which is distinct from the population produced in adults (Yang et al 2015). Taken together, the above literature indicates that defects in thymus function could be an underlying contributor to the development of AA due to incomplete deletion of autoreactive T cells and/or improper education of thymic Tregs.

3.1.2 Therapeutic approaches targeting central tolerance

Since the thymus goes through age-related involution which influences thymic output and size, re-establishment of central tolerance by thymic rejuvenation is an approach with therapeutic potential for autoimmunity. For example, it has been shown in mice that IL-22 restores thymic function through signalling in mTECs, which leads to proliferation and survival of these cells (Dudakov et al 2012). Another age related thymic change is the loss of ghrelin and its receptor. Infusion of ghrelin into the thymus restores thymic architechture, numbers of thymocytes, numbers of recent thymic emigrants (Dixit et al 2007) and indicates potential for small molecule ghrelin agonists. A second approach to harness tolerance induction in the thymus is intrathymic injection of antigens. This strategy has shown promising results in different autoimmune models in for example a rat model of autoimmune emphysema (Hanaoka et al 2010) and in experimental autoimmune encephalomyelitis (EAE) where Lewis rats were intrathymically injected with myelin basic protein (Okura et al 2003). With regards to AA, the autoantingen is however still not confirmed, which limits this feasibility at present. Therapeutic options directed towards the thymus are included in Figure 1.

3.2 Peripheral tolerance and immune privilege of the hair follicle structure

In addition to central tolerance performed in the thymus, mechanisms of peripheral tolerance are of pivotal importance to avoid autoimmunity. For AA, an extra layer of tolerance is the hair follicle itself which has properties of an immune privileged (IP) site. Primarily, this privilege is based upon down-

regulated MHC class 1a, beta(2)-microglobulin and MHC class II in the hair bulb (Harrist et al 1983, Meyer et al 2008). Hence, breakage of the hair follicle structure and IP makes MHC class I and class II molecules accessible for interaction with immune cells and is considered a central event in the course of AA (Gilhar and Kalish 2006). The local mediator and cytokine milieu is another component in maintaining a tolerogenic milieu in the hair follicles. It has been shown that the immune suppressing alpha-melanocyte stimulating hormone, transforming growth factor-beta2 (TGFβ2), macrophage migration inhibitory factor and indoleamine-2,3-dioxygenase (IDO) are all up-regulated in $CD200^+$ stem cell-rich hair follicle bulge regions (Meyer et al 2008). The role of IDO is to make DCs turn tolerogenic by exerting metabolic control over effector and Tregs (Munn et al 2013).

3.2.1 Treg function and induction in AA

Induction of $Foxp3^+$ Treg function has been an area of great focus since the discovery of these cells and their importance in the prevention of autoimmune disease development (Sakaguchi et al 2008, Sakaguchi et al 2009). The Treg population is a key layer of peripheral tolerance and it has been demonstrated that these cells are especially abundant inside the hair follicles (Sanchez Rodriguez et al 2014). Their specific local function, apart from suppression of effector T-cells, has yet not been established although it was speculated that they are guardians of the stem cells. Tregs contribute directly by tolerance to specific autoantigens but also indirectly to a tolerogenic milieu; the latter because in contrast to effector T cells, the Treg IL-2 receptor contains the high affinity IL-2Rα chain CD25 and hence outcompetes effector T cells for IL-2 (Sakaguchi et al 2008). Recently, the single nucleotide polymorphisms allelic variant (rs2294020) for the FOXP3 gene as well as the single nucleotide polymorphisms allelic variant (rs378299) of the inducible costimulator ICOSLG have been associated to AA (Conteduca et al 2014). These genetic associations highlight the role of Tregs in AA.
To our knowledge, there is only one report demonstrating impaired function in Tregs from circulating blood of AA patients (Shin et al 2013) and one report of the same in alopecic mice (Zöller et al 2002), however both of these studies evaluated Treg function *in vitro* under non-inflammatory conditions. Except for the indirect study of Tregs in five patients with different degrees of AA given a low dose of recombinant IL-2 (9 week pulsed protocol of Aldesleukin, Castela et al 2014), the function of Tregs in the AA inflammatory milieu (*in vivo* and *in situ*) has to our knowledge not been evaluated. The fact that four of five patients responded to Aldesleukin with hair growth and concomitant increased numbers of Tregs in the skin indicates that induction of Treg function might be a feasible therapeutic option in this disease.
The activation of Tregs but not effector T-cells by low dose IL-2 supplementation or engineered small molecules or antibodies that target the IL-2 receptor might be a safe and effective future therapy for AA patients (Mitra et al 2015, Spangler et al 2015). Recent technical achievements in the *ex vivo* expansion of Tregs with maintained stability and function and their re-administration to patients with autoimmune disorders demonstrate that Treg cell therapy might be feasible in the future (Perdigoto et al 2016). Targeting epigenetic modifications within Treg functionality may also be a future therapeutic tactic (Morikawa & Sakaguchi 2014). Moreover, understanding the role of PTPN22 in AA, the most strongly associated allele outside of the HLA regions in autoimmunity, might reveal druggable targets (Bhanusali et al 2014, Salinas-Santander et al 2015, Maine et al 2012, Brownlie et al 2012).

3.2.2 Tolerogenic DCs in autoimmunity

Dendritic cells are critical in determining the outcome of the immune response. Depending on their maturation state, DCs either can elicit an inflammatory response or a tolerogenic response. Migratory semi-mature DCs have been identified as a potent tolerogenic DC population (Azukizawa et al 2011, Vitali et al 2012, Idoyaga et al 2013). These migratory DCs can among other sites be found in the skin,

and they induce the formation of peripheral Tregs. Their tolerogenic property is a promising mechanism that could be targeted to resolve autoimmune disease. Recently, Doñas and colleagues showed that inhibition of the histone demethylase JMJD3 made DCs adopt a more migratory/semi-mature/tolerogenic phenotype with lower expression of the costimulatory molecules CD80 and CD86 (Doñas et al 2016). Tolerogenic DC-induction therapy uses patient-derived precursors *ex vivo* to manipulate these cells to differentiate into tolerogenic DCs which are then injected back into the patients. So far, this type of approach has reached phase I clinical trials for T1D (Giannoukakis et al 2011) and RA (Benhem et al 2015, Bell et al 2016) but to our knowledge not yet for AA.

3.3 Highlighted promising present/future work of autoimmune models, AA autoantigens and stem cell therapeutics

In analogy with AA as an autoimmune disease where immunologic privilege has been broken, a recent publication showed that in addition to Aire-induced central tolerance, expression of Lyn in eye-draining lymph node DCs is an important layer of peripheral tolerance to avoid autoimmune uveitis (Proekt et al 2016). Here, functional expression of Lyn could protect against autoimmune uveitis despite dominant-negative Aire mutation. Similar studies investigating multiple layers of tolerance that protect the hair follicle against autoimmune attack would greatly enhance the understanding of AA etiology. Other important work in the near future will include efforts to uncover autoantigens involved in AA which to date is an area of scarce knowledge (Sette et al 2015, Wang et al 2016). Therapeutic promise of skewing the immune system by using autoantigens to induce peripheral tolerance in AA has been provided by Erb and colleagues (Erb et al 2013). Using hair specific keratins as autoantigens (keratin 71 and keratin 31) they showed that subcutaneous injection of keratin-peptides induced tolerance via T-cell anergy in the C3H/HeJ mouse model of AA and partly prevented disease progression.
Another interesting approach to induce or restore immune regulation/tolerance in autoimmune disease, such as AA, is the Stem Cell Educator Therapy (Tianhe Stem Cell Biotechnologies®, Jinan, China). This was successfully tested in 9 moderate to severe AA patients (average age 20 years and >5 years of disease) who received one treatment in which their own mononuclear cells were separated from whole blood and circulated through a closed-loop to briefly interact with adherent human cord blood-derived multipotent stem cells, after which they were returned educated to the patient's circulation (Li et al 2015). Eight of nine patients responded with improved hair growth and quality of life within 4-12 weeks until the 2 year follow up. The investigators noted restoration of the IP around the hair follicles (as "a ring of TGF-1β"), an upregulation of Th2 cytokines, no change in the amounts of circulating Tregs but importantly less cytotoxic $CD8^+NKG2D^+$ cells. The exact mechanisms of this treatment are not known. In the choice between targets to induce tolerance, central or peripheral, in AA patients and to restore hair growth, thorough patient segmentation is the way forward in order to reveal the underlying molecular mechanisms of this heterogeneous disease.

4 Gut microbiota and helminths, autoimmunity and tolerance

4.1 Intestinal inflammation, gut microbiota dysbiosis and autoimmune disease

There are several studies supporting the hypothesis that abrogation of the intestinal epithelial barrier function induces local or remote inflammation and also autoimmunity (Arrieta et al 2006, Fasano & Shea-Donohue 2005, Öhman et al 2015). Interestingly, the clustering of diseases with increased intestinal permeability (T1D, Crohn's disease, CeD, atopic dermatitis, RA, irritable bowel disease, ankylosing spondylitis) is very similar to the genetic clustering of AIDs including AA, strengthening the hypothesis that a compromised intestinal barrier and hence compromised mechanisms of intestinal tolerance contributes to these diseases.

Oral tolerance is a separate branch of peripheral tolerance and is specific to the oral route, i.e. tolerogenic processes in the gut and intestine. While central tolerance aims to tolerize against self-structures, oral tolerance is primarily important for induction of tolerance towards food and microbiota antigens. Hence break of tolerance in the gut with regards to autoimmune disease is probably more an effect of dysbiosis of the microbiota than failed oral tolerance *per se*. The appreciation that the microbiota in the gastrointestinal tract is of high importance for the development of the immune system has been around for some time (Kelly et al 2007). The first study that revealed some of the underlying biology came in 2010 from the Benoist-Mathis lab. They showed that a strain of segmented filamentous bacteria is enough to drive autoimmune arthritis in a Th17-dependent manner in mice (Wu et al 2010). Recently is has been demonstrated that a subpopulation of RA patients has a Prevotella copri dominated microbiota. Here, transfer of microbiota from RA patients into germ free arthritis-prone SKG mice resulted in increased number of Th17 cells compared to controls. Severe arthritis also developed in the mice receiving Prevotella copri dominated RA microbiota (Maeda et al 2016). The role of dysbiosis of the gut microbiota and its role in RA has recently been reviewed elsewhere (Coit and Sawalha 2016). Using the relapsing-remitting mouse model of spontaneously developing EAE, Berer and colleagues have investigated the role of the microbiota (Berer et al 2011). Here, the authors propose a two-step model: firstly, autoreactive T cells in the gut associated lymphoid tissue are activated by the microbiota followed by recruitment of autoantibody-producing B cells. Secondly, the communicating autoimmune T- and B-cells trigger a demyelinating encephalomyelitis. Also, germ-free MOG-Tg mice harbouring segmented filamentous bacteria alone as gut microbiota have been shown to be highly susceptible to EAE compared to mice without segmented filamentous bacteria (Lee et al 2011).
Another example of microbiota-mediated autoimmunity was published by Horai and colleagues. They showed that a non-cognat antigen in the intestine originating from the microbiota activated autoimmune retina-specific T-cells and caused autoimmune uveitis, independently of endogenous retinal autoantigen (Horai et al 2015). Again, as with the hair sack, the eye is an immune privileged site, hence such an environment does not seem to be excluded from this type of autoimmune process.
Adding to the complexity of the role of microbiota in autoimmune disease, its absence due to antibiotic treatment can trigger type 1 diabetes in mice (Livanos et al 2016) and its role is increasingly speculated about with regards to human autoimmunity (Luckey et al 2013, Belkaid & Hand 2014, McLean et al 2015). Highlighted therapeutic options towards the gut are included in Figure 1.

4.1.1 Therapeutic approaches to alter the gut microbiota – immune system interaction

So far, there is scarce evidence that a dysregulated microbiota of the intestine is a contributing factor to AA, but with the huge gut commensal variety in mind, it is not implausible that this could be the case in a genetically susceptible population. The dietary contribution to immunological homeostasis including the role of Tregs is starting to emerge (Maslowski & Mackay 2011). The effect of short-chain fatty acids (SCFAs) produced by the gut microbiota as a result of fiber consumption has been shown beneficial in experimentally induced colitis (Smith et al 2013). In addition, SCFA-induced Treg effect does not seem limited to the gut but has also been illustrated in EAE mice where addition of SCFAs ameliorated disease via lamina propria-derived Tregs (Haghikia et al 2015). The effect of SCFAs seems to be driven by inhibition of histone deacetylase (HDAC), at least in the case of butyrate and propionate (Arpaia et al 2013, Furusawa et al 2013).
Several druggable targets within this field, like e.g. HDAC6 and 9 (Smith et al 2013, Arpaia et al 2013, Furusawa et al 2013) and GPR43/41 (Ulven 2012, Kim et al 2014, Macia et al 2015, Bolognini et al 2016), have been elucidated from the SCFA interaction with the immune system, especially with regards to Treg function. Also, an altered diet in itself is possibly a non-medical therapeutic approach to re-establish a homeostatic health-promoting gut microbiota, e.g. by a more fiber-rich diet (Bollrath &

Powrie 2013). Several attempts in finding small molecule approaches to heal intestinal permeability are ongoing, e.g. zonulin inhibitors or PAR-2 antagonists (Fasano 2012, Patel et al 2010).
Recently, a previously unknown layer of peripheral tolerance was revealed by Hepworth and colleagues. They could show that in the gut, a population of type 3 innate lymphoid cells (ILCs) with intrinsic expression of MHC class II promote tolerance by inducing cell death of commensal-specific T-cells (Hepworth et al 2015). Since commensal antigens are not part of the thymus TRA-repertoire, this ILC3-mediated mechanism represents a major step forward in the understanding of tolerance induction towards the commensal microbiota and is a new possibility to exploit for therapeutic intervention to avoid microbiota dysbiosis.

4.2 Helminth's induction of tolerance

Helminth infections are still common in many developing countries, affecting more than 2 billion worldwide (de Silva et al 2003). Interestingly the decline of helminth infections in developed countries correlates with the increased incidences of inflammatory disorders (Rook 2012). As part of their chronic nature, helminths induce a situation of immunological tolerance to enable their survival alongside the host. This immunotolerance is generally associated with a shift of immunological response from Th1 to Th2, inhibition of DC activation and induction of Tregs (Maizels et al 2009). In addition to enabling establishment and survival of the helminth, this induced immunological tolerance could have potential benefit to the host, particularly with regards to dampening aberrant immunological and allergic responses. In recent years, several studies in both humans and mice have sought to investigate the protective effect of helminth infection (or that of helminth secretory products) on various autoimmune diseases. These have included Crohn's disease, ulcerative colitis, MS, and allergic rhinitis in humans, and have been extended to include models of asthma, psoriasis, transplant rejection, diabetes, gastritis and hepatitis in rodents (Leonardi et al 2015). Many of these studies showed promising results with regards to the ability of helminths to modulate a wide range of autoimmune/chronic inflammatory disorders. Interestingly, these studies highlight the fact that intestinal helminths are capable of eliciting immune responses that are not only limited to their local environment, but are systemic in nature.
A number of mechanisms are suggested to be involved in this systemic control (Mishra et al 2014). Initially, the presence of the helminths within the gut causes damage to the mucosal epithelial cells which in response release various cytokines including IL-25, IL-33 and thymic stromal lymphoprotein (TSLP). These cytokine alarmins activate ILC2s and basophils within the lamina propria of the gut (alongside other inflammatory cell populations), which either indirectly through ILC2 secretion of IL-5 activate eosinophils, or directly through basophil production lead to IL-4 release, thereby driving Th2 and Treg cell differentiation (Sonnenberg & Artis 2015, von Moltke et al 2016). Increased understanding of the mechanistic pathways through which helminths affect immune balance within the body may open up the potential for the design of small molecules and biologics targeting the specific pathways described above. It should be noted that the above mentioned mechanisms associated with the support of immune tolerance in the context of helminth infections are present in the pathology of allergic mucosal inflammation, which raise some concerns for therapeutic development.

4.2.1 Helminth based therapeutic approaches

Whilst there have been a number of clinical trials that have investigated the therapeutic potential of helminths in various autoimmune conditions (in particular irritable bowel disease) the ingestion or transdermal administration of live parasites in therapy comes with a number of caveats including potential pathogenicity, dissemination and elimination from the human host (Leonardi et al 2015). These could be potentially overcome by shifting the focus of potential therapeutics towards mimicking the

observed anti-inflammatory properties of helminth excreted/secreted (ES) products. To date there have been very few trials which specifically address this. Whilst the use of these proteins may result in undesirable immunogenic effects it may be possible to develop small molecule mimetics. In 2013 a phosphorylcholine analogue (11a) was reported to be as efficacious as ES-62 in preventing the development of collagen-induced arthritis in a mouse model, and supressing the development of antinuclear antigens and kidney pathology in a murine SLE model. In both cases it was shown that this small molecule mirrored the mechanism of action of ES-62 in downregulating the TLR/IL-1R transducer MyD88 (Al-Riyami et al 2013, Rodgers et al 2015). Additionally Tuftsin-PC and FhHDM-1 are both parasite-derived assets in pre-clinical development for the treatment of inflammatory and autoimmune diseases (Ben-Ami Shor et al 2015, Bashi[a] et al 2015, Bashi et al 2016, US 2016/0256516 A1, Robinson et al 2011, Thivierge et al 2013).

To date, there are no studies examining the effect of helminth exposure in models or clinical cases of AA so we can only hypothesize regarding their potential effect in this disease. As described, helminth therapy has been shown to be effective in immunological disorders that are distant to the gut. To our knowledge there is only one study demonstrating immunomodulation within the skin, this being prevention of the formation of psoriatic lesions in the skin of fsn/fsn mice following subcutaneous exposure to the helminth glycan LNFPIII (Atochina et al 2006). In this paper, the authors describe a decrease in IFNγ levels and an increase in IL-13 within skin cells, as well as a reduction in $CD8^+$ T cells within lymph nodes, these being pathways which are likely to also be of importance in AA. Additionally, the increased generation of $Foxp3^+$ Treg cells (probably through induction of TGFβ) has been documented following exposure of mice to various helminths as well as to S.mansoni soluble egg antigens (Bashi[b] et al 2015). This may be relevant in AA with its circulating Th1/Th2 cytokine profile and due to the fact that follicle-associated $Foxp3^+$ cells have been shown to be decreased in patients in comparison to healthy controls (Han et al 2015).

5 Concluding remarks

We have highlighted the role of defective immune tolerance at several levels in AA and that the development of AA is likely a result of these factors working in concert. Break of the immune privilege around the hair follicle *per se* is not expected to induce AA unless cytotoxic immune cells have been misdirected by mechanisms of molecular mimicry, e.g. with triggering by microbial antigen and/or defective mechanisms of central tolerance. The association of AA with atopic dermatitis supports the view that an inherent deficiency in resolving the local skin inflammation may be a factor contributing to AA. Then again, it is still not completely understood why some people remain in telogen-placode even if the local inflammation is more or less completely resolved. This indicates that triggering anagen from any of these resting phases takes another, non-immunogenic, signal.

The unravelling of patients underlying deficiencies may help to develop personalized treatment options to efficaciously resolve flares and maintain remission in patients.

Conflict of Interest

GS, SJ and AÅ are employees of AstraZeneca and declare no conflicts of interest.

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