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iNKT cells ameliorate human autoimmunity: Lessons from alopecia areata

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ABSTRACT

Alopecia areata (AA) is understood to be a CD8+/NKG2D+ T cell-dependent autoimmune disease. Here, we demonstrate that human AA pathogenesis of is also affected by iNKT10 cells, an unconventional T cell subtype whose number is significantly increased in AA compared to healthy human skin. AA lesions can be rapidly induced in healthy human scalp skin xenotransplants on Beige-SCID mice by intradermal injections of autologous healthy-donor PBMCs pre-activated with IL-2. We show that in this *in vivo* model, the development of AA lesions is prevented by recognized the iNKT cell activator, α-galactosylceramide (α-GalCer), which stimulates iNKT cells to expand and produce IL-10. Moreover, in pre-established humanized mouse AA lesions, hair regrowth is promoted by α-GalCer treatment through a process requiring both effector-memory iNKT cells, which can interact directly with CD8+/NKG2D+ T cells, and IL-10. This provides the first *in vivo* evidence in a humanized model of autoimmune disease that iNKT10 cells are key disease-protective lymphocytes. Since these regulatory NKT cells can both prevent the development of AA lesions and promote hair re-growth in established AA lesions, targeting iNKT10 cells may have preventive and therapeutic potential also in other autoimmune disorders related to AA.

1. Introduction

Alopecia areata (AA) is a common, T cell-dependent, organ-specific autoimmune disease that predominantly attacks growing hair follicles (HF) that have lost their relative immune privilege [1–4]. Lesions can contain many types of lymphocytes, including $CD4^+$ and $CD8^+$ T cells, invariant (i)NKT cells and NK cells [5,6]. While a central AA-promoting role of NKG2D+ $CD8^+$ T cells is now well-appreciated [3,7–12], other cells such as NK cells, unconventional T cells and mast cells likely contribute to the pathobiology of AA [5,6,9,12,13].

Since iNKT cells have become a recent focus of interest in autoimmune disease research [14–19], we used the best currently available humanized mouse model for AA [20–23] to assess whether iNKT cells may also play a role in human AA. Animal models are routinely used for the study of human autoimmune diseases, including psoriasis, atopic dermatitis, and multiple sclerosis [24,25]. Unfortunately, differences between human and animal immune systems often severely limit the ability of animal models to mimic the human autoimmune condition (24). However, some humanized mouse models (including those for psoriasis and AA) that employ human skin xenotransplants, which are intracutaneously injected with defined, autologous human immunocytes present characteristic lesions of these two skin diseases that very closely mimic the human disease phenotype [20,21,23,26].

For example, we have previously demonstrated that the humanized mouse model of AA leads to rapid and predictable development of focal hair loss, which demonstrates all the clinical, histological, and immunohistochemical features used for the clinical diagnosis of AA [21,22]. Therefore, this humanized mouse model ideally suited to delineate intralesional immune responses and interactions of human tissue (skin) with defined human

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immunocyte populations *in vivo* in an autologous setting. Furthermore, they can be used to dissect common immunopathology mechanisms shared between mammalian species, to test novel therapeutic strategies, and to understand mechanisms of drug action within the human target tissue in question [20–23,26,27]. We previously demonstrated that the AA humanized mouse model leads to rapid and predictable development of focal hair loss lesions that display all the characteristic clinical, histological and immunohistochemical features used for the clinical diagnosis of AA [21].

Recently, impressive regulatory effects of iNKT10 were described by Sag et al. [28]. These authors demonstrated the expansion of iNKT10 by repeated stimulation with the potent glycolipid antigen, alpha-galactosylceramide (α-GalCer). Therefore, we were particularly interested in probing the effects of α-GalCer on experimentally induced human AA *in vivo*, using the humanized mouse model of AA [21,22,27].

Our data generated in this model strongly support the hypothesis that iNKT10 cells with regulatory function play an important, previously unappreciated role in the pathobiology of human AA, and that targeting their function pharmacologically may represent a promising novel management strategy for both preventing and treating human AA. To the best of our knowledge, these translationally highly relevant preclinical data also provide the first evidence available in the literature that iNKT10 cells can exert an immunoregulatory effect in a human disease model [29]. Since autoimmunity in AA has tended to serve as a model for other T cell-dependent autoimmune diseases [1,5,8,30], our study also encourages a more systematic dissection of the potential role for iNKT10 cells and their therapeutic targeting in other autoimmune diseases.

2. Materials and Methods

2.1. Animals

In this study, 90 C.B-17/IcrHsd-scid-bg (Beige-SCID) mice (Harlan Laboratories Ltd., Jerusalem, Israel) were used at 2–3 months of age. NOD-scid IL2rγnull (NSG) mice (Jackson laboratories) were also used. Both mouse strains demonstrated good integrity following autologous human skin and PBMC engraftment. The mice were housed in the pathogen-free animal facility of the Rappaport Faculty of Medicine, Technion – Israel Institute of Technology. Animal care and research protocols were in accordance with institutional guidelines and were approved by the Institutional Committee on Animal Use.

2.2. Donors

Thirteen healthy donors aged 43 ± 10 years were included in this study. Full thickness healthy scalp skin (diameter of 3 mm) was transplanted onto Beige-SCID or NSG mice. Twenty ml of venous blood was collected from the same donors. For immunohistochemical analysis, biopsies were obtained from lesional areas of six patients with alopecia areata (age 37 ± 8). The study was approved by our institute's Institutional Ethics Committee.

2.3. Transplantation procedure

Full thickness biopsies were taken from healthy donors undergoing plastic surgery in the scalp. Biopsies from each donor were dissected horizontally to generate pieces with a diameter of 3 mm. Three 3 mm pieces were grafted orthotopically into the subcutaneous layer of each mouse. The transplantation of the skin biopsies was performed as previously described [20–23]. Seven days following surgery, mice were treated with Minoxi-5 (hair regrowth treatment for men containing 5% Minoxidil active ingredient) by spreading it on the grafts twice a day until we received optimal expedited hair growth (period of two months).

2.4. Culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by centrifugation of Ficoll/Hypaque (Pharmacia, Amershem Pharmacia Biotech, Uppsala, Sweden). The PBMCs were cultured for 14 days with 100 U/ml human IL-2 (PROSPEC, protein specialists) alone or in combination with α-GalCer 100 ng/ml (Abcam) in filtered medium composed of RPMI 1640, 10% human AB serum (Sigma-Aldrich Co. LLC), 1% L-glutamine and 1% Penicillin-Streptomycin antibiotics (media components; biological industries, Kibbutz Beit Ha'Emek, Israel). The medium was changed as needed. After culture, PBMCs were injected intradermally in the skin grafts. In our previous work, we used a high concentration (7×10^6) of injected cells [20–23]. In this work, we investigated the use of lower cell concentration (3×10^6). The latter demonstrated the same phenotypic characterization as was observed in the previously published grafts where 7×10^6 cells had been injected.

Photo-documentation of the grafts was made. The grafts were harvested after 75 days, and then the skin tissues were fixed and embedded in paraffin. The mice were humanely sacrificed.

2.5. Research groups

Mice were divided randomly into the following 11 treatment groups:

Group 1 (18 mice) – PBMCs were incubated with a high dose of IL-2 (100 U/ml). After 14 days of incubation, cells were injected into the grafts.

Group 2 (8 mice) – PBMCs were activated nonspecifically with phytohemagglutinin (PHA) (10 μg/1 ml). After 14 days of incubation, cells were injected into the grafts.

Group 3 (8 mice) – PBMCs were incubated with a high dose of IL-2 (100 U/ml) combined with α-GalCer (100 ng/ml) for 14 days. Cells were injected into the grafts.

Group 4 (8 mice) – PBMCs were incubated with a high dose of IL-2 (100 U/ml). After 14 days of incubation, cells were injected into the grafts. Subsequent to PBMC injection, intragraft injection of α-GalCer (2 μg, 2Xweek) was performed.

Group 5 (6 mice) – PBMCs were incubated with a high dose of IL-2 (100 U/ml) in combination with α-GalCer (100 ng/ml) for 14 days. Cells were then bound with anti-iNKT cell (6B11) antibody conjugated to ferromagnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and directed through a cell separation column containing a magnetic field (Miltenyi Biotec). Depleted cells were collected for FACS analysis and were injected into the implanted grafts.

Group 6 (6 mice) – PBMCs were incubated with a high dose of IL-2 (100 U/ml) in combination with α-GalCer (100 ng/ml) for 11 days. Then, cells were incubated with 6B11 anti-TCR Vα24 neutralization antibody (IgG1, Beckman Coulter Inc., 10 μg/ml) for an additional three days. Cells were subsequently injected into the grafts.

Group 7 (6 mice) – (Comparator for Group 6) PBMCs were incubated with a high dose of IL-2 (100 U/ml) in combination with α-GalCer (100 ng/ml) for 11 days. Then, instead of 6B11 anti-TCR Vα24 neutralization antibody, cells were incubated with isotype control (nonspecific IgG1, Beckman Coulter Inc., 10 μg/ml(for an additional three days. Cells were subsequently injected into the grafts.

Group 8 (6 mice) – PBMCs were incubated with a high dose of

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