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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Blocking Potassium Channels (Kv1.3): A New Treatment Option for Alopecia Areata?

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TO THE EDITOR

T lymphocytes express a variety of voltage-gated potassium channels (Kv) that greatly affect T-cell function (Szabò *et al.*, 2010; Feske *et al.*, 2012). Namely, Kv1.3 channels regulate autoreactive effector memory T cells, which are implicated in the pathogenesis of several autoimmune diseases (Beeton *et al.*, 2001, 2006, 2008*; s4*). Kv1.3 channel blockers may therefore offer a therapeutic option in several autoimmune disorders (Wulff *et al.*, 2003a). This may include alopecia areata (AA), a T-cell–dependent autoimmune disease, in which hair follicles (HFs) are attacked by a dense lymphocytic infiltrate of activated CD4+ and CD8+ T cells (*s9*, *s17*, *s15*, Gilhar *et al.*, 2012).

After grafting to SCID mice, lesional (alopecic) scalp skin from AA patients regrows hair when the infiltrating scalp lymphocytes are lost (*s14, s15,* Gilhar *et al.*, 2012), whereas AA-like hair loss can be induced in this humanized mouse model of AA by injecting lesional T cells (*s13, s15*). Previous work by us and others had suggested that NK cells and NKG2D ligands have a major role in AA (Ito *et al.*, 2008; Petukhova *et al.*, 2010). Moreover, recently we demonstrated that it is possible to induce AA lesions in healthy human scalp transplants on SCID mice only by injection of peripheral blood mononuclear cells (PBMCs) greatly enriched for NKG2D+ and CD56+ cells, which includes NK, NKT, and some CD8+ T cells (Gilhar *et al.*, 2013).

Therefore, we hypothesized that inhibiting T-cell function in AA by well-tolerated Kv1.3 blockers may constitute a therapeutic approach to AA management, and asked the following questions: (1) Are Kv1.3+ T cells increased in lesional AA skin? (2) Does Kv1.3-selective potassium channel blockade alter the development of AA lesions in a humanized mouse model of AA (Gilhar *et al.*, 2013)?

First, we obtained biopsies from lesional skin of 10 patients with AA after informed consent and institutional review board approval. Second, normal scalp skin from healthy volunteers was grafted onto beige SCID mice. Three

Abbreviations: AA, alopecia areata; HFs, hair follicles; PBS, phosphate-buffered saline; PBMCs, peripheral blood mononuclear cells

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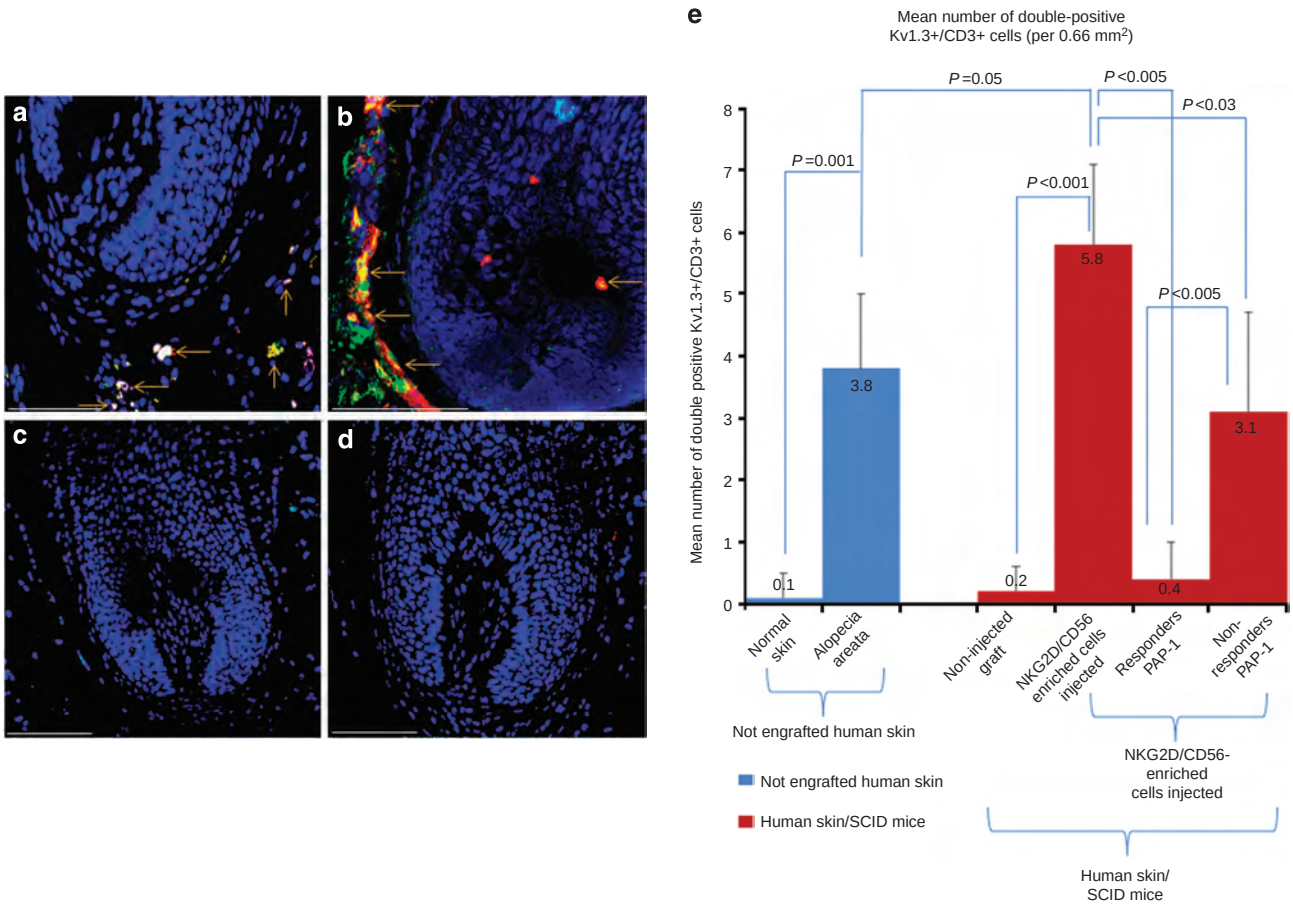

Figure 1. Kv1.3/ CD3+ T cells in alopecia areata (AA) and human skin grafts transplanted onto SCID mice. (**a**) Double immunofluorescence demonstrates the presence of Kv1.3+/CD3+ cells around hair lesional hair follicles of AA patients and (**b**) in 11/15 human skin grafts injected with peripheral blood mononuclear cells (PBMCs) greatly enriched for NKG2D+ and CD56+ cells. Yet this was not seen in either normal skin of healthy volunteers (**c**) or in (**d**) 10/13 grafts injected with the same PBMC preparation but also injected with PAP-1 instead of vehicle alone. Counterstain was performed with ToPro3 (blue nuclei), Kv1.3 (green), and CD3 (red). (**e**) A significant increase in the number of Kv1.3/CD3 double-positive cells was found in lesional skin of AA patients compared with healthy scalp skin controls (3.8 ± 1.2 vs. 0.1 ± 0.4, $P<0.001$). Similarly, a significantly increased number of Kv1.3/CD3 double-positive cells was found in the humanized AA mouse model, as compared with lesional human AA scalp skin ($P=0.05$) and with the PAP-1-treated responders ($P<0.05$). Kv1.3+/CD3+ cells were also largely absent in 7/8 human skin grafts injected with PHA-stimulated control PBMCs that had not developed AA lesions, as expected (Gilhar *et al.*, 2013).

months after skin engraftment and hair regrowth, autologous PBMCs (from the same healthy volunteers) that had been enriched for NKG2D+/CD56+ cells, which were stimulated *in vitro* with IL-2, were injected intradermally into the grafts. Within 4–6 weeks, this induced a hair loss phenotype in the human skin grafts that showed the characteristic clinical and histological features of human AA (Gilhar *et al.*, 2013).

Double-immunofluorescence confocal microscopy demonstrated the presence of multiple Kv1.3+ cells around and within lesional HFs, both in the skin of AA patients (Figure 1a) and in lesional human skin in the humanized AA mouse model (Figure 1b). The fact that the perifollicular Kv1.3+ cells were also CD3+ (Figures 1a, b) confirmed that the Kv1.3+ cells are T lymphocytes. In both lesional skin of AA patients and in human skin transplanted onto SCID mice with experimentally induced AA, the number of Kv1.3+ T cells was greatly increased compared with normal skin of healthy volunteers and normal hair graft ($P<0.001$) (Figure 1c).

Previous studies have demonstrated that disease-associated autoreactive T cells in type 1 diabetes mellitus, multiple sclerosis, and rheumatoid arthritis are mainly negative for the chemokine receptor, CCR7, but express Kv1.3 (Wulff *et al.*, 2003b; Beeton *et al.*, 2006; s21). This phenotype may allow one to distinguish autoreactive effector memory T cells in patients with autoimmune disease (*s14*). It is therefore important to note that many of these $Kv1.3^+$ cells were $CCR7^-$, both in AA patients (47 ± 2% of the total number of Kv1.3+ cells) and in the humanized AA mouse model (69 ± 15%).

This suggests that most $Kv1.3^+$ cells in the inflammatory infiltrate in human AA lesions represent autoreactive effector memory T cells, which may have undergone repeated antigen stimulation *in vivo* (*s14*). As Kv1.3 blockers preferentially suppress autoreactive $CCR7^-$ effector memory T cells that arise as a consequence of repeated autoantigen stimulation (Beeton *et al.*, 2006), blocking the Kv1.3 channel therefore promises greater specificity for suppressing autoreactive

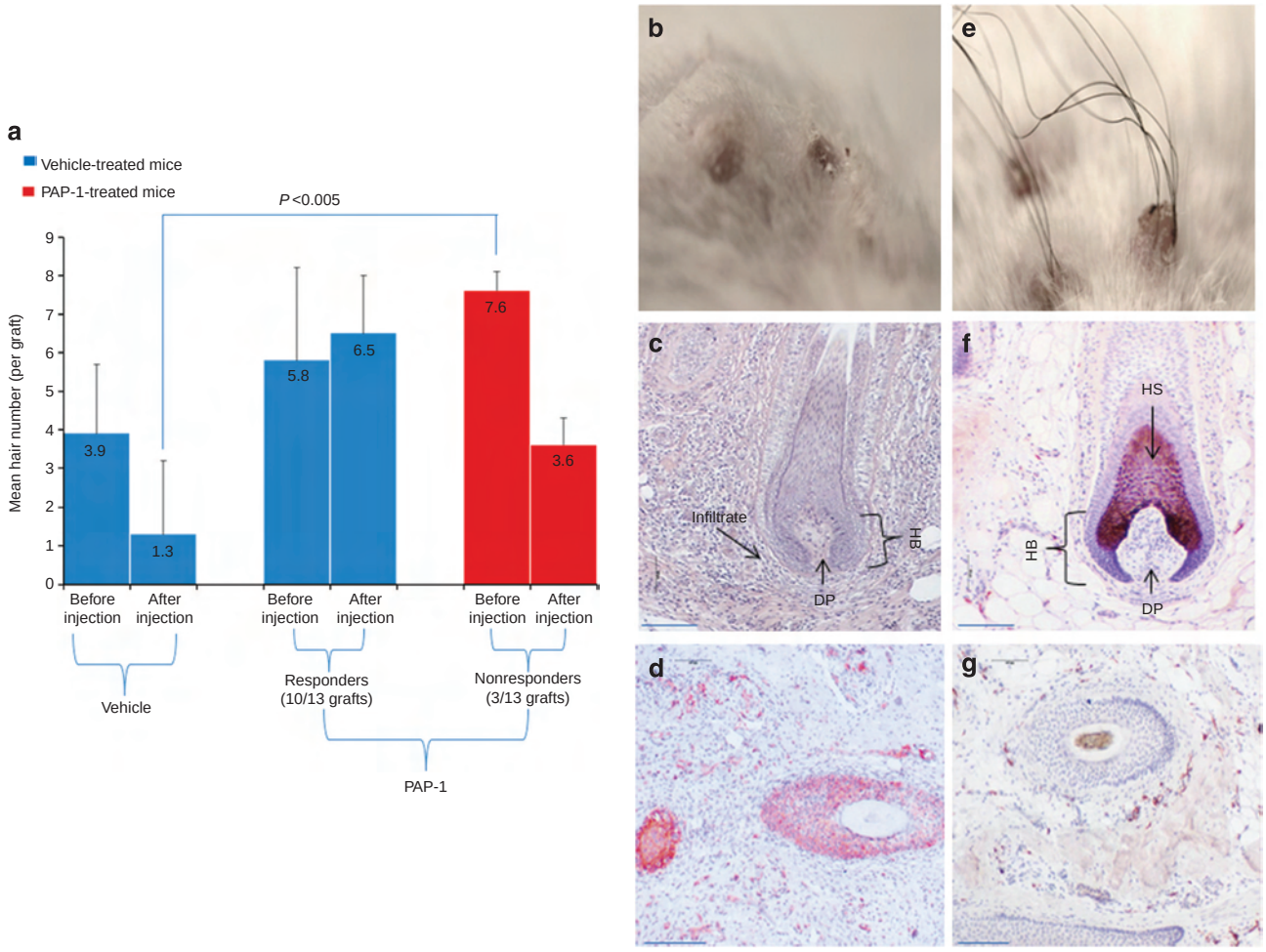

Figure 2. The Kv1.3 blocker PAP-1 suppresses AA development in human skin grafts. Administration of Kv1.3 blocker inhibited the activity of NKG2D+/CD56+-enriched cells and thereby suppressed the development of AA. A stable number of hairs in the responder grafts (treated with both NKG2D+/CD56+ cells and thereafter with PAP-1) clearly indicates a therapeutic benefit of PAP-1 injection in the humanized mouse of AA. Ten SCID mice, grafted with normal human skin and injected with autologous NKG2D+/CD56+-enriched cells, were used in this pilot study. Each mouse was grafted with three scalp skin transplants obtained from the same donor ($n=30$ grafts in total). Five mice were treated with the Kv1.3 blocker, PAP-1; the remaining five mice served as a control group and were injected with vehicle only (Cremophor EL/PBS). (**a**) The mean hair number is increased after Kv1.3 blockade. A significantly increased mean number of hairs was observed in PAP-1-treated responder grafts as compared with vehicle controls and with nonresponders (mean number of 5.8 ± 2.1 vs. 3.9 ± 1.5, $P=0.05$, respectively). A similar mean number of hairs was observed in the responder grafts (11/15) before and after treatment with PAP-1. However, significant hair loss was noted in grafts injected with NK-enriched cells and vehicle only ($P<0.005$), and in the nonresponder grafts from the PAP-1-treated group (3/11, $P<0.05$). (**b**) As expected, complete AA-like hair loss was observed in human skin grafts injected with peripheral blood mononuclear cells (PBMCs) greatly enriched for NKG2D+ and CD56+ cells and treated only with vehicle (Gilhar et al., 2013). (**c**) Histological evaluation confirmed the clinical observation of AA-like lesions by demonstrating the characteristic dense perifollicular lymphocytic infiltrates. (**d**) By performing immunohistochemistry, ectopic HLA-DR expression within the hair follicle epithelium was demonstrated. (**e**) In sharp contrast to control mice only injected with NKG2D+/CD56+ cells and vehicle, hair growth was observed in 3-mm grafts after treatment with PAP-1. This was accompanied by (**f**) histological and (**g**) immunohistochemical normalization of the phenotype of PAP-1-treated grafts. DP, dermal papilla; HB, hair bulb; HS, hair shaft. Bar = 100 μm.

T cells than targeting other molecules expressed on all T lymphocytes.

Encouraged by these findings, we then probed in the humanized AA mouse model (Gilhar *et al.*, 2013) whether the Kv1.3-selective potassium channel blocker, 5-(4-phenoxybutoxy) psoralen (PAP-1), has an impact on the development of AA lesions *in vivo*. PAP-1 is a selective small-molecule blocker of Kv1.3, which blocks Kv1.3 (EC_{50}:2 nM) and potently inhibits the proliferation of human $CCR7^-$ T cells while sparing naïve and central memory T cells (Schmitz *et al.*, 2005, s18).

In line with our previously reported results (Gilhar *et al.*, 2013), after intradermal injection of NKG2D/CD56-enriched, IL-2-activated PBMCs, 11 out of 15 control grafts showed the expected, characteristic focal alopecia (Figure 2a, b), along with the histological (Figure 2c) and immunohistochemical (Figure 2d) features of AA. Double-immunofluorescence microscopy revealed that most Kv1.3+ cells were $CCR7^-$ (Supplementary Figure S1A–E online). PAP-1 or control vehicle injections were given daily for 31 days, intradermally into each graft, following injection of the NKG2D/CD56-enriched cells.

In striking contrast to control mice, 10 out of 13 grafts in the five mice that had

been treated with PAP-1 (6 mg kg^{-1}, 0.2 ml injection volume per graft) showed complete hair regrowth (Figure 2a, e) and complete histological (Figure 2f) and immunohistological normalization of the skin phenotype (Figure 2g). In addition, quantitative immunohistomorphometry revealed that the number of Kv1.3+/CD3+ cells was significantly reduced in the responder grafts (Figure 1d), compared with either phosphate-buffered saline (PBS)-treated control grafts or nonresponder grafts (Figure 1e). Interestingly, Kv1.3 blockade by PAP-1 significantly reduced the number of perifollicular and intrafollicular Kv1.3+ cells even in the three nonresponder grafts, as compared with PBS-treated control AA lesions (Figure 1e). As the cellular infiltrate in spontaneously developed human AA lesions and in the injected grafts that developed AA lesions strongly expressed Kv1.3, it is reasonable to assume that the therapeutic effect of PAP-1 and its reduction of the number of NKG2D+/CD56+ cells were owing to its Kv1.3-blocking effects.

Taken together, these pilot data suggest that Kv1.3^{+}/CCR7^{-} T_{EM} cells may be both important elements in human AA pathogenesis *and* promising targets for more selective future immunotherapy of AA. Evidently, the robustness of anti-AA effects of Kv1.3 blockers needs to be confirmed in larger cohorts of humanized AA mice (Gilhar *et al.*, 2013). However, the limited currently available data are in line with promising results obtained with Kv1.3 blockers in other autoimmune disease *(s10, s4;* Wulff *et al.*, 2003b, 2009, Beeton *et al.*, 2006) and psoriasis models (Gilhar *et al.*, 2011). This encourages one to systematically explore Kv1.3 blockade as a therapeutic strategy in human AA (for additional background, methods, discussion, and supplementary references, see Supplementary Text online).

CONFLICT OF INTEREST

A patent on the use of Kv1.3 in AA has been filed by A.G. and A.K.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Pharmacological Inhibition of Stearoyl CoA Desaturase in the Skin Induces Atrophy of the Sebaceous Glands

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TO THE EDITOR

Acne is a multifactorial disease of the pilosebaceous unit resulting from increased sebum production, altered keratinization, inflammation, and bacterial hypercolonization. With respect to hyperseborrhea as one of the contributing factors, it is of interest to study pathways that regulate sebaceous gland differentiation. Interestingly, mouse strains deficient in the enzyme stearoyl-CoA desaturase 1 (SCD1) exhibit severe hypoplasia of sebaceous

Abbreviation: SCD, stearoyl-CoA desaturase

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