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Identification of Autoantigen Epitopes in Alopecia Areata

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Alopecia areata (AA) is believed to be a cell-mediated autoimmune hair loss disease. Both CD4 and cytotoxic CD8 T cells (CTLs) are important for the onset and progression of AA. Hair follicle (HF) keratinocyte and/or melanocyte antigen epitopes are suspected potential targets of autoreactive CTLs, but the specific epitopes have not yet been identified. We investigated the potential for a panel of known epitopes, expressed by HF keratinocytes and melanocytes, to induce activation of CTL populations in peripheral blood mononuclear cells. Specific synthetic epitopes derived from HF antigens trichohyalin and tyrosinase-related protein-2 induced significantly higher frequencies of response in AA CTLs compared with healthy controls (IFN-gamma secretion). Apoptosis assays revealed conditioned media from AA peripheral blood mononuclear cells stimulated with trichohyalin peptides elevated the expression of apoptosis markers in primary HF keratinocytes. A cytokine array revealed higher expression of IL-13 and chemokine ligand 5 (CCL5, RANTES) from AA peripheral blood mononuclear cells stimulated with trichohyalin peptides compared with controls. The data indicate that AA affected subjects present with an increased frequency of CTLs responsive to epitopes originating from keratinocytes and melanocytes; the activated CTLs secreted soluble factors that induced apoptosis in HF keratinocytes. Potentially, CTL response to self-antigen epitopes, particularly trichohyalin epitopes, could be a prognostic marker for human AA.

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INTRODUCTION

Alopecia areata (AA) is one of the most common autoimmune diseases in humans with a lifetime risk of 1.7–2.1% in the United States (Mirzoyev et al., 2014; Safavi et al., 1995). AA skin lesions are characterized by $CD4^+$ (T-helper lymphocytes) and $CD8^+$ (cytotoxic T lymphocytes; CTLs) cell infiltration around hair follicles (HFs) (Todes-Taylor et al., 1984; Whiting, 2003). Notably, CTLs infiltrate into the HFs based on previous observations (McElwee et al., 2003; Ranki et al., 1984; Zhang et al., 2013). The recovery of hair growth for some patients when treated with immunosuppressants, and the reduction of infiltrating CTLs in the recovered HFs, suggests that T cells are functionally important in AA development (Alkhalifah et al., 2010a, 2010b; Van Scott, 1958; Wang and McElwee, 2011).

In rodent models, in vivo depletion of $CD8^+$ cells can restore hair growth in dundee experimental bald rats (McElwee et al., 1996), whereas depletion of $CD4^+$ cells resulted in partial hair regrowth (McElwee et al., 1999), indicating the importance of both CTLs and T-helper lymphocytes cells in the persistence of AA. In C3H/HeJ mice, AA can be induced by transfer of T cells (McElwee et al., 2005; Wang et al., 2015). Furthermore, transfer of human peripheral blood mononuclear cells (PBMCs) to scalp explants grafted to severe combined immunodeficient mice can also induce AA (Gilhar et al., 2013). Although the role of CTLs is evident in AA rodent models, the exact autoantigen epitopes that are involved in eliciting CTL responses that cause and/or perpetuate AA lesions are still debated.

HF melanocyte- and keratinocyte-derived antigens may be involved in the pathogenesis of AA (Erb et al., 2013; Gilhar et al., 2001; Messenger and Bleehen, 1984, 1985; Paus et al., 1993). Apoptosis of hair bulb melanocytes is observed in acute AA (Tobin et al., 1990). Shedding of pigmented hair during the onset of AA and redevelopment of nonpigmented hair after the resolution of AA is also observed (Finner, 2011). T cells stimulated with melanocyte-derived epitopes reinduced AA in human AA scalp explants grafted to severe combined immunodeficient mice (Gilhar et al., 2001).

A close association of infiltrating T cells with HF root sheaths (McElwee et al., 2003; Messenger and Bleehen, 1984) circumstantially suggests that keratinocyte-expressed epitopes may also be autoreactive CTL targets. Autoantibodies against keratinocyte-derived trichohyalin (TCHH) and cytokeratin-16 are increased in the serum of patients with AA (Leung et al., 2010), though sera injections are unable to alter hair growth in rodent studies (Gilhar et al., 1992).

We utilized the collective knowledge of potential AA-associated antigens presented in previous studies to

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Abbreviations: AA, alopecia areata; CM, conditioned media; CTL, cytotoxic T lymphocytes; ELISpot, enzyme-linked immunoSpot; GP100, premelanosome protein analog; HF, hair follicle; ICS, intracellular cytokine stain; MART1, melanoma antigen recognized by T cells 1 Leu27 analog; PBMC, peripheral blood mononuclear cell; SFC, spot-forming cell; TCHH, trichohyalin; TYR, tyrosinase; TYRP2, tyrosinase-related protein-2

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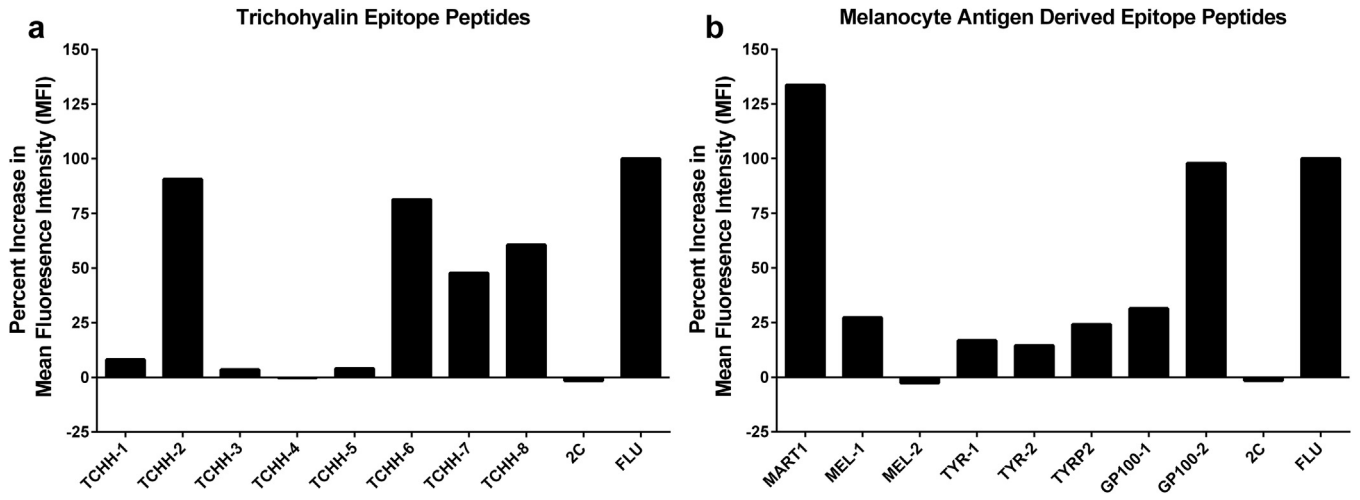

Figure 1. HLA-A*0201 stabilization assay performed on candidate epitope peptides revealed different ability to stabilize the expression of HLA-A*0201 on T2 cells. TCHH peptides 2, 6, and 8 had the ability to stabilize >50% of the binding affinity of the positive control FLU peptide (**a**). The MART1 (Leu27 analog) peptide had stronger affinity to HLA-A*0201 compared with the FLU peptide (**b**). High affinity peptides were most likely able to induce responses in vitro and in vivo. 2C, SIY-K^b negative control peptide; FLU, influenza matrix 1; GP100, premelanosome protein analog; MART1, melanoma antigen recognized by T cells 1 (Leu27 analog); MEL, MART1 analog; TCHH, trichohyalin; TYR, tyrosinase; TYRP2, tyrosinase-related protein-2.

identify candidate autoantigen epitopes that may be able to induce high-frequency CTL responses in AA-affected human subjects. Successful identification of antigen epitopes that are able to induce significant responses from AA subjects' CTLs could lead to the development of disease biomarkers and specific therapeutic modalities targeting antigen-specific CTLs.

RESULTS

In silico-designed peptides displayed varying degrees of affinity to HLA-A*0201

High-ranking peptides predicted in silico indicate the likelihood of them being potential high-affinity epitopes for HLA-A*0201 (on $CD8^+$ T cells) and were selected for further screening. As with other CTL-associated diseases, the predominance of the HLA-A*0201 haplotype in the general population makes it ideal for screening for peptides that can be presented to the immune system in patients with AA (Ellis et al., 2000; Liu et al., 2012; Panagiotopoulos et al., 2003). Some of the predicted peptide sequences did not have strong affinity to HLA-A*0201 (as reflected by their low mean fluorescence intensity) on T2 cells, despite their high predicted scores, and vice versa. Three of eight peptides (Nos. 2, 6, and 8) from TCHH stabilized the HLA-A*0201 expression at ≥50% of the maximum level stabilized by the positive control influenza matrix 1 (FLU) peptide. One melanocyte expressed premelanosome protein analog (GP100) peptide had more than 90% stabilization, whereas melanoma antigen recognized by T cells 1 Leu27 analog (MART1) peptide was able to stabilize HLA-A*0201 better than FLU (>100%) (Figure 1a and b). Conversely, several peptides derived from cytokeratin-16, pro-opiomelanocortin, melanoma-associated antigen 3, and tyrosine hydroxylase-B were eliminated because of their overall inability to stabilize HLA-A*0201 in vitro (data not shown). This does not discount the relevance of these peptides for subsets of patients with AA. For example, melanoma-associated antigen 3 elicited responses in HLA-A*2402 positive patients' CTLs (Ito et al., 2013); the same peptide may have higher affinity for HLA-A*2402, but not for HLA-A*0201.

TCHH, tyrosinase and tyrosinase-related protein 2 peptides induced a higher frequency of CTL activation in AA PBMCs

Both negative control peptides and background control wells had very low numbers of $IFN\gamma^+$ spot-forming cells (SFCs) produced by AA and control PBMCs indicating a low frequency of CTL activation (Figure 2a). SFC numbers from AA PBMCs above the threshold were in general higher than control subject PBMCs (Figure 2b and c). However, peptide group TCHH-G1 (trichohyalin peptide 1−4) and TCHH-G2 (trichohyalin peptide 5−8) induced significantly higher activated CTL frequencies in AA PBMCs, resulting in higher SFC numbers (Figure 2b). In addition, melanocyte antigen-derived epitopes tyrosinase (TYR) and tyrosinase-related protein-2 (TYRP2) were also able to induce higher SFC numbers in AA versus control PBMCs (Figure 2c). Several control subjects also showed very high CTL activation frequencies after TYR stimulation.

Antigen epitope peptide activation of CTLs correlated with the extent and duration of AA as well as concurrent treatments

Subjects receiving topical treatments revealed overall lower CTL activation compared with those who were not on any treatments at the time of blood collection (Figure 3a). Patients with a current AA episode of less than 2-year duration had higher frequencies of CTL activation compared with longer term AA (Figure 3b). Furthermore, AA patients with body hair and nail involvement had higher overall PBMC activation frequencies as compared with AA limited to the scalp (Figure 3c). Patients with body hair and nail involvement also showed relatively higher frequencies of CTL activation after TCHH peptide stimulation similar to those with only scalp hair involvement. Investigation into PBMC activation by individual TCHH peptides yielded variable results, and no

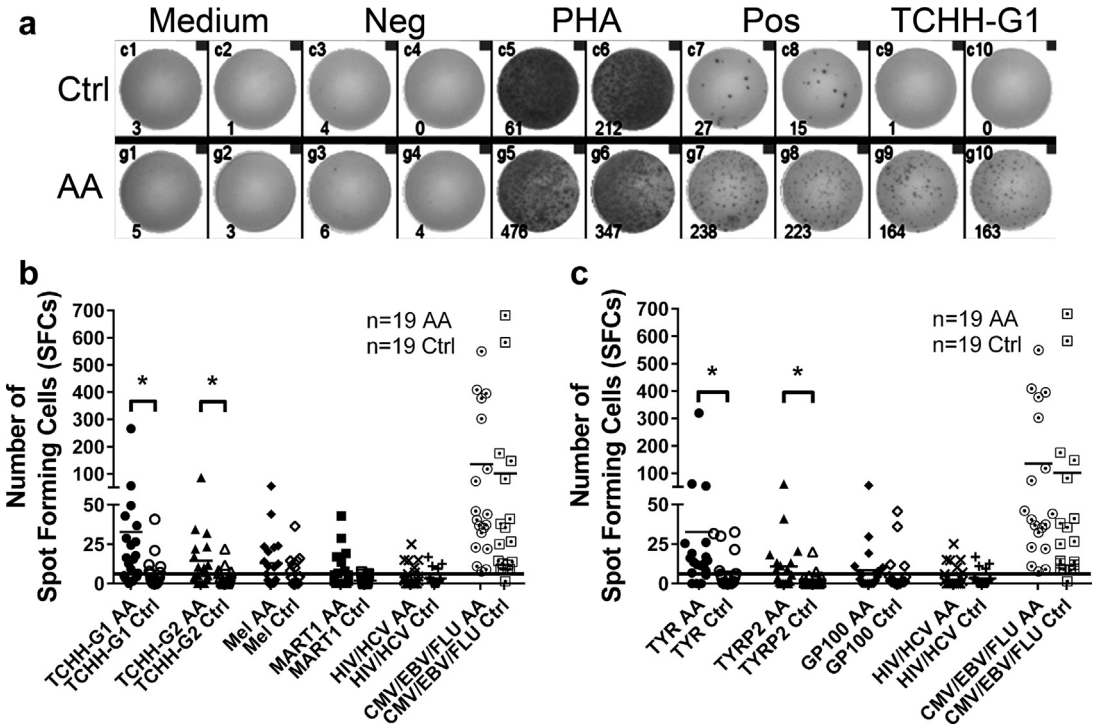

Figure 2. TCHH, TYR, and TYRP2 peptides significantly increased frequencies of AA PBMC activation. A representative picture from an ELISpot plate is shown; each single spot is a result of IFNγ production and represents one single activated PBMC (**a**). Both groups of TCHH peptides, TCHH-G1, and TCHH-G2, were able to induce significantly higher frequencies of PBMC activation compared with similarly stimulated control PBMCs (**b**). Melanocyte-derived antigen epitopes TYR and TYRP2 were also able to induce significantly higher frequencies of AA PBMC activation (**c**). The threshold line denotes true positive activation and was determined by 3× SE of SFCs from the negative controls. Significance of difference was determined by the nonparametric Mann-Whitney *U*-test where $*P \leq 0.05$. Neg: HIV/HCV, Pos: FLU/CMV/EBV. AA, alopecia areata; CMV, cytomegalovirus; EBV, Epstein-Barr virus; FLU, influenza matrix 1; GP100, premelanosome protein analog; HCV, hepatitis C virus; HIV, human immunodeficiency virus; MART1, melanoma antigen recognized by T cells 1 (Leu27 analog); Mel, MART1 analogs 1 and 2; PBMC, peripheral blood mononuclear cell; SE, standard error; SFC, spot-forming cell; TCHH, trichohyalin; TYR, tyrosinase; TYRP2, tyrosinase-related protein-2.

significant difference was observed between AA and controls. Potentially, the highly heterogeneous population recruited in this study could mask the effect of individual epitope peptides (Figure 3d).

IFNγ intracellular cytokine staining confirmed CTL activation after stimulation by specific peptides

Intracellular cytokine stain (ICS) performed on randomly selected PBMC samples confirmed enzyme-linked immunoSpot (ELISpot) assay results. The number of $CD8^+/IFN\gamma^+$ cells increased after stimulation with specific peptides in subjects with AA. AA PBMCs exposed to TCHH-G1 resulted in significantly higher $CD8^+/IFN\gamma^+$ cell numbers compared with negative control peptide-treated PBMCs. Although other peptides did not induce statistically significant increases of $CD8^+/IFN\gamma^+$ cells in AA PBMCs, the pattern of response levels was similar to that of ELISpot assays. TYR peptide stimulation induced very high levels of $CD8^+/IFN\gamma^+$ cells in both AA and control subject PBMCs, similar to ELISpot assay results, suggesting that responses to TYR epitopes were not AA specific (Figure 4).

TCHH peptides induced AA PBMCs to secrete proapoptotic factors harmful to primary HF keratinocytes

Conditioned media (CM) derived from peptide-stimulated PBMC from AA or control subjects had no significant differential effect on proliferation marker Ki67 in HF keratinocytes (Figure 5a and b). However, AA PBMC cell-derived CM induced higher keratinocyte expression of early (annexin-V) and late stage (fixable viability dye) apoptosis markers compared with control CM (Figure 5a). TCHH-G1 and TCHH-G2 stimulated AA CTLs to secrete soluble CM factors that induced significantly higher keratinocyte expression of annexin-V (Figure 5c). A similar, but not statistically significant, trend was observed with the level of fixable viability dye in keratinocytes (Figure 5d).

Differential release of inflammatory cytokines into culture media by AA and control PBMCs after TCHH peptide stimulation

Inflammatory cytokines such as IFNγ and tumor necrosis factor-α were not detected in CM by a cytokine array. However, IL-13 and CCL5 (regulated on activation normal T expressed and secreted, RANTES) were elevated in the CM of AA PBMCs stimulated with both TCHH-G1 and TCHH-G2 (Figure 6a). Conversely, CCL4 (macrophage inflammatory protein 1beta, MIP1β) and matrix metalloproteinase 9 (MMP9) were higher in the CM of control PBMCs stimulated with TCHH peptides (Figure 6b).

DISCUSSION

Various studies with AA models have suggested several different autoantigens that may be the source of epitope peptide targets for AA-specific CTLs (Gilhar et al., 2001;

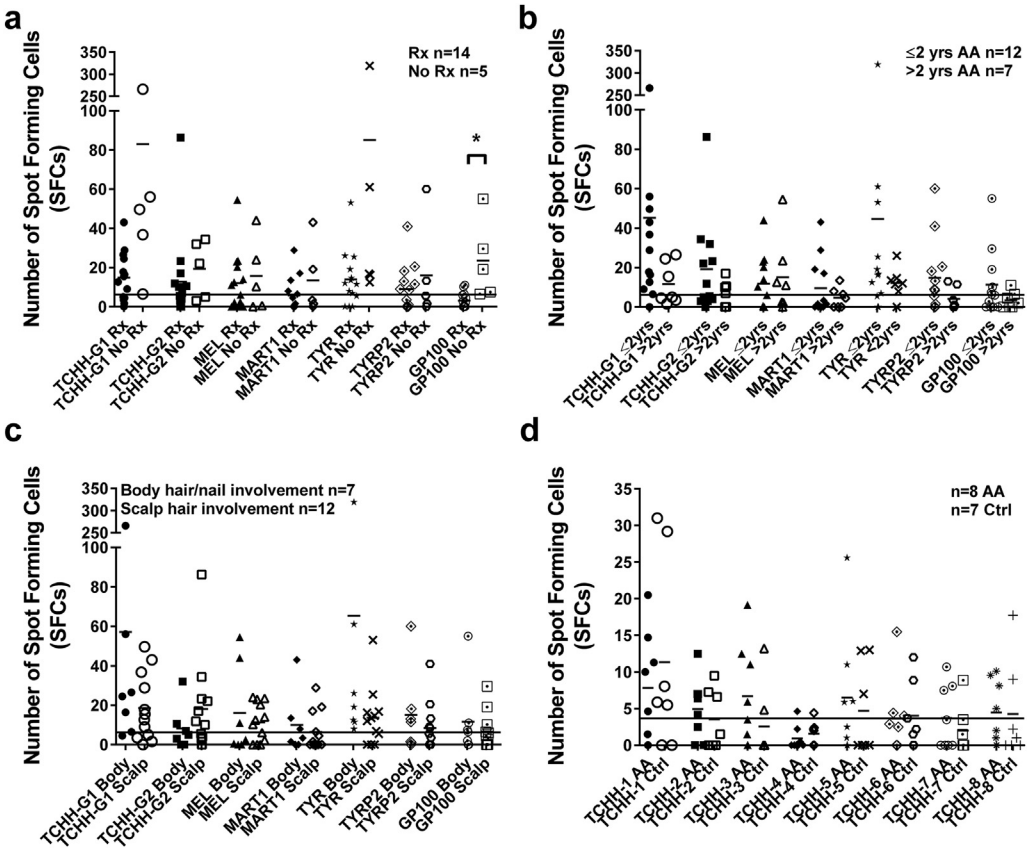

Figure 3. Patients with AA not currently receiving treatments, with recent onset and more extensive body hair/nail involvement, have higher frequencies of PBMC activation while PBMCs stimulated with individual trichohyalin peptides did not show significant differences. Patients with AA who were not receiving any treatments at the time of blood collection showed overall higher frequencies of PBMC activation after peptide stimulation compared with those who were being treated (**a**). In addition, patients who developed AA within the past 2 years showed overall higher PBMC activation compared with those with more long-standing AA (**b**). Furthermore, patients with more chronic forms of AA, such that body hair loss and nail abnormalities were observed, showed overall higher frequencies of PBMC activation (**c**). However, stimulation of AA and control subject PBMCs with individual trichohyalin peptides did not yield significant differences unlike when stimulating as peptide cocktails (**d**). Significance of difference was determined by the nonparametric Mann-Whitney *U*-test where $*P \leq 0.05$. AA, alopecia areata; GP100, premelanosome protein analog; MART1, melanoma antigen recognized by T cells 1 (Leu27 analog); MEL, MART1 analogs 1 and 2; PBMC, peripheral blood mononuclear cell; TCHH, trichohyalin; TYR, tyrosinase; TYRP2, tyrosinase-related protein-2.

Kemp et al., 2011; Leung et al., 2010). Here we examined both melanocyte- and keratinocyte-associated peptide activation responses in human AA PBMC samples.

We used online matrix-assisted algorithms to predict HLA-A*0201 binding peptide sequences (Table 1). However, a direct correlation between the predicted HLA-A*0201 binding scores of a peptide in silico and the degree of HLA-A*0201 stabilization in vitro was not observed (Figure 1) indicating some peptides designed in silico may not be functional and cannot actually induce CTL responses in vitro or in vivo. However, peptide sequence prediction using computer algorithms was still a logical starting point to narrow down the number of candidate peptides based on their likelihood to bind to HLA-A*0201. This approach has been used to screen for antigenic epitope sequences for other autoimmune diseases (Johnston et al., 2004; Martinez et al., 2003) and cancers (Bredenbeck et al., 2005; Hansson et al., 2003). We used such data as a basis for subsequent in vitro validation assays.

IFNγ release after stimulation with the candidate peptides was more similar to the results obtained with the T2 stabilization assay. Some of the peptides that were able to stabilize the highest levels of HLA-A*0201, like MART1 and GP100 (Figure 1b), only induced moderate levels of IFNγ secretion in ELISpot assays and ICS (Figures 2 and 3). Meanwhile, the peptide sequences with modest ability to stabilize HLA-A*0201, like TCHH-G1 and TCHH-G2, and TYRP2, induced much higher frequencies of CTL activation from AA PBMCs compared with respective controls (Figure 2b and c). Because negative selection during T-cell maturation involves the survival of T cells with rapid binding and disengagement of self-peptides, this process could enable the survival of small populations of autoreactive CTLs with moderate affinity (Liu et al., 1995). In several autoimmune diseases such as multiple sclerosis (Muraro et al., 1997) and type I diabetes (Chang and Unanue, 2009), immunodominant epitopes are often revealed to be peptides with moderate or low affinity to HLA. Our ELISpot and ICS assays reveal potential autoreactive CTLs (against TCHH-G1, TCHH-G2, TYRP2, and potentially MART1) in patients with AA that could have escaped deletion. Notably, TYR could be a nonspecific CTL activator as it induced relatively high frequencies of CTL activation in both AA and control PBMCs (Figures 2c and 3).

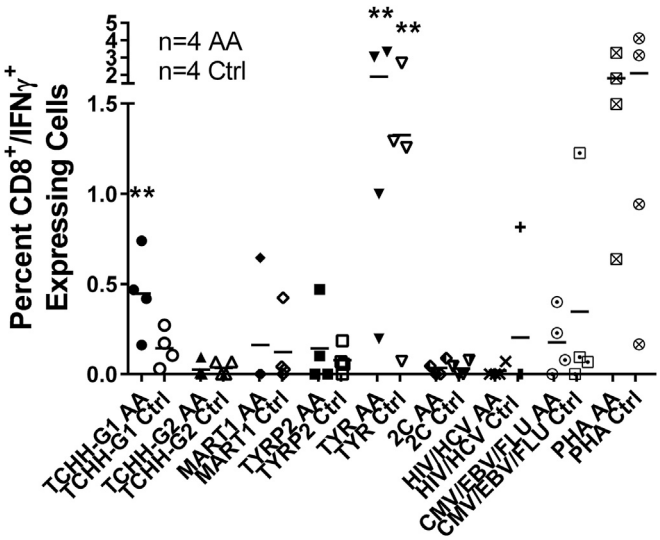

Figure 4. Intracellular cytokine staining confirmed CD8$^+$ T-cell-specific activation by the synthesized peptides. The percentage of CD8$^+$/IFNγ$^+$ cells in AA PBMC populations after stimulation with peptides was higher than the control PBMCs. TYR peptides showed stimulation of CD8$^+$ cells (CTLs) to secrete IFNγ in both AA and control subjects. Significance of difference compared with baseline (no treatment) was determined by the nonparametric Mann-Whitney *U*-test where $^{**}P \leq 0.05$. AA, alopecia areata; CTL, cytotoxic T lymphocytes; CMV, cytomegalovirus; EBV, Epstein-Barr virus; FLU, influenza matrix 1; HCV, hepatitis C virus; HIV, human immunodeficiency virus; MART1, melanoma antigen recognized by T cells 1 (Leu27 analog); PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; TCHH, trichohyalin; TYR, tyrosinase; TYRP2, tyrosinase-related protein-2.

Highly variable and weaker CTL responses were observed when analyzing each of the eight individual TCHH peptides with a small subset of AA patients and controls (Figure 4d). Each subject with AA could have different primary or preferential epitopes within each antigen, which could explain why TCHH peptide cocktails induced higher responses. In addition, multiple epitope sequences can contribute to the activation of AA CTLs, and the primary target may vary between subjects with different extents and stages of AA, similar to observations with CTL epitopes in type I diabetes (Panagiotopoulos et al., 2003; Standifer et al., 2006). Epitope spreading is often observed in the chronic phase of autoimmune diseases (von Herrath et al., 2007; You and Chatenoud, 2006), and may affect epitope specificity between individuals. AA is a complex disease and factors such as disease extent (Figure 4a), treatment regimens (Figure 4b), age, and gender (not shown) could have a significant impact on CTL activation. Our results revealed that AA patients with body hair loss and nail abnormalities tended to have higher frequencies of CTL activation across multiple antigen epitopes, compared with patients with just scalp involvement, potentially reflecting higher disease severity (Figure 4c). Small sample size here could also be a limiting factor, but potentially the epitope peptides identified are part of the complex of pathway(s) leading to AA.

We found that although PBMCs of patients with AA can be activated at higher frequencies on HF epitope challenge, the cytokines secreted by the activated PBMCs did not have significant differential effects on HF keratinocyte proliferation compared with controls (Figure 5a and b). However, CM from AA PBMCs, particularly when challenged with TCHH peptides, was able to significantly increase annexin-V expression and moderately increased keratinocyte numbers in late apoptosis (Figure 5a, c, d). This result is consistent with ELISpot assay results where AA PBMCs showed higher frequencies of activation after TCHH peptide challenge (Figure 2b). Although antigens derived from melanocytes have been suggested as potential targets by AA CTLs (Gilhar et al., 2001), the melanocyte-derived antigen epitopes in our panel did not show very consistent results in inducing AA CTL responses as with TCHH epitopes (Figures 2 and 5). Activated CTLs are secretors of multiple proinflammatory cytokines; their close proximity with HF keratinocytes in vivo could be harmful to the health of keratinocytes even without direct cell-cell contact.

The involvement of TCHH in AA has previously been suggested by the discovery of specific autoantibodies in the sera of patients with AA. TCHH is found in the HF keratinocyte inner root sheath, as well as in nails and tongue epithelium (Lee et al., 1993; O'Keefe et al., 1993). It is relatively large (250 kDa) and many CTL-specific epitopes can potentially be derived from this antigen. Patients with AA often develop a "pitted nail" presentation (Alkhalifah et al., 2010a). Potentially, this could be due to targeted disruption of nail keratinocytes by activated TCHH -specific CTLs. Although the transfer of TCHH autoantibodies yielded mixed disease induction results in mouse models (Gilhar et al., 1992; Tobin et al., 1998), the presence of TCHH-specific CTLs strongly suggest that AA is autoantigen driven.

IFNγ was used here to define CTL activation, though whether IFNγ is a direct inducer of AA is unclear (Gilhar et al., 2005; Sundberg, 2007). A variety of proinflammatory cytokines could contribute to the death of HF keratinocytes, such as tumor necrosis factor-α (Wong and Pamer, 2003). By using a multiplex cytokine array, we found differential production of cytokines between AA and control PBMCs stimulated with TCHH peptides, such as increased IL-13 and RANTES production by AA PBMCs (Figure 6a) and elevated MIP1β and MMP9 by control PBMCs (Figure 6b). However, several inflammatory cytokines were below the minimal detection limit of the array. In addition, due to the small sample size and large variations between each subject's PBMC cytokine expression, statistical significance could not be determined.

Atopic dermatitis, in which IL-13 and RANTES are elevated (Boguniewicz and Leung, 2011), is often concomitant with AA (Mohan and Silverberg, 2015). However, we screened PBMCs from individuals who did not report having atopy at the time of blood draw. In recent large-scale genome-wide association studies, IL-13 was identified at a candidate AA susceptibility locus (Barahmani et al., 2006; Betz et al., 2015; Petukhova et al., 2010). IL-13 expression is significantly elevated in serum from patients with AA (Tembhre and Sharma, 2013). Further, serum levels of RANTES positively correlate to AA activity and severity, while nonsignificantly associated with atopy in patients with AA (Kuwano et al., 2007). Although IL-13 and RANTES are classically T-helper lymphocyte 2-associated cytokines, they may yet be shown to have a significant role in CTL-mediated AA pathogenesis.

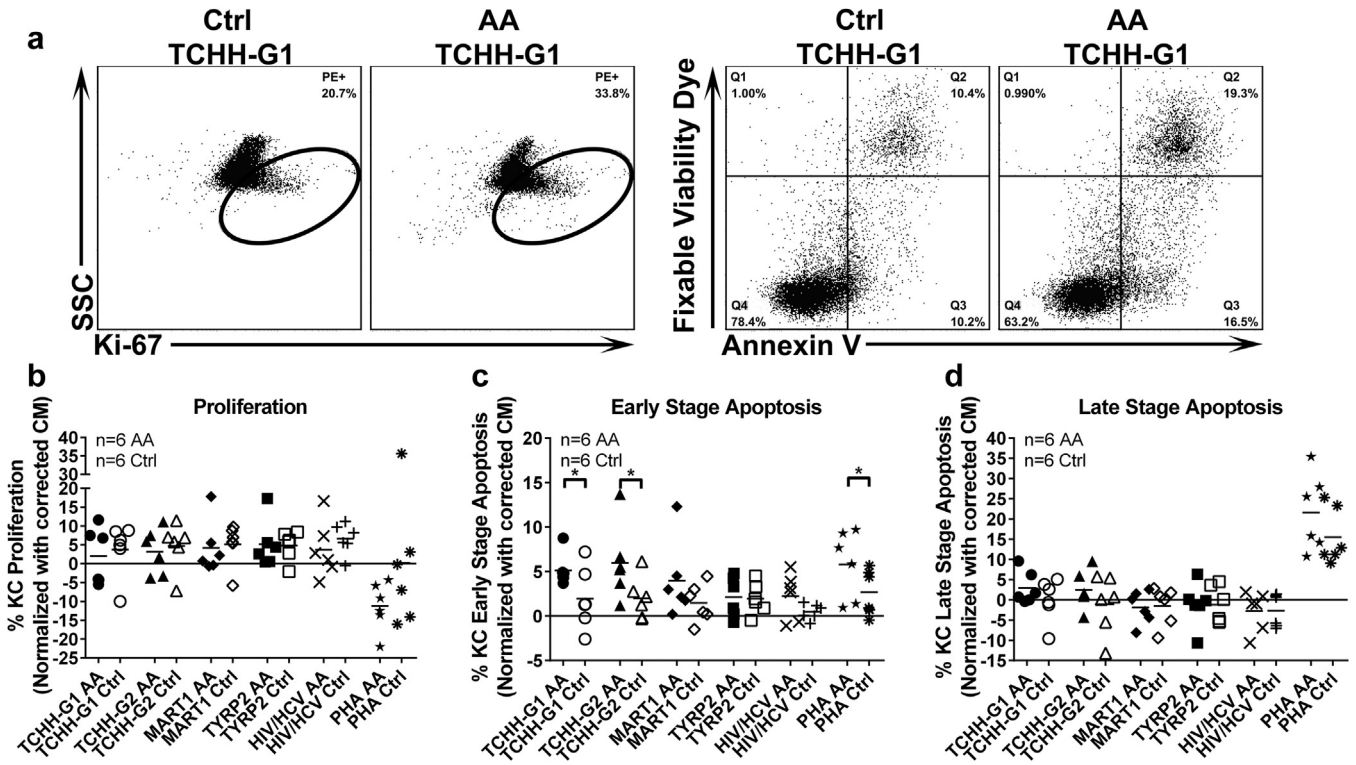

Figure 5. CM derived from AA PBMCs stimulated with TCHH-G1 peptides can induce apoptosis in HF keratinocytes (KC). Representative flow cytometry scatter plots for the evaluation of proliferation (Ki67), early stage apoptosis (annexin-V), and late stage apoptosis (fixable viability dye; FVD) markers on HF keratinocytes (**a**). The effect of CM from both AA and control PBMCs stimulated with different peptides was variable and showed no significant difference between the two subject groups (**b**). CM from TCHH-G1 and TCHH-G2 stimulated AA PBMCs significantly increased annexin-V^+/FVD^- HF keratinocyte numbers compared with CM from control PBMCs, indicating higher ability to induce early-stage apoptosis (**c**). Keratinocytes in late-stage apoptosis (annexin-V^+/FVD^+) after culture with either AA or control CM showed no statistically significant difference, but exhibited a similar trend as observed for early-stage apoptosis (**d**). Significance of difference was determined by Fisher's least significant difference test where $*P \leq 0.05$. AA, alopecia areata; CM, conditioned media; CMV, cytomegalovirus; EBV, Epstein-Barr virus; FLU, influenza matrix 1; HCV, hepatitis C virus; HF, hair follicle; HIV, human immunodeficiency virus; MART1, melanoma antigen recognized by T cells 1 (Leu27 analog); PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; TCHH, trichohyalin; TYR, tyrosinase; TYRP2, tyrosinase-related protein-2.

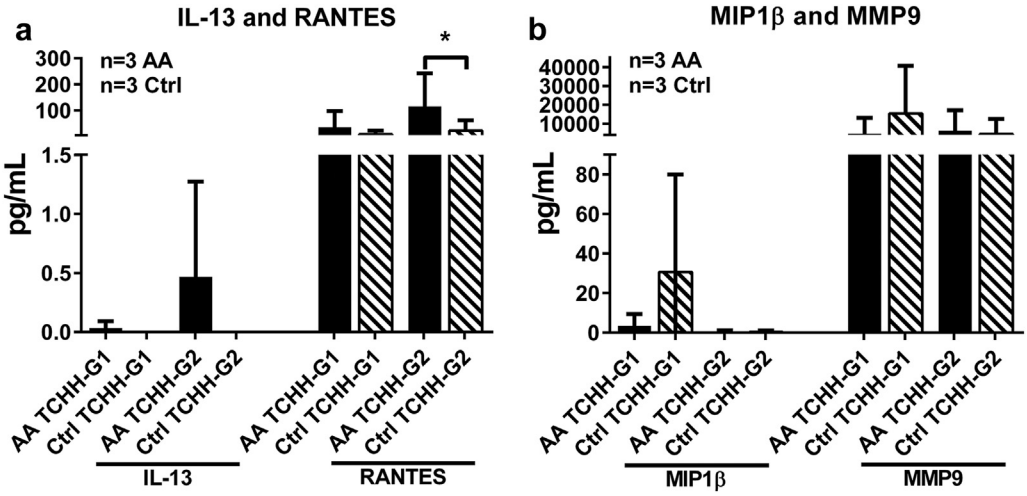

Figure 6. AA PBMCs secreted more IL-13 and RANTES on trichohyalin peptide simulation, whereas control PBMCs secreted more MIP1β and MMP9. Analysis of CM, after PBMCs were stimulated by trichohyalin (TCHH) peptides, was evaluated via a cytokine array. The data revealed AA PBMCs, taken from individuals who did not report atopy, secreted more IL-13 and RANTES after stimulation by both TCHH-G1 and G2 (**a**). However, control PBMCs also secreted certain inflammatory cytokines and factors such as MIP1β and MMP9 at a higher degree compared with AA PBMCs (**b**). Significance of difference was determined by one-way ANOVA where $*P \leq 0.05$; n = 3 for each group. AA, alopecia areata; CM, conditioned media; MIP1β, macrophage inflammatory protein 1beta; MMP9, matrix metalloproteinase 9; PBMC, peripheral blood mononuclear cell; RANTES, regulated on activation, normal T expressed and secreted.

Table 1. List of human autoantigen epitope peptide sequences used

Identity	Position	Sequence	BIMAS	SYFPEITHI	Score
Trichohyalin-1 (TCHH-1)	35	NLLEREFGA	437.114	19	8305.17
Trichohyalin-2 (TCHH-2)	835	LLQEEEEEL	72.959	26	1896.93
Trichohyalin-3 (TCHH-3)	4	FLLFIFKVA	65.869	15	988.04
Trichohyalin-4 (TCHH-4)	778	QLQEEEDGL	42.917	23	987.09
Trichohyalin-5 (TCHH-5)	895	KLQQKEEQL	36.637	22	806.01
Trichohyalin-6 (TCHH-6)	52	KTVDLILEL	9.793	24	235.03
Trichohyalin-7 (TCHH-7)	182	VLRKEEEKL	1.352	23	31.10
Trichohyalin-8 (TCHH-8)	55	DLILELLDL	3.685	26	95.81
MelanA/MART1 analog-1 (Ala26) (MEL-1)	26	AAAGIGILTV	–	–	–
MelanA/MART1 analog-2 (MEL-2)	27	AAGIGLTV	–	–	–
MelanA/MART1 analog-3 (Leu27) (MART1)	26	ELAGIGILT	–	–	–
Tyrosinase-1 (TYR-1)	1	MLLAVLYCL	309.050	27	8344.35
Tyrosinase-2 (TYR-2)	369	YMNGTMSQV	531.455	23	12223.47
Tyrosinase-related protein 2 (TYRP2)	180	SVYDFFVWL	973.849	21	20450.83
Modified GP100-1 209m (GP100-1)	209	IMDQVPSFV	–	–	–
Modified GP100-2 280.9v (GP100-2)	280	YLEPGPVTV	–	–	–
SIY-K^b 2C TCR (2C)		SIYRYYGL	–	–	–
Cytokeratin 16-1 (KRT16-1)	402	ILLDVKTRL	550.915	25	13772.875
Cytokeratin 16-2 (KRT16-2)	111	GLLVGSEKV	126.098	27	3404.646
Cytokeratin 16-3 (KRT16-3)	127	RLASYLDKV	78.385	29	2273.165
Melanoma-associated-antigen 3 (MAGE3)	195	IMPKAGLLI	12.809	19	243.371
Pro-opiomelanocortin-1 (POMC-1)	16	LLLQASMEV	437.482	26	11374.532
Pro-opiomelanocortin-2 (POMC-2)	12	LLLALLLQA	71.872	24	1724.928
Tyrosine hydroxylase isoform B-1 (THB-1)	299	FLASLAFRV	1855.647	24	44535.528
Tyrosine hydroxylase isoform B-2 (THB-2)	365	KLSTLYWFT	1613.824	16	25821.584
Tyrosine hydroxylase isoform B-3 (THB-3)	292	GLLSARDFL	213.015	23	4899.345
Cytomegalovirus pp65 (CMV)	495	NLVPMVATV	–	–	–
Epstein-Barr virus BMFL1 (EBV)	259	GLCTLVAML	–	–	–
Influenza matrix 1 (FLU)	58	GILGFVFTL	–	–	–
Human immunodeficiency virus p17 (HIV)	77	SLYNTVATL	–	–	–
Hepatitis C virus (HCV)	132	DLMGYIPLV	–	–	–

Abbreviation: BIMAS, bioinformatics and molecular analysis section.

The success of the methods employed here to narrow down a large panel of candidate antigen epitopes suggests that further AA epitopes could be identified using similar approaches. Knowledge of inciting antigen epitopes might also be used as a potential diagnostic tool for understanding variations in disease pathogenesis in patients with AA. We observed consistent activation of AA CTLs by TCHH peptides followed by indirect killing of keratinocytes. Potentially, these TCHH peptide-specific CTLs in patients with AA may serve as a prognostic marker for the disease. Ultimately, knowing the key antigen targets in AA may allow new targeted treatments to be developed.

MATERIALS AND METHODS

Study subject recruitment and blood cell isolation

The study was approved by the University of British Columbia (UBC) Clinical Research Ethics Board. After written informed consent, AA-affected subjects and controls were recruited from Vancouver General Hospital-Skin Care Centre and the Clinical Research Unit. Subjects with AA ranged from those with <25% patchy hair loss to alopecia totalis and/or universalis. Control subjects comprised individuals with either no hair loss or androgenetic alopecia. Clinical data were recorded for each individual regarding the subject's hair loss and background information at the time of blood collection (Supplementary Tables S1 and S2 online). PBMCs were obtained by the Ficoll-paque density gradient centrifugation method.

Subject sample haplotyping

PBMC samples from both AA and control subjects with major histocompatibility complex-I allele HLA-A*0201 were identified via FACS for further analysis. The HLA-A2 family is the largest allele family of the HLA-A locus with HLA-A*0201 being the most predominant allele present in 50% of the general population (Ellis et al., 2000). In our cohorts, the HLA-A*0201 frequency was 48% for subjects with AA and 47% for control subjects.

Peptide sequence prediction

Computer-based algorithms were used to predict the nonamer peptide antigen sequences that would bind to HLA-A*0201. The combined results from two algorithms, SYFPEITHI (University of Tuebingen) (Rammensee et al., 1999) and Matrix-assisted algorithms from the National Institutes of Health, BioInformatics and Molecular Analysis Section (Parker et al., 1994), were used to design a panel of candidate peptide sequences. The score of each peptide from both prediction algorithms were multiplied together to give a list of peptide sequences with highest avidity (Table 1). All peptides were synthesized (Kinexus Bioinformatics, Vancouver, BC) with 60–80%

purity, controlled by high-performance liquid chromatography and mass spectrometry (Ouyang et al., 2006). Epitope peptide sequences for selected antigens, TCHH peptides (TCHH-1−8), cytokeratin-16-1−3 peptides, melanoma-associated antigen 3 (Ito et al., 2013), pro-opiomelanocortin-1, 2, and tyrosine hydroxylase-B-1−3 were predicted from full-length protein sequences. MelanA/MART1 analogs (MART1, MEL-1, 2) (Mehrotra et al., 2004; Valmori et al., 1998a, 1998b), TYR-1, 2 (Wolfel et al., 1994), TYRP2 (Kawakami et al., 2000), modified GP100 (GP100-1, 2) (Parkhurst et al., 1996), cytomegalovirus pp65, Epstein-Barr virus BMFL1, influenza matrix (Cellerai et al., 2010), human immunodeficiency virus p17 (Ferrari et al., 2004), and hepatitis C virus (Spangenberg et al., 2005) sequences were obtained from the published literature. SIY-K^b(2C), a nonrelevant peptide (Udaka et al., 1996), was used as an additional control for determining the background signal.

HLA-A*0201 (T2) stabilization assay

T2 cells (174xCEM.T2) from the American Type Culture Collection were used to screen and confirm stable presentation of each peptide by the HLA-A*0201 complex (major histocompatibility complex-I) in vitro. T2 cells are TAP-1 and -2 (transporter associated with antigen processing) deficient and only express stable HLA-A2 in the presence of high-affinity peptides; therefore, HLA-A2 stability correlates to peptide biological activity and affinity in vitro. T2 stabilization assays were performed following protocols described elsewhere (Forero et al., 1998; Zeh et al., 1994) with slight modifications (Supplementary Materials online). HLA-A2 restrictive Influenza M1 58-66 peptide (GILGFVFTL) was used as a positive control to indicate maximum HLA-A2 expression levels. The stabilization values (mean fluorescence intensity) for each of the epitope peptides were normalized with reference to maximum HLA-A2 expression induced by the high-affinity positive control peptide.

Human PBMC IFNγ ELISpot assay

The numbers of autoantigen-specific CTLs from frozen human PBMC samples were quantified with IFNγ ELISpot assays using the manufacturer's protocols (Mabtec, Cincinnati, OH). PBMC samples were suspended in complete medium and seeded into each ELISpot plate well (200,000 cells/well) and different peptides were added at 5 μg/ml and incubated at 37°C, 5% CO_2 for 48 hours. The number of spots quantified (iSpot FluoroSpot Reader System; Autoimmun Diagnostika GmbH, Strassberg, Germany) denoted the number of CTLs activated against the respective peptides. The cutoff for positive responses against each epitope peptide was defined by 3× standard error above the mean number of the SFCs of negative control epitopes (human immunodeficiency virus, hepatitis C virus) after correcting for the background control (no peptide stimulation), and the Mann-Whitney *U*-test was performed as described in a similar study (Panagiotopoulos et al., 2003).

Intracellular cytokine stain

The production of IFNγ by the CTLs on stimulation with various peptides was confirmed with ICS plus $CD8^+$ and $CD4^+$ cell staining (detailed in Supplementary Materials). All reagents and antibodies were purchased from eBioscience (San Diego, CA). $CD4^+$/IFNγ$^+$ or $CD8^+$/IFNγ$^+$ populations were identified and significance was determined with the Mann-Whitney *U*-test (de Almeida et al., 1998).

Hair follicle microdissection and root sheath keratinocyte culture

Human scalp tissue was obtained as waste material (approved by the UBC Clinical Research Ethics Board) from hair transplant surgeries (not patients with AA). Outer root sheath and inner root sheath cells were obtained from HF units. The outer root sheath/inner root sheath cells were cultured until passage five and harvested for apoptosis assays. See Supplementary Materials.

Apoptosis induction via peptide-activated PBMC conditioned media

PBMCs from AA and control subjects were cultured with 5 μg/ml of peptides that showed significant ability to activate AA PBMCs (TCHH-G1 and G2, MART1, TYRP2) for 3 days. CM from PBMCs stimulated with different peptides was then transferred to the cultured HF keratinocytes and incubated for 8 hours at 37°C, 5% CO_2. HF keratinocytes were labeled with fixable viability dye, annexin-V and Ki67 (eBioscience) for late-, early-stage apoptosis and proliferation, respectively. See Supplementary Materials.

Human cytokine array analysis of cell culture supernatant from PBMCs stimulated with epitope peptides

Cell culture supernatant CM samples (n = 3 for AA and n = 3 for controls) were sent for quantitative analysis for inflammatory cytokine content (Quantibody Human Cytokine Array Q1; RayBiotech, Norcross, GA). The signals from the CM samples were compared with standard curves and concentration was determined automatically by software (Q-Analyzer, RayBiotech). Statistical calculations were performed with one-way ANOVA. See Supplementary Materials.

CONFLICT OF INTEREST

KJM is a shareholder and consultant for Replicel Life Sciences. All other authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.04.004.

REFERENCES

Alkhalifah A, Alsantali A, Wang E, McElwee KJ, Shapiro J. Alopecia areata update: part I. Clinical picture, histopathology, and pathogenesis. J Am Acad Dermatol 2010a;62:177−88, quiz 89−90.

Alkhalifah A, Alsantali A, Wang E, McElwee KJ, Shapiro J. Alopecia areata update: part II. Treatment. J Am Acad Dermatol 2010b;62:191−202, quiz 3−4.

Barahmani N, de Andrade M, Slusser JP, Zhang Q, Duvic M. Major histocompatibility complex class I chain-related gene A polymorphisms and extended haplotypes are associated with familial alopecia areata. J Invest Dermatol 2006;126:74−8.

Betz RC, Petukhova L, Ripke S, Huang H, Menelaou A, Redler S, et al. Genome-wide meta-analysis in alopecia areata resolves HLA associations and reveals two new susceptibility loci. Nat Commun 2015;6:5966.

Boguniewicz M, Leung DY. Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. Immunol Rev 2011;242:233−46.

Bredenbeck A, Losch FO, Sharav T, Eichler-Mertens M, Filter M, Givehchi A, et al. Identification of noncanonical melanoma-associated T cell epitopes for cancer immunotherapy. J Immunol 2005;174:6716−24.

Cellerai C, Perreau M, Rozot V, Enders FB, Pantaleo G, Harari A. Proliferation capacity and cytotoxic activity are mediated by functionally and phenotypically distinct virus-specific CD8 T cells defined by interleukin-7R {alpha} (CD127) and perforin expression. J Virol 2010;84:3868−78.

Chang KY, Unanue ER. Prediction of HLA-DQ8beta cell peptidome using a computational program and its relationship to autoreactive T cells. Int Immunol 2009;21:705−13.

de Almeida AB, Silva MC, Braga C, Freedman DO. Differences in the frequency of cytokine-producing cells in antigenemic and nonantigenemic individuals with bancroftian filariasis. Infect Immun 1998;66:1377–83.

Ellis JM, Henson V, Slack R, Ng J, Hartzman RJ, Katovich Hurley C. Frequencies of HLA-A2 alleles in five U.S. population groups. Predominance of A*02011 and identification of HLA-A*0231. Hum Immunol 2000;61:334–40.

Erb U, Freyschmidt-Paul P, Zoller M. Tolerance induction by hair-specific keratins in murine alopecia areata. J Leukoc Biol 2013;94:845–57.

Ferrari G, Neal W, Ottinger J, Jones AM, Edwards BH, Goepfert P, et al. Absence of immunodominant anti-Gag p17 (SL9) responses among Gag CTL-positive, HIV-uninfected vaccine recipients expressing the HLA-A*0201 allele. J Immunol 2004;173:2126–33.

Finner AM. Alopecia areata: clinical presentation, diagnosis, and unusual cases. Dermatol Ther 2011;24:348–54.

Forero L, Zwirner NW, Fink CW, Fernandez-Vina MA, Stastny P. Juvenile arthritis, HLA-A2 and binding of DEK oncogene-peptides. Hum Immunol 1998;59:443–50.

Gilhar A, Kam Y, Assy B, Kalish RS. Alopecia areata induced in C3H/HeJ mice by interferon-gamma: evidence for loss of immune privilege. J Invest Dermatol 2005;124:288–9.

Gilhar A, Keren A, Shemer A, d'Ovidio R, Ullmann Y, Paus R. Autoimmune disease induction in a healthy human organ: a humanized mouse model of alopecia areata. J Invest Dermatol 2013;133:844–7.

Gilhar A, Landau M, Assy B, Shalaginov R, Serafimovich S, Kalish RS. Melanocyte-associated T cell epitopes can function as autoantigens for transfer of alopecia areata to human scalp explants on Prkdc(scid) mice. J Invest Dermatol 2001;117:1357–62.

Gilhar A, Pillar T, Assay B, David M. Failure of passive transfer of serum from patients with alopecia areata and alopecia universalis to inhibit hair growth in transplants of human scalp skin grafted on to nude mice. Br J Dermatol 1992;126:166–71.

Hansson L, Rabbani H, Fagerberg J, Osterborg A, Mellstedt H. T-cell epitopes within the complementarity-determining and framework regions of the tumor-derived immunoglobulin heavy chain in multiple myeloma. Blood 2003;101:4930–6.

Ito T, Bertolini M, Funakoshi A, Ito N, Takayama T, Biro T, et al. Birth, life, and death of the MAGE3 hypothesis of alopecia areata pathobiology. J Dermatol Sci 2013;72:327–30.

Johnston A, Gudjonsson JE, Sigmundsdottir H, Love TJ, Valdimarsson H. Peripheral blood T cell responses to keratin peptides that share sequences with streptococcal M proteins are largely restricted to skin-homing CD8(+) T cells. Clin Exp Immunol 2004;138:83–93.

Kawakami Y, Suzuki Y, Shofuda T, Kiniwa Y, Inozume T, Dan K, et al. T cell immune responses against melanoma and melanocytes in cancer and autoimmunity. Pigment Cell Res 2000;13(Suppl 8):163–9.

Kemp EH, Sandhu HK, Weetman AP, McDonagh AJ. Demonstration of autoantibodies against tyrosine hydroxylase in patients with alopecia areata. Br J Dermatol 2011;165:1236–43.

Kuwano Y, Fujimoto M, Watanabe R, Ishiura N, Nakashima H, Ohno Y, et al. Serum chemokine profiles in patients with alopecia areata. Br J Dermatol 2007;157:466–73.

Lee SC, Kim IG, Marekov LN, O'Keefe EJ, Parry DA, Steinert PM. The structure of human trichohyalin. Potential multiple roles as a functional EF-hand-like calcium-binding protein, a cornified cell envelope precursor, and an intermediate filament-associated (cross-linking) protein. J Biol Chem 1993;268:12164–76.

Leung MC, Sutton CW, Fenton DA, Tobin DJ. Trichohyalin is a potential major autoantigen in human alopecia areata. J Proteome Res 2010;9:5153–63.

Liu GY, Fairchild PJ, Smith RM, Prowle JR, Kioussis D, Wraith DC. Low avidity recognition of self-antigen by T cells permits escape from central tolerance. Immunity 1995;3:407–15.

Liu W, Zhai M, Wu Z, Qi Y, Wu Y, Dai C, et al. Identification of a novel HLA-A2-restricted cytotoxic T lymphocyte epitope from cancer-testis antigen PLAC1 in breast cancer. Amino Acids 2012;42:2257–65.

Martinez NR, Augstein P, Moustakas AK, Papadopoulos GK, Gregori S, Adorini L, et al. Disabling an integral CTL epitope allows suppression of autoimmune diabetes by intranasal proinsulin peptide. J Clin Invest 2003;111:1365–71.

McElwee KJ, Freyschmidt-Paul P, Hoffmann R, Kissling S, Hummel S, Vitacolonna M, et al. Transfer of CD8(+) cells induces localized hair loss whereas CD4(+)/CD25(−) cells promote systemic alopecia areata and CD4(+)/CD25(+) cells blockade disease onset in the C3H/HeJ mouse model. J Invest Dermatol 2005;124:947–57.

McElwee KJ, Silva K, Boggess D, Bechtold L, King LE, Jr, Sundberg JP. Alopecia areata in C3H/HeJ mice involves leukocyte-mediated root sheath disruption in advance of overt hair loss. Vet Pathol 2003;40:643–50.

McElwee KJ, Spiers EM, Oliver RF. In vivo depletion of CD8+ T cells restores hair growth in the DEBR model for alopecia areata. Br J Dermatol 1996;135:211–7.

McElwee KJ, Spiers EM, Oliver RF. Partial restoration of hair growth in the DEBR model for Alopecia areata after in vivo depletion of CD4+ T cells. Br J Dermatol 1999;140:432–7.

Mehrotra S, Chhabra A, Chakraborty A. Antigen presentation by MART-1 adenovirus-transduced interleukin-10-polarized human monocyte-derived dendritic cells. Immunology 2004;113:472–81.

Messenger AG, Bleehen SS. Alopecia areata: light and electron microscopic pathology of the regrowing white hair. Br J Dermatol 1984;110:155–62.

Messenger AG, Bleehen SS. Expression of HLA-DR by anagen hair follicles in alopecia areata. J Invest Dermatol 1985;85:569–72.

Mirzoyev SA, Schrum AG, Davis MD, Torgerson RR. Lifetime incidence risk of alopecia areata estimated at 2.1% by Rochester Epidemiology Project, 1990–2009. J Invest Dermatol 2014;134:1141–2.

Mohan GC, Silverberg JI. Association of vitiligo and alopecia areata with atopic dermatitis: a systematic review and meta-analysis. JAMA Dermatol 2015;151:522–8.

Muraro PA, Vergelli M, Kalbus M, Banks DE, Nagle JW, Tranquill LR, et al. Immunodominance of a low-affinity major histocompatibility complex-binding myelin basic protein epitope (residues 111–129) in HLA-DR4 (B1*0401) subjects is associated with a restricted T cell receptor repertoire. J Clin Invest 1997;100:339–49.

O'Keefe EJ, Hamilton EH, Lee SC, Steinert P. Trichohyalin: a structural protein of hair, tongue, nail, and epidermis. J Invest Dermatol 1993;101:65S–71S.

Ouyang Q, Standifer NE, Qin H, Gottlieb P, Verchere CB, Nepom GT, et al. Recognition of HLA class I-restricted beta-cell epitopes in type 1 diabetes. Diabetes 2006;55:3068–74.

Panagiotopoulos C, Qin H, Tan R, Verchere CB. Identification of a beta-cell-specific HLA class I restricted epitope in type 1 diabetes. Diabetes 2003;52:2647–51.

Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol 1994;152:163–75.

Parkhurst MR, Salgaller ML, Southwood S, Robbins PF, Sette A, Rosenberg SA, et al. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. J Immunol 1996;157:2539–48.

Paus R, Slominski A, Czarnetzki BM. Is alopecia areata an autoimmune-response against melanogenesis-related proteins, exposed by abnormal MHC class I expression in the anagen hair bulb? Yale J Biol Med 1993;66:541–54.

Petukhova L, Duvic M, Hordinsky M, Norris D, Price V, Shimomura Y, et al. Genome-wide association study in alopecia areata implicates both innate and adaptive immunity. Nature 2010;466:113–7.

Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 1999;50:213–9.

Ranki A, Kianto U, Kanerva L, Tolvanen E, Johansson E. Immunohistochemical and electron microscopic characterization of the cellular infiltrate in alopecia (areata, totalis, and universalis). J Invest Dermatol 1984;83:7–11.

Safavi KH, Muller SA, Suman VJ, Moshell AN, Melton LJ, 3rd. Incidence of alopecia areata in Olmsted County, Minnesota, 1975 through 1989. Mayo Clin Proc 1995;70:628–33.

Spangenberg HC, Viazov S, Kersting N, Neumann-Haefelin C, McKinney D, Roggendorf M, et al. Intrahepatic CD8+ T-cell failure during chronic hepatitis C virus infection. Hepatology 2005;42:828–37.

Standifer NE, Ouyang Q, Panagiotopoulos C, Verchere CB, Tan R, Greenbaum CJ, et al. Identification of novel HLA-A*0201-restricted

epitopes in recent-onset type 1 diabetic subjects and antibody-positive relatives. Diabetes 2006;55:3061–7.

Sundberg JP, Kathleen A, Edwards K, Black S, Bennett Jenson A, King LE. Failure to induce alopecia areata in C3H/HeJ mice with exogenous interferon gamma. J Exp Animal Sci 2007;43:265–70.

Tembhre MK, Sharma VK. T-helper and regulatory T-cell cytokines in the peripheral blood of patients with active alopecia areata. Br J Dermatol 2013;169:543–8.

Tobin DJ, Alhaidari Z, Olivry T. Equine alopecia areata autoantibodies target multiple hair follicle antigens and may alter hair growth. A preliminary study. Exp Dermatol 1998;7:289–97.

Tobin DJ, Fenton DA, Kendall MD. Ultrastructural observations on the hair bulb melanocytes and melanosomes in acute alopecia areata. J Invest Dermatol 1990;94:803–7.

Todes-Taylor N, Turner R, Wood GS, Stratte PT, Morhenn VB. T cell subpopulations in alopecia areata. J Am Acad Dermatol 1984;11:216–23.

Udaka K, Wiesmuller KH, Kienle S, Jung G, Walden P. Self-MHC-restricted peptides recognized by an alloreactive T lymphocyte clone. J Immunol 1996;157:670–8.

Valmori D, Fonteneau JF, Lizana CM, Gervois N, Lienard D, Rimoldi D, et al. Enhanced generation of specific tumor-reactive CTL in vitro by selected Melan-A/MART-1 immunodominant peptide analogues. J Immunol 1998a;160:1750–8.

Valmori D, Gervois N, Rimoldi D, Fonteneau JF, Bonelo A, Lienard D, et al. Diversity of the fine specificity displayed by HLA-A*0201-restricted CTL specific for the immunodominant Melan-A/MART-1 antigenic peptide. J Immunol 1998b;161:6956–62.

Van Scott EJ. Morphologic changes in pilosebaceous units and anagen hairs in alopecia areata. J Invest Dermatol 1958;31:35–43.

von Herrath M, Sanda S, Herold K. Type 1 diabetes as a relapsing-remitting disease? Nat Rev Immunol 2007;7:988–94.

Wang E, McElwee KJ. Etiopathogenesis of alopecia areata: Why do our patients get it? Dermatol Ther 2011;24:337–47.

Wang EH, Khosravi-Maharlooei M, Jalili RB, Yu R, Ghahary A, Shapiro J, et al. Transfer of Alopecia Areata to C3H/HeJ Mice Using Cultured Lymph Node-Derived Cells. J Invest Dermatol 2015;135:2530–2.

Whiting DA. Histopathologic features of alopecia areata: a new look. Arch Dermatol 2003;139:1555–9.

Wolfel T, Van Pel A, Brichard V, Schneider J, Seliger B, Meyer zum Buschenfelde KH, et al. Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. Eur J Immunol 1994;24:759–64.

Wong P, Pamer EG. CD8 T cell responses to infectious pathogens. Annu Rev Immunol 2003;21:29–70.

You S, Chatenoud L. Proinsulin: a unique autoantigen triggering autoimmune diabetes. J Clin Invest 2006;116:3108–10.

Zeh HJ, 3rd, Leder GH, Lotze MT, Salter RD, Tector M, Stuber G, et al. Flow-cytometric determination of peptide-class I complex formation. Identification of p53 peptides that bind to HLA-A2. Hum Immunol 1994;39:79–86.

Zhang B, Zhao Y, Cai Z, Caulloo S, McElwee KJ, Li Y, et al. Early stage alopecia areata is associated with inflammation in the upper dermis and damage to the hair follicle infundibulum. Australasian J Dermatol 2013;54:184–91.