Autoimmune Disease Induction in a Healthy Human Organ: A Humanized Mouse Model of Alopecia Areata

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TO THE EDITOR

In this study, we present an animal model that swiftly recreates one of the most frequent human autoimmune disorders, alopecia areata (AA) (Safavi *et al.*, 1995; Harries *et al.*, 2010; Alli *et al.*, 2012; Gilhar *et al.*, 2012), within transplanted healthy human skin (Figure 1a–c,g).

AA is a CD8+ T cell–dependent autoimmune hair loss disorder, which is associated with other autoimmune diseases. It results from inflammatory cell infiltrates that attack growing (anagen) hair follicles in association with a collapse of the normal immune privilege (IP) that healthy hair follicles enjoy (Gilhar *et al.*, 1998; McElwee *et al.*, 2005; Paus *et al.*, 2005; Alli *et al.*, 2012; Gilhar *et al.*, 2012). As in other autoimmune diseases, IFN-γ and substance P are key inducers of hair follicle IP collapse (Ito *et al.*, 2004; Peters *et al.*, 2007; Siebenhaar *et al.*, 2007; Gilhar *et al.*, 2012).

AA patients demonstrate excessive natural killer (NK) cell activities, as well as overexpression of NK-activating receptors (NKG2D) and NKG2D-activating ligands (MICA and ULBP3) (Ito *et al.*, 2008; Petukhova *et al.*, 2010). NKG2D is expressed on NK and NKT cells, as well as on some T lymphocytes, and its ligands are implicated in other common autoimmune diseases (Gambelunghe *et al.*, 2007; Andersson *et al.*, 2011).

Therefore, we asked whether the transfer of human peripheral blood mononuclear cells (PBMCs) greatly enriched for NKG2D+ and CD56+ cells suffices to induce AA-like hair loss in healthy human skin transplanted onto immunocompromised mice, even when PBMCs are derived from healthy donors.

Fifty-five C.B-17/IcrHsd-scid-bg mice were used in this study. Punch biopsies from normal hairy human scalp were obtained from each donor during routine face-lift surgery with written informed consent and ethics approval, and were grafted onto SCID mice. Thereafter, either allogeneic or autologous PBMCs enriched for NKG2D+ or CD56+ T cells (CD4, CD8) from *healthy* donors were injected once subcutaneously (Figure 1g).

Briefly, PBMCs were isolated from healthy donors and then cultured with 100 U ml^{-1} IL-2 (2 weeks)/phytohemagglutinin (PHA) (10 μg ml^{-1}). Alternatively, PBMCs were cultured with IL-2 (100 U ml^{-1}). After 14 days of incubation, NKG2D/CD56-enriched cells were incubated with anti-CD56 antibodies conjugated to ferromagnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and directed through a cell separation column containing a magnetic field (Miltenyi Biotec GmbH). In addition, CD56-depleted cells were prepared for injection.

The NKG2D/CD56 cell population expressed the surface markers shown in Figure 2a, including heterogeneous NK cell markers, and exhibit NK cytotoxicity (Gilhar *et al.*, 2002). Treatment with high-dose IL-2 (100 U ml^{-1}) led to a significantly greater enrichment of NKG2D+ and CD56+ following culture of PBMCs than PHA treatment (Supplementary Figure S1 online).

Three months after human skin graft transplantation, 7×10^6 of these NKG2D/CD56-enriched cells were injected (note that in the humanized mouse model for psoriasis these cells are given intradermally into split-thickness skin grafts (Gilhar *et al.*, 2002, 2011), whereas they are injected into the subcutis of 3-mm full-thickness scalp skin grafts in the present model).

Strikingly, 3–5 weeks after cell injection, all the characteristic clinical and histological features of human AA were reproduced. Although impressive hair growth was observed in human scalp skin grafts before cell injection (Figure 1b), marked, localized hair loss was seen exclusively in scalp grafts treated with PBMCs enriched for NKG2D+ and CD56+ cells (Figure 1c), but in none of the control groups (i.e., PBMCs non-specifically activated with PHA, and activated PBMCs depleted for CD56+ cells) (Supplementary Figure S2 online).

Exactly as in human AA (Gilhar *et al.*, 2012), dense perifollicular infiltrates of CD4+ or CD8+T cells around proximalanagen hair follicles (Supplementary Figure S3 A–F online) and collapse of hair follicle IP (Figure 2b–e) were seen. Importantly, hair loss occurred irrespective of whether the mice had been injected with autologous or allogeneic NKG2D+/CD56+ cell–enriched PBMCs. Only injection of stimulated PBMCs that were highly enriched for activated NK/NKT cells mimicked human AA (Supplementary Figure S3 A–F online). Besides their IP-collapse–promoting activities, the IFN-γ-secreting NK/T cells may directly inhibit hair growth as IFN-γ potently induces catagen (Ito *et al.*, 2005).

Although CD56+ cells are critical for inducing the AA phenotype in this model, the key pathogenic subpopulation(s) in the injected cell collective, and the spectrum of cytokines secreted by them (e.g., IFN-γ) (Gilhar *et al.*, 1998, 2012) (Figure 2f,g), remain unknown. However, this model already offers a superior preclinicial AA research tool and allows the preclinical testing of new candidate AA therapies (see supplementary text online). Moreover, this model provides functional support for the concept that NKG2D+ and/or CD56+ cells (and likely NKG2D-

Abbreviations: AA, alopecia areata; IP, immune privilege; NK, natural killer; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin

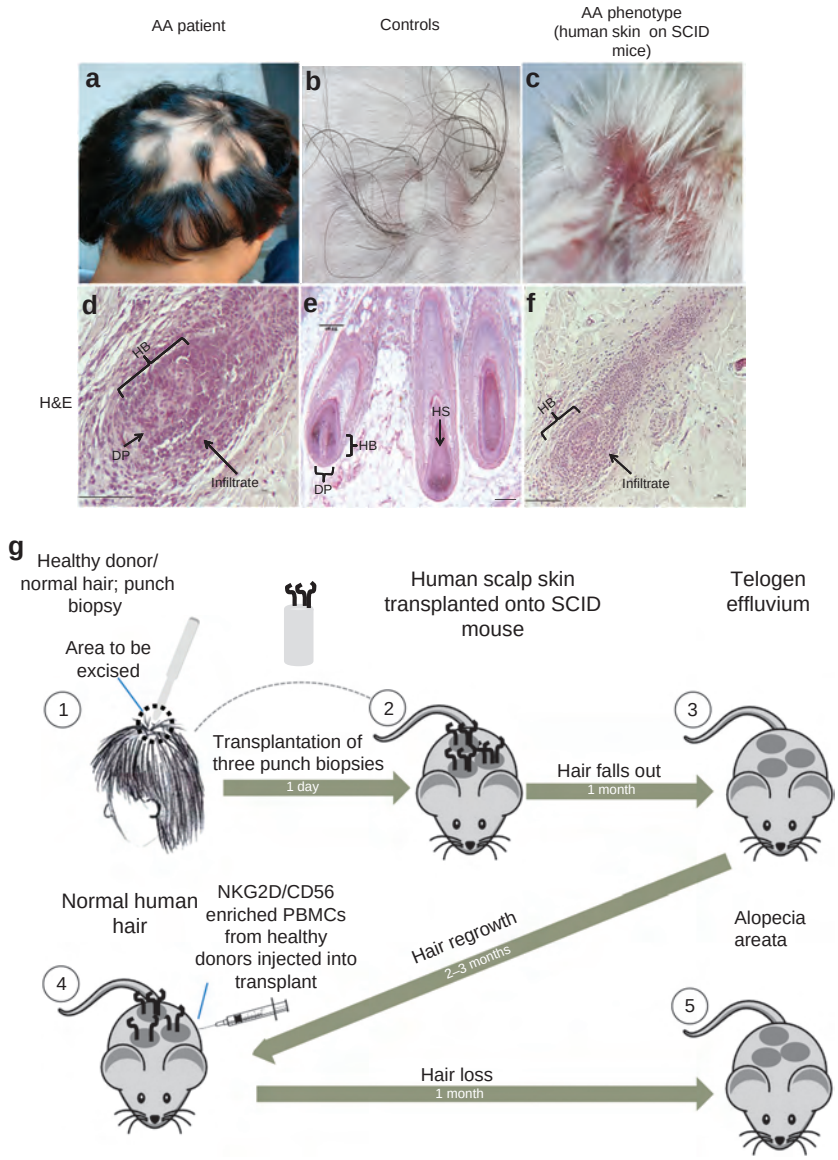

Figure 1. Alopecia areata (AA) in patients and in the humanized AA mouse model. (**a**) Characteristic hair phenotype of a female patient with AA (Gilhar *et al.*, 2012). (**b**) Normal hair growth in a human scalp skin transplanted onto beige SCID mice. (**c**) Complete AA-like hair loss in human skin graft injected with peripheral blood mononuclear cells (PBMCs) greatly enriched for NKG2D+ and CD56+ cells (details: see Figure 2a). (**d, f**) Dense perifollicular and intrafollicular lymphocytic infiltrate in AA patients (**d**). The same is seen in human skin graft injected with PBMCs enriched for NKG2D+ and CD56+ cells (**f**). (**e**) Normal human scalp hair follicles in a control graft injected with phytohemagglutinin-activated PBMCs. DP, dermal papilla; HB, hair bulb; HS, hair shaft. Bar = 100 μm. (**g**) Overview of the humanized AA mouse model. (1) Punch biopsies (3 mm) are taken from a healthy donor with normal hair growth under local anesthesia. (2) Three biopsies are inserted into small incisions made on the back of a beige SCID mouse. (3) About 2–4 weeks after the transplantation, the expected hair loss (telogen effluvium) is seen. After that, the transplants are treated twice daily with 5% topical minoxidil to enhance hair regrowth (Harries *et al.*, 2010). (4) Optimal hair regrowth is seen 2–3 months after scalp skin transplantation. (5) Allogeneic or autologous PBMCs from peripheral blood from healthy donors are cultured with high-dose IL-2 (100 U ml^{-1}). Lymphocyte lines with natural killer (NK) activity and heterogeneous T cells with NK phenotype, including CD56+ and NKG2D+, are generated by culture of PBMCs from normal donors in 100 U ml^{-1}. This enriching culture with CD56+ and NKG2D+ cells (47%) is injected (7×10^6) once into the lower part of the full-thickness skin grafts. This induces the characteristic macroscopic and microscopic features of AA (Gilhar *et al.*, 2012) within previously normal human scalp skin transplants.

stimulating ligands such as MICA and ULPB3) have an important role in AA pathogenesis (Ito *et al.*, 2008; Petukhova *et al.*, 2010).

This humanized mouse model of AA, which uses healthy human skin and PBMCs from healthy donors, questions whether certain genetic and environmental factors predisposing to the development of AA are really indispensable for inducing the AA phenotype. Certainly, the current model offers to welcome new opportunities to dissect the specific contributions of genetic and environmental factors to AA pathobiology (for additional background, discussion, and references, see Supplementary text online).

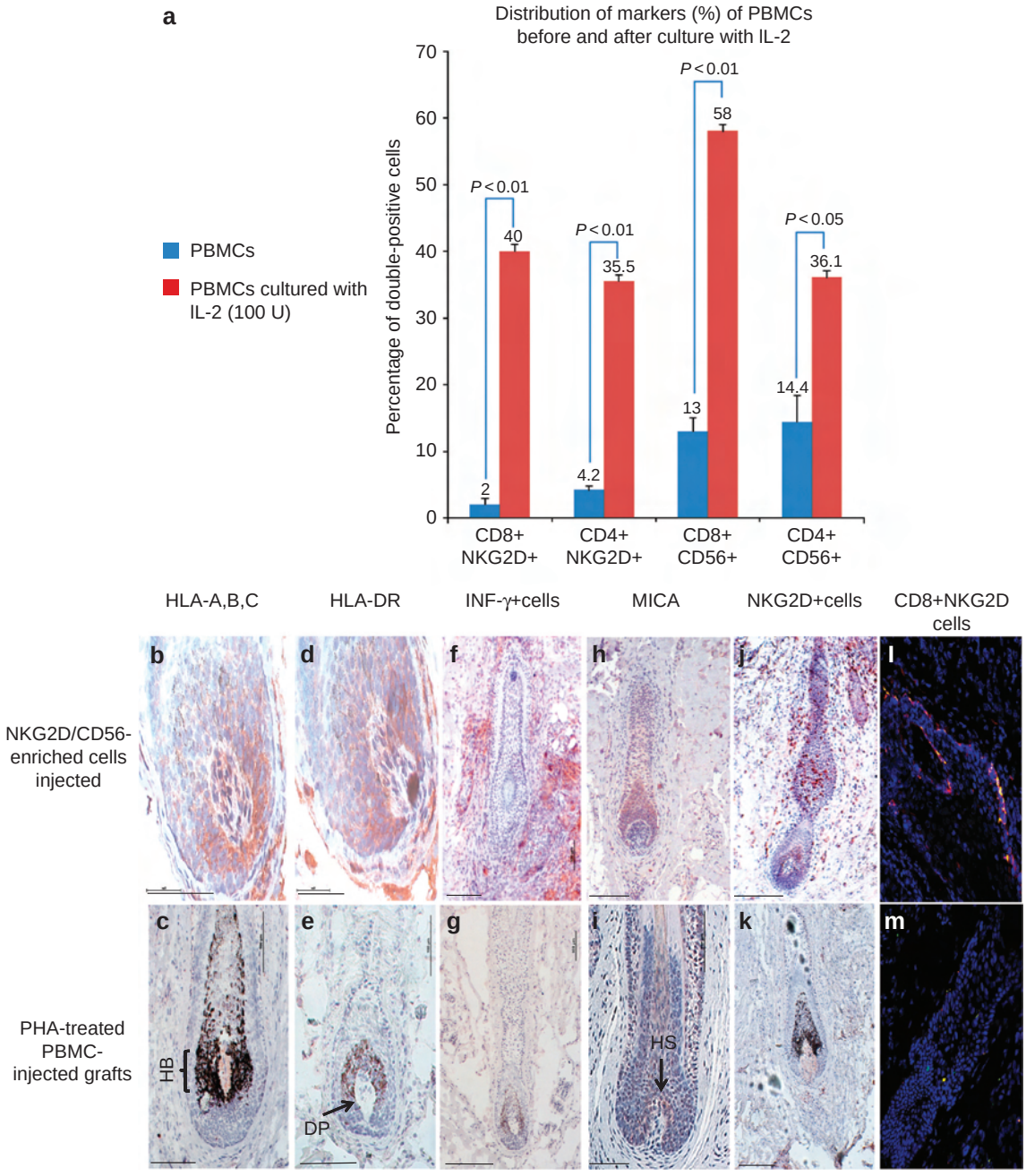

Figure 2. The role of CD56+ and NKG2D+ cells in the induction of alopecia areata in the humanized mouse model. (**a**) Flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) cultured with high-dose IL-2 (100 U ml^{-1}) demonstrated a significant enrichment in NKG2D+ and CD56+ cells among CD4+ and CD8+ T cells. (**b, c**) Immunohistochemistry revealed the characteristic signs of hair follicle immune privilege collapse (Ito *et al.*, 2008; Gilhar *et al.*, 2012) in hair follicles affected by alopecia areata (AA) after injection of PBMCs enriched for NKG2D+ and CD56+ cells, as indicated by ectopic major histocompatibility complex (MHC) class I expression (Ito *et al.*, 2008). (**d, e**) Immune privilege collapse was also evident from the ectopic expression of MHC class II (Ito *et al.*, 2008). (**f, g**) The fact that many of the perifollicular lymphocytic cells in the test mice were strongly positive for IFN-γ supports the widely accepted central role of IFN-γ in AA pathogenesis (Gilhar *et al.*, 2012). (**h, i**) Interestingly, the injection of activated PBMCs enriched for NKG2D+ cells was associated with a prominent upregulation of one classical NKG2D ligand, MICA (Ito *et al.*, 2008; Petukhova *et al.*, 2010), possibly as a result of excessive IFN-γ secretion (see **f, g**). (**j, k**) Indeed, a dense intrafollicular and perifollicular NKG2D+ cells infiltrate is observed in the treated mice, which enables NKG2D/MICA engagement (see **h, i**). (**l, m**) Double immunostaining revealed CD8+/NKG2D+ T cells around lesional hair follicles (arrows) after injection of PBMCs enriched for NKG2D+ and CD56+ cells. Bar = 100 μm. DP, dermal papilla; HB, hair bulb; HS, hair shaft.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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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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High Anti-Staphylococcal Antibody Titers in Patients with Epidermolysis Bullosa Relate to Long-Term Colonization with Alternating Types of *Staphylococcus aureus*

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TO THE EDITOR

Patients with the blistering disease epidermolysis bullosa (EB) develop wounds that are highly susceptible to bacterial colonization. Recently, we reported that over 75% of the EB patients sampled at one particular point of time were colonized with *Staphylococcus aureus* (van der Kooi-Pol *et al.*, 2012). To determine possible changes in *S. aureus* colonization over time, swabs were collected from the nares, throats, and wounds of 61 EB patients at three time points during a period of ~2 years. All *S. aureus* isolates were typed by multiple-locus variable number of tandem repeats analysis (MLVA) and *spa* typing. This revealed major fluctuations in the *S. aureus* types sampled from individual EB patients. In addition, blood samples were obtained from 13 EB patients to determine their IgG levels against 43 virulence factors or whole cells of *S. aureus*. Overall, the sera of EB patients contained higher anti-staphylococcal IgG levels than those of healthy individuals. Specifically, this applied to IgGs against nine important virulence factors, including the superantigens (SAgs) staphylococcal enterotoxin M (SEM), SEN, and SEO. Notably, EB patients carrying different *S. aureus* types contained higher levels of anti-staphylococcal antibodies than EB patients colonized by only one type.

Abbreviations: EB, epidermolysis bullosa; ET, exfoliative toxin; HlgB, gamma-hemolysin B; IsaA, immunodominant antigen A; Isd, iron-responsive surface determinant; Luk, leukocidin; LytM, peptidoglycan hydrolase; MFI, median fluorescence intensity; MLVA, multiple-locus variable number of tandem repeats analysis; Nuc, endonuclease; SAgs, superantigens; SasG, S. aureus surface protein G; SCIN, staphylococcal complement inhibitor; SE, staphylococcal enterotoxin