Letter to the Editor

Presence of MAGE-A3 specific T cells in alopecia areata - study for the possibility of AA antigens -

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Dear Editor,

In this distinguished journal, we routinely read discovery stories that have been ultimately crowned by success. Instead, here we would like to share our trials and tribulations along a less felicitous, yet very instructive and educational research journey that brought us close to what we hoped to be a clinically important advance in understanding the pathobiology of alopecia areata (AA), a tissue-specific, T cell-dependent autoimmune disease [1].

Most currently available evidence suggests that, upon interferon (IFN)-γ-induced collapse of the hair follicle's (HF's) physiological immune privilege (IP), as yet unidentified follicular autoantigens are exposed to preexisting autoreactive CD8$^+$ T cells by ectopically expressed major histocompatibility (MHC) class I molecules within the epithelium of anagen hair bulbs [1,2]. Peptides derived from melanogenesis-associated autoantigens expressed only by melanin-producing anagen HFs are persuasive candidates as key autoantigens in AA [3]. Therefore, focusing on well-investigated MHC class I-restricted melanocyte-related antigens known to be recognized by CD8$^+$ T cells is a sensible AA research strategy (supplementary text S1).

We thus hypothesized that it should be possible to detect cytotoxic CD8$^+$ T cells (CTLs) directed against MHC class-I restricted autoantigens (tyrosinase, MAGE-A2, and MAGE-A3 (MBL)), using pentamer technology [4] (Supplemental text S2). To test this hypothesis, peripheral blood mononuclear cells (PBMCs) were obtained from

Japanese healthy controls and AA patients (Supplementary Table S1).

Initially, this approach yielded auspicious results: MAGE-A3-reactive CD8$^+$ T cells were found to be significantly increased in PBMCs in the acute phase of AA with multifocal lesions (AAM) and alopecia areata totalis (AAT) compared to healthy controls, chronic phase of AAM, or AAT/alopecia areata universalis (AU) (Figure 1a and S1a,b, Supplementary Text S3) (p=0.025 by Kruskal-Wallis ANOVA). Furthermore, skin infiltrating T cells of an acute phase AA lesion from one patient, which were isolated as previously described [5], also showed an increased number of MAGE-A3 specific CTLs (Figure 1b) as that in peripheral blood nuclear cells (PBMCs) from the same AA patients (Figure 1c) compared to the average frequency of MAGE-A3$^+$ T cells in PBMCs from control subjects (Figure 1a). This pilot finding suggested an enrichment of MAGE-A3$^+$ CTLs in lesional AA skin.

Next, we probed whether such CTLs can produce IFN-γ after stimulation with MAGE-A3 (supplementary text S4). Indeed, IFN-γ protein expression was significantly increased in CD8$^+$ T cells from acute phase AA patients co-cultured with MAGE-A3 compared to healthy controls (Figure 1d and supplementary Figure S1c,d). Moreover, the percentage of MAGE-A3 specific CTLs in PBMCs was also monitored in an acute phase AAT patient (n=1, patient 16) during the treatment with oral 20 mg/day prednisolone for 60 days. Before the treatment, the patient suffered from AAT and 2.30% of CD8$^+$ T cells reacted with MAGE-A3 (Figure 1e). Sixty days after the

treatment, marked hair regrowth had occurred while the percentage of MAGE-A3-reactive CTLs decreased to 0.49% (Figure 1f).

At this point, we wondered whether, contrary to the conventional wisdom that MAGE-A3 is only expressed by cancer cells [6,7], MAGE-A3 is also expressed in human HFs, at least under inflammatory conditions. This had been encouraged by the report that testis, placenta, fetal ovary and wounded skin may also express MAGE family members, besides melanoma and other cancers [8,9]. Finally, first immunohistological analyses conducted in one Japanese AA patient (Supplementary text S5) suggested that AA-affected HFs expressed MAGE-A3-like immunoreactivity with anti-human MAGEs (Y-18) (Figure 2a).

At this point we got quite excited: Not only seemed the adopted short cut-strategy to identify both, one hypothesis-driven, carefully selected putative key autoantigen as well as the autoreactive CTLs that recognize it, to have worked. But this also appeared to generate further experimental support for the IP collapse hypothesis of AA pathogenesis, which stipulates a key role for MHC class-I presented, melanocyte-related autoantigens recognized by CTLs. Finally, this would have been the first demonstration that MAGE-A3 is expressed also by adult human scalp HFs, at least under proinflammatory conditions. Given, however, that the demonstration of intrafollicular MAGE-A3 gene and protein expression is a cornerstone supporting the scenario sketched above, we decided to run additional analyses and controls.

First, by qRT-PCR analysis of mRNA extracted from either healthy human scalp HFs or lesional skin, using appropriate primers and controls (Supplementary text S6), failed to reveal MAGE-A3 transcripts above the detection threshold of our assay (Figure 2b). Next, when two different, MAGE-A3-specific primary antibodies (6C1 and 57B) were systematically employed by immunohistochemistry and an appropriate MAGE-A3[+] normal human tissue (testis) [9,10] was used as positive control, along with rigorously negative controls (Supplementary text S7), the previously detected "MAGE-A3-like" immunoreactivity of AA HFs turned out to be negative (Figure 2c-f, Supplementary Figure S2d-f, S2i-l) (Supplementary Text S8). Finally, also a final, semiquantitative RT-PCR using different MAGE-A3 primers (Supplementary text S6) failed to show MAGE-A3 mRNA in healthy or AA skin (Figure 2g). Therefore, under physiological or pathological conditions, human anagen scalp HFs do not express MAGE-A3 on the mRNA or protein level, and the MAGE-A3-reactive CTLs found in AA patients are likely to have preexisted before disease development.

In pursuing it, we provide the first evidence of CD8^{+} CTLs that do react with melanocyte-associated proteins (here: MAGE-A3) in HLA-A2402^{+} AA patients and show that CD8^{+} T cells from AA patients do express the potent IP collapse inducer, IFN-γ [2], upon stimulation with MAGE-3A.

The appealing hypothesis that, after HF-IP collapse, ectopic MAGE-A3 expression to

autoreactive CD8$^+$ T cells triggers a CTL-attack on the HF, thus inducing the AA phenotype, may seem obsolete now. Yet, that more MAGE-A3-reactive CTLs are indeed present in acute AAT and AAM patients than in healthy controls or chronic AA patients begs the question whether these T cells are involved in AA pathobiology, and may thus be a worthwhile therapeutic target for future AA management, after all.

Conflicts of interest: None declared.

References

1. Gilhar A, Etzioni A, Paus R. Alopecia areata. N Engl J Med 2012;366:1515-25.

2. Ito T, Ito N, Saathoff M, Stampachiacchiere B, Bettermann A, Bulfone-Paus S, et al. Collapse and restoration of MHC class-I-dependent immune privilege: exploiting the human hair follicle as a model. Am J pathol 2004;164:623-34.

3. 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.

4. Yagi H, Hashizume H, Horibe T et al. Induction of therapeutically relevant cytotoxic T lymphocytes in humans by percutaneous peptide immunization. Cancer Res 2006;66:10136-44.

5. Ito T, Hashizume H, Shimauchi T, Funakoshi A, Ito N, Fukamizu H, et al. CXCL10 produced from hair follicles induces Th1 and Tc1 cell infiltration in the acute phase of alopecia areata followed by sustained Tc1 accumulation in the chronic phase. J Dermatol Sci 2013;69:140-7.

6. Russo V, Pilla L, Lunghi F, Crocchiolo R, Greco R, Ciceri F, et al. Clinical and immunologic responses in melanoma patients vaccinated with MAGE-A3-genetically modified lymphocytes. Int J Cancer2013; 132:2557-66.

7. Moeller I, Spagnoli GC, Finke J, Veelken H, Houet L. Uptake routes of tumor-antigen MAGE-A3 by dendritic cells determine priming of naïve T-cell subtypes. Cancer Immunol Immunother 2012;61:2079-90.

8. Jungbluth AA, Silva WA Jr, Iversen K, Frosina D, Zaidi B, Coplan K, et al. Expression of cancer-testis (CT) antigens in placenta. Cancer Immun 2007; 7:15.

9. Becker JC, Gillitzer R, Bröcker EB. A member of the melanoma antigen-encoding gene (MAGE) family is expressed in human skin during wound healing. Int J Cancer 1994;58:346-8.

10. Rimoldi D, Salvi S, Schultz-Thater E, Spagnoli GC, Cerottini JC. Anti-MAGE-3 antibody 57B and anti-MAGE-1 antibody 6C1 can be used to study different proteins of the MAGE-A family. Int J Cancer 2000;86:749-51.

Figure legends

Figure 1

Frequency of MAGE-A3-specific autoreactive CD8$^+$ T cells is significantly increased in PBMCs and skin infiltrating T cells in alopecia areata and is correlated with IFN-γ-production and clinical course of AAT.

(**a**) Frequency of MAGE-A3-specific autoreactive CD8$^+$ T cells was analyzed by MHC class I-specific pentamers in PBMCs from healthy controls (n=10), acute phase multifocal AA (AAM, n=8), acute phase alopecia areata totalis (AAT, n=7), chronic phase AAM (n=7), and chronic phase alopecia areata totalis/universalis patients (AAT, n=6; AAU, n=2) *p≤0.05, p value was calculated by Kruskal-Wallis ANOVA. Representative results of flowcytometric analysis on MAGE-A3-specific autoreactive CD8$^+$ T cells.

(b) Biopsied skin samples from an acute phase AA patient (Patient No. 16) were cultured in RPMI with 50IU/ml IL-2 and anti CD3 antibody for 1 week. Then, CD8+ T cells were analyzed with MHC class I-specific pentamers for MAGE-A3.

(c) This was compared to that of PBMCs from the same patient.

(d) PBMCs obtained from acute phase AAM patients (n=3) and healthy controls (n=3) were incubated with MAGE3 for 8 hours, followed by flowcytometric analysis for the detection of intracytoplasmic IFN-γ expression. *p≤0.05, p value was calculated by Mann-Whitney-U-Test.

(e) Clinical picture of a patient suffering from AAT (Patient No. 25) before the

treatment with prednisone, who displayed 2.3% MAGE-A3 specific CTLs of PBMC CD8^{+} T cells.

(f) Clinical picture of the same patient 60 days after glucocorticosteroid treatment (note the marked hair regrowth). At this time, only 0.65% of PMBC CD8+ T cells represented MAGE-A3 specific CTLs (FACS).

Figure 2

Protein and mRNA expression of MAGE-A3 in healthy and AA skin.

(a) Immunohistochemical staining was demonstrated on lesional scalp skin of one Japanese AA patient, using polyclonal goat anti-human MAGEs (Y-18) [Santa Cruz Biotechnology]. This immunoreactivity turned out to be non-specific (see Fig. S2a).

(b) Relative mRNA expression of MAGE-3 analyzed by quantitative RT-PCR in anagen HFs from 2 healthy subjects (1,2), catagen HFs from 1 healthy subject (3), whole human skin punches from 1 healthy subject (4) and from one AA patient (5). Human melanoma cell lines (m1, ht199, wm35) were used as positive control.

(c-f) Specific MAGE-3 immunostaining in AA lesional (c, e) and healthy (d, f) scalp skin was performed using a mouse anti-human MAGE-1 Ab-6 (6C1) [Thermo Fisher Scientific] and a goat anti-MAGE-3 monoclonal antibody 57B [by courtesy of Prof. Giulio C. Spagnoli]), both of which are reported to recognize human MAGE-A3 antigen (see supplement text 7, Figure S2b-l).

Size bars: 50 μm.

(g) mRNA expression of MAGE-3 analysed by semiquantittaive RT-PCR in anagen

scalp HFs from 2 healthy subjects (1,2), catagen HFs from 1 healthy subject (3), whole skin punches from 2 healthy subjects (4,6) and AA patient (5). Human melanoma cell lines (M1, HT199, and WM35) were used as positive control.
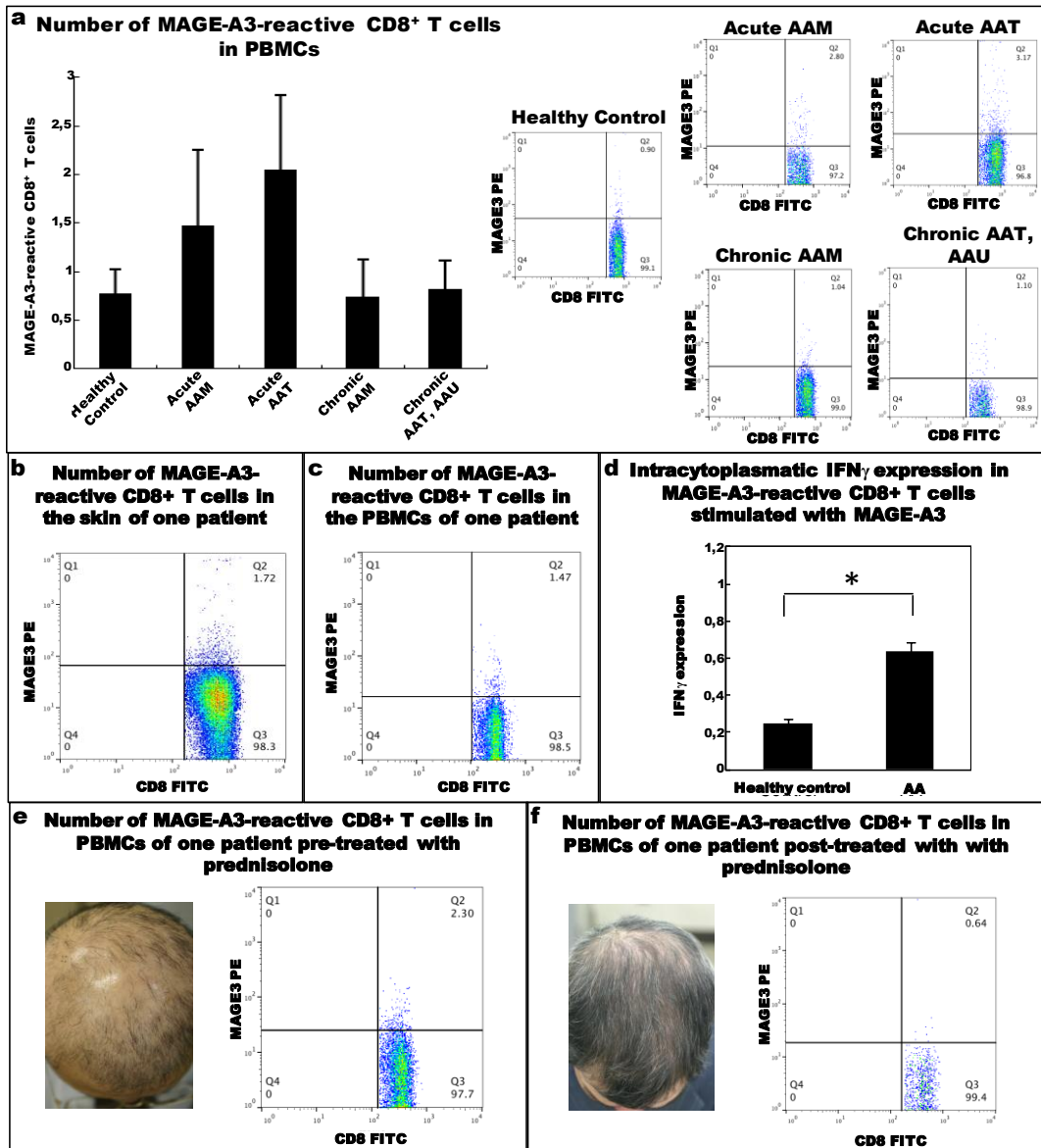
Figure 1

Figure 2