Mediation of Alopecia Areata by Cooperation Between CD4+ and CD8+ T Lymphocytes

Transfer to Human Scalp Explants on Prkdcscid Mice

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Objective: To determine the role of CD4+ and CD8+ T lymphocytes in the pathogenesis of alopecia areata.

Design: Relapse of alopecia areata was induced in autologous human scalp grafts on Prkdcscid mice by injection of activated T lymphocytes derived from lesional skin. CD4+ and CD8+ T cells were separated by magnetic beads before injection.

Setting: University-based dermatology practice.

Participants: Eleven patients with either alopecia totalis or severe alopecia areata.

Main Outcome Measures: Hair regrowth, hair loss, and immunohistochemical findings of scalp explants.

Intervention: Transfer of scalp T cells to autologous lesional scalp explants on Prkdcscid mice.

Results: Injection of unseparated T cells and mixed CD4+ plus CD8+ T cells resulted in significant hair loss (P<.01) in 5 of 5 experiments. However, injection of purified CD4+ or CD8+ T cells alone did not result in reproducible hair loss. CD4+ and CD8+ T cells induced follicular expression of intercellular adhesion molecule 1 (CD54), HLA-DR, and HLA-A, HLA-B, and HLA-C after injection into scalp grafts.

Conclusions: CD4+ and CD8+ T cells have a role in the pathogenesis of alopecia areata. It is hypothesized that CD8+ T cells act as the effector cells, with CD4+ T cell help. It is now necessary to look for HLA-A, HLA-B, and HLA-C associations with alopecia areata. Therapeutic manipulations that interfere with CD8+ activity should be examined.

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Alopecia areata (AA) is a hair loss disorder characterized by the autoimmune destruction of hair follicles. It is a chronic, relapsing disease with a variable clinical course. The disease affects both men and women, with a peak incidence in children and young adults. Alopecia areata is often accompanied by other autoimmune conditions, such as vitiligo and thyroiditis. The disease is thought to be mediated by autoreactive T cells that recognize hair follicle antigens. The pathogenesis of AA involves the activation of CD4+ and CD8+ T cells, which contribute to the destruction of hair follicles. CD4+ T cells may provide help to CD8+ effector T cells, while CD8+ T cells are thought to be the primary effector cells in the disease process.

In this study, the researchers aimed to determine the role of CD4+ and CD8+ T lymphocytes in the pathogenesis of AA. They induced relapse of AA in autologous human scalp grafts on Prkdcscid mice by injecting activated T lymphocytes derived from lesional skin. CD4+ and CD8+ T cells were separated by magnetic beads before injection.

The study included 11 patients with either alopecia totalis or severe alopecia areata. The main outcome measures were hair regrowth, hair loss, and immunohistochemical findings of scalp explants.

The results showed that injection of unseparated T cells and mixed CD4+ plus CD8+ T cells resulted in significant hair loss (P<.01) in 5 of 5 experiments. However, injection of purified CD4+ or CD8+ T cells alone did not result in reproducible hair loss. CD4+ and CD8+ T cells induced follicular expression of intercellular adhesion molecule 1 (CD54), HLA-DR, and HLA-A, HLA-B, and HLA-C after injection into scalp grafts.

The conclusions of the study are that CD4+ and CD8+ T cells have a role in the pathogenesis of AA. It is hypothesized that CD8+ T cells act as the effector cells, with CD4+ T cell help. It is now necessary to look for HLA-A, HLA-B, and HLA-C associations with AA. Therapeutic manipulations that interfere with CD8+ activity should be examined.

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PATIENTS AND METHODS

PATIENTS

Eleven patients with either alopecia totalis or severe alopecia areata were studied. Severe alopecia areata was defined as large areas of alopecia with small residual areas of hair. These patients were categorized as S4 (76%-90% hair loss) by the alopecia areata investigational assessment guidelines.17 Duration of alopecia areata, age, sex, and clinical characteristics are listed in Table 1. There were no other preselection criteria, and these 11 patients were not described previously in the literature. Informed consent was obtained after the nature and possible consequences of the studies were explained. Protocols were approved by the institutional review board (Technion Institute of Technology). None of the patients had any therapy in the 60 days before the scalp biopsy specimens were taken.

SKIN BIOPSY SPECIMENS

Nineteen 2-mm punch biopsy specimens from involved areas were obtained from each patient. Four biopsy specimens from each patient were used for isolation of autologous cutaneous T cells. Two biopsy samples were snap frozen in liquid nitrogen for immunoperoxidase staining. One frozen biopsy specimen was sectioned vertically, and the other was sectioned horizontally. An additional biopsy specimen from each patient was submitted for routine histological examination with hematoxylin-eosin and was horizontally sectioned in its entirety. Twelve punch biopsy specimens (2 mm) were grafted in sets of 3 or 4 onto SCID mice, depending on the number of treatment groups (3 or 4 mice per treatment group per patient) (Table 2).

ANIMALS

C.B-17/scid females obtained from Charles River Laboratories (Kent, England), and used at 2 to 3 months of age. These mice have the Prkdcscid mutation, which results in severe combined immunodeficiency (SCID). The mice were raised in the pathogen-free animal facility of the B. Rapaport Faculty of Medicine, Technion Institute of Technology. Animal care and research protocols were in accordance with institutional guidelines.

RESULTS

PHENOTYPE OF CULTURED SCALP T CELLS

T cells retrieved from the cultures were phenotyped by cytofluorographic analysis. Culture with hair follicle homogenate resulted in an increase in the proportion of CD8+ T cells from 6% to 89%. No increase in the proportion of CD8+ T cells was noted in the scalp T cells cultured with nonfollicular homogenate, suggesting that the hair follicle–associated autoantigen has a preferential stimulatory effect on CD8+ T cells.

CD4+ VS CD8+ T CELLS IN THE TRANSFER OF ALOPECIA AREATA TO HUMAN GRAFTS ON SCID MICE

Gilhar et al12 previously demonstrated the ability of human scalp T cells to transfer alopecia areata to autologous human lesional scalp grafts on SCID mice. Transfer requires activation of the scalp T cells by culture with hair follicle homogenate. This transfer system was used to investigate the relative roles of CD4+ and CD8+ T cells in alopecia areata.

SKIN GRAFTING

Graft transplantation to SCID mice was performed as described elsewhere.13,14 Each 2-mm graft was inserted through an incision in the skin into the subcutaneous tissue over the lateral thoracic cage of each mouse and was covered with a standard bandage. The dressing was removed on day 7.

ISOLATION OF T LYMPHOCYTES FROM SCALP PUNCH BIOPSY SPECIMENS

Four punch biopsy specimens from each patient were used for T-cell isolation. Tissue-infiltrating lymphocytes were isolated from scalp punch biopsy specimens using collagenase (Sigma-Aldrich Corp, St Louis, Mo), as described elsewhere.12 The average number of cells retrieved from each patient for each experiment after culture is listed in Table 2.

ISOLATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

Peripheral blood mononuclear cells were isolated from heparinized whole blood samples by centrifugation over Hypaque 1077 (Amersham Pharmacia Biotech, Uppsala, Sweden).

HAIR FOLLICLE HOMOGENATE PREPARATION AND NONFOLLICULAR SCALP HOMOGENATE

Anagen hair follicles were isolated as described elsewhere,12 under a stereodissecting microscope from normal scalp biopsy specimens obtained from healthy individuals who underwent plastic surgery procedures. Tissue was obtained from occipital scalp with no clinical evidence of male pattern alopecia. The protein content of each homogenate was assayed by a colorimetric assay.19 The samples were stored at −20°C and diluted with culture medium to a final protein concentration of 10 µg/mL.

After preparation of the hair follicle homogenerate by microdissection, the residual nonfolicular scalp tissue was homogenized and handled as described previously to generate a control nonfolicular scalp homogenerate.

Continued on next page
CULTURE OF T LYMPHOCYTES

Lymphocytes were plated at 1 x 10^5 cells/well in RPMI-FCS, along with irradiated (5000 rad [50 Gy]) peripheral blood mononuclear cells (1 x 10^6 cells/well) in 24 plate wells (Greiner of America, Lake Mary, Fla) as feeders. After 3 days of culture, recombinant interleukin 2 (IL-2) (Genzyme Diagnostics, San Carlos, Calif), 10 U/mL, was added. Every 5 days the T cells were restimulated with feeders, for a total culture time of 30 days. T cells were cultured for 40 days for the previous study. Lymphocytes were stimulated with follicular homogenate preparations using a modification of this protocol in which follicular homogenate (10 µg/mL) was added along with the feeder cells at each stimulation.

PHENOTYPIC CHARACTERIZATION OF CULTURED T LYMPHOCYTES

The phenotype of the cultured T-cell lines was determined by cytfluorographic analysis (FACSCalibur; Becton Dickinson, San Jose, Calif). The following monoclonal antibodies were used: anti-CD3 fluorescein isothiocyanate conjugated (clone SK 7; Becton Dickinson), anti-CD4 (immunoglobulin G [IgG] 2a, S3.5), anti-CD8 (IgG2a, 3B5) (Caltag Laboratories, Burlingame, Calif), and anti-HLA-DR (IgG2a, DK22) (Dako A/S, Glostrup, Denmark). Subtype-matched mouse IgG–fluorescein isothiocyanate conjugated (Becton Dickinson) was used as a negative control. When indicated, T cells were phenotyped by immunohistochemical analysis of cytospin preparations.

ISOLATION OF CD4+ AND CD8+ T LYMPHOCYTES FROM SCALP T-LYMPHOCYTE CULTURES

Magnetic beads conjugated with CD4 or CD8 (Dynal, Lake Success, NY) were used to isolate CD4+ and CD8+ T cells using the supplied protocol. As indicated in Table 2, for the first 4 experiments, either the CD4+ or CD8+ T cells would be isolated by positive selection and the reciprocal subset would be isolated by negative selection. Phenotyping of isolated T cells by immunohistochemical analysis of cytospin preparations indicated that the negatively selected population contained less than 2% of the positively selected population (CD8− cells contained less than 2% CD8+ cells, and CD4− cells contained less than 2% CD4+ cells). Negatively selected cells also were more than 95% pure for the reciprocal phenotype (CD4+ cells were 97% CD8− and CD8+ cells were 95% CD4−). Thus, the negatively selected populations were highly purified. However, the positively selected populations contained double-positive CD4+ and CD8+ T cells, with the reciprocal phenotype varying up to 26%. For this reason, all data are shown for negatively selected T cells. In the final experiment (3 donors), CD4+ and CD8+ T cells were isolated by negative selection to allow for mixing experiments and direct comparisons.

INJECTION OF CULTURED T LYMPHOCYTES INTO GRAFTS ON SCID MICE

The SCID mice bearing grafts of lesional scalp were divided into groups as indicated for each experiment. Between the 23rd and 30th days, the grafts were injected intradermally (0.1 mL) with autologous lymphocytes as noted for each experiment. On day 82 the mice were humanely killed and grafts were harvested for immunohistochemical and histological analyses. The percentage of grafts with hair and the number of hairs per graft were recorded. The details of each experiment, including number of T cells injected per graft, number of patients, number of mice, and recovery of T cells from culture, are given in Table 2.

IMMUNOHISTOCHEMICAL STAINING

Staining was performed as reported elsewhere. Monoclonal antibodies to human antigens used were as follows: anti-HLA-DR, anti-CD54 (ICAM-1) (Biosdesign Inc, Carmel, NY), anti-CD3, anti-CD4, anti-CD8, anti-CD25 (Dako A/S), and anti-HLA class 1 (Dako A/S). Each specimen was coded at the time of biopsy and was evaluated by 2 observers (A.G. and B.A.) who were masked to the coding.

STATISTICAL ANALYSIS

Statistical analysis was carried out using analysis of variance for multiple comparisons or the 2-tailed t test for single comparisons.

Three experimental protocols were used to confirm the validity of the results (Table 2). In 2 experiments (5 patients), CD8+ cells were isolated by negative selection. In the second protocol (3 patients), this was reversed, and CD4+ T cells were negatively selected to control for artifact introduced by the selection procedure. In the fifth experiment, both the CD4+ and CD8+ populations were purified by negative selection. Mixing experiments were also performed with CD4+ and CD8+ T cells combined at a 1:1 ratio.

Scalp grafts were placed in SCID mice and injected intradermally with cultured scalp T cells. Grafts with no injected T cells served as negative controls. The positive control for transfer of alopecia areata was injection of unseparated T cells, or mixed CD4+ and CD8+ T cells, cultured with hair follicle homogenate. Specificity of the response was controlled by injection of T cells cultured with nonfollicular homogenate. Experimental groups were injected with scalp CD4+ or CD8+ T cells cultured with hair follicle homogenate. The details of each experiment, including number of T cells injected per graft, number of patients, number of mice, and recovery of T cells from culture, are given in Table 2.

Culture of T cells with nonfollicular scalp homogenate did not induce loss of hair (experiment 1) (Figure 1), indicating that induction of hair loss had specificity for hair follicle–associated autoantigens. These T cells proliferated in the presence of IL-2, indicating a degree of activation, indicating that nonspecifically activated T cells do not induce hair loss.

Injection of scalp T cells cultured with hair follicle homogenate induced a significant decrease in the number of hairs per graft in all experiments. This was detected as a decrease in the number of hairs per graft and
as a decrease in the number of grafts with hair (in experiment 1, from 100%-33%). Histological analysis revealed that hair loss was associated with dystrophic anagen follicles and with an increase in catagen follicles. In all 5 experiments (11 donors), injection of a mixture of CD4+ and CD8+ cells or unfractionated T cells provided reproducible, significant hair loss (P < .01 by analysis of variance). Injection of purified CD8+ cells alone did not induce a significant decrease in hair growth in any of 3 experiments, with a slight, nonsignificant decrease in experiments 1 and 2. CD4+ cells alone induced significant hair loss in 1 of 3 experiments. The reproducible hair loss after injection of CD4+ plus CD8+ T cells supports a collaboration between CD4+ and CD8+ T cells in inducing hair loss.

**INDUCTION OF FOLLICULAR HLA-DR AND ICAM-1 (CD54) BY T-CELL INJECTIONS**

Concurrent with hair loss, the injected grafts demonstrated immunohistological findings seen with active alopecia areata, including expression of HLA-DR, ICAM-1, and HLA-A, HLA-B, and HLA-C (Figure 2). Expression of HLA-DR and ICAM-1 dropped to low levels in grafts not injected with T cells. Injection of scalp T cells incubated with hair follicle homogenate resulted in expression of both antigens in 50% of grafts. CD4+ and CD8+ T cells induced similar expression of ICAM-1 and HLA-DR, with optimal expression in the presence of unfractionated T cells (Figure 3). The ability of CD4+ T cells to induce ICAM-1 and HLA-DR supports a helper role for CD4+ cells. Interfollicular CD8+ T cells were found in grafts injected with CD8+ T cells (Figure 3), supporting an effector role for CD8+ T cells. Alopecia areata relapse can be transferred to human scalp explants by injection of autoantigen-activated autologous T cells. Evidence that this effect is specific for a hair follicle–associated autoantigen was provided by the finding that culture of T cells with nonfollicular scalp homogenate did not allow transfer of alopecia areata. The role of CD8+ and CD4+ T cells in this system was investigated by fractionation of the T cells before transfer. Unseparated T cells and mixed CD4+ plus CD8+ T cells induced significant hair loss in all experiments (5/5), with a total of 11 donors. CD4+ T cells alone induced hair loss in 1 of 3 experiments, whereas CD8+ T cells alone induced only marginal hair loss in 2 of 3 experiments. It is possible that the variability in the effects of CD4+ cells alone results from residual CD8+ T cells in the scalp grafts. These data indicate a requirement for cooperation between CD4+ and CD8+ T cells for optimal hair loss.

Because the T cells were activated in vitro before injection, it may be expected that CD8+ effector cells would induce hair loss without the need for CD4+ help. The finding that CD4+ and CD8+ T cells are required for optimal hair loss suggests that both T-cell subsets have a role in the induction of pathologic findings. CD4+ T cells are generally more effective at cytokine production. Interferon γ produced by CD4+ cells may facilitate the effects of CD8+ T cells by inducing HLA-A, HLA-B, and HLA-C as well as ICAM-1 (CD54) on follicular epithelium.

The observation that alopecia areata can be transferred by scalp T cells activated by hair follicle homogenate has proven to be highly reproducible. The effect was observed in this series of 11 patients and in the initial published series13 of 6 patients, for a total of 17 patients. The patients were not screened or selected for any criteria other than the clinical findings listed. The ability of T cells to transfer alopecia areata is specific for T cells activated by culture with hair follicle homogenate. The requirement for scalp T cells is believed to reflect a greater precursor frequency in lesional skin than peripheral blood. Scalp T cells incubated with nonfollicular homogenate plus IL-2 did not have this effect despite activation as evidenced by pro-

**Table 2. Experiment Details**

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<th>Experiment No.</th>
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*ND indicates not done.

**COMMENT**
liferation in response to IL-2. Scalp and peripheral blood T cells activated by IL-2 alone and peripheral blood T cells activated by phytohemagglutinin antigen were unable to transfer hair loss. Thus, transfer of hair loss is not a nonspecific effect of injection of activated T cells.

It was necessary to use lesional (bald) scalp for the explants because transplantation of noninvolved scalp, or scalp from healthy donors, results in loss of hair with suboptimal regrowth. Hair loss after grafting occurs with grafts of human scalp to humans (transplants) or mice. In the published experience of Gilhar and Krueger with grafts of human scalp to immunodeficient mice, the number of hairs regrowing from a 2-mm graft is insufficient for analysis, whereas there is adequate hair regrowth from grafts of involved alopecia scalp. For this reason, it was not possible to perform these experiments with noninvolved, or normal, scalp explants. This model thus represents relapse of alopecia areata in previously involved scalp rather than induction. This is an appropriate model for this condition, which is marked by multiple relapses. The experiments were designed so that T lymphocytes were always injected into autologous scalp explants, thereby ensuring major histocompatibility complex compatibility.

Evidence is accumulating for the role of CD8+ T cells in the pathogenesis of skin diseases as diverse as allergic contact dermatitis, drug eruptions, and psoriasis. There is additional support for an effector role for CD8+ T cells in the pathogenesis of alopecia areata. The intrafollicular T-cell infiltrate is predominantly composed of CD8+ T cells. Inflammatory intrafollicular T cells of alopecia areata are cytotoxic and possess both the Fas/Fas ligand and granzyme B cytotoxic mechanisms. In contrast, the perifollicular infiltrate is composed primarily of CD4+ T cells.

![Figure 1](image1.png)

Figure 1. Effects of injection of purified CD4+ or CD8+ T cells into autologous human scalp explants on Prkdcscid mice. Asterisk indicates P<.01 by t test, single comparison relative to control; dagger, P<.01 by analysis of variance, multiple comparisons. Error bars represent SD.

![Figure 2](image2.png)

Figure 2. Human lesional skin grafted to Prkdcscid mice. A, Control graft not injected with T cells (hematoxylin-eosin histological analysis). B, Graft injected with CD8+ T cells demonstrating lymphocytic infiltrate (hematoxylin-eosin histological analysis). C, CD4+ staining of a graft injected with CD4+ and CD8+ T cells demonstrating perifollicular infiltrate. D, Intrafollicular infiltrates of CD8+ T cells in a graft injected with CD8+ T cells. E, Control graft not injected with T cells stained for HLA-A, HLA-B, and HLA-C. The black pigment is melanin. F, Graft injected with CD8+ T cells demonstrating follicular HLA-A, HLA-B, and HLA-C. G, Graft injected with CD8+ T cells stained for HLA-DR. H, Graft injected with CD8+ T cells stained with intercellular adhesion molecule 1.

![Figure 3](image3.png)

Figure 3. Induction of follicular HLA-DR and intercellular adhesion molecule 1 (ICAM-1) after injection of CD8+ cells into human scalp explants. T cells were cultured with hair follicle homogenate and antigen-presenting cells.
Depletion of either CD8+ or CD4+ T cells can reverse alopecia areata in the Dundee experimental bald rat, indicating a synergy or cooperation between CD8+ and CD4+ T cells. One of the features of alopecia areata is aberrant expression of HLA-A, HLA-B, and HLA-C on the follicular epithelium of the hair bulb. Paus et al14 hypothesized that this expression of class I major histocompatibility complex allows an autoaggressive response by CD8+ T cells. The researchers suggested that the CD8+ cells cause the induction of major histocompatibility complex class II by the affected hair follicles, resulting in a second wave of CD4+ cells. Kalish et al35 previously reported the presence of CD4+ autoreactive T cells in the infiltrate of alopecia areata.

CD4+ T cells are well known to provide “help” for CD8+ T-cell activation and generation of cytotoxicity.36 This help is mediated in large part by cytokines such as IL-2. Memory CD45RO+ CD4+ T cells are most efficient in providing this help for alloantigen-specific CD8+ cytotoxicity.37 In addition to cytotoxicity, CD8+ T cells may induce pathologic effects by the secretion of cytokines.38 Lesional T cells inhibit proliferation of keratinocytes through production of cytokines, including interferon γ and tumor necrosis factor α.39 Alopecia areata has HLA associations with DQB1*03,40-44 as well as HLA-B1845 and possibly HLA-A2.44 Because HLA-DR,D,DP,DQ molecules present antigen to CD4+ T cells, and HLA-A, HLA-B, and HLA-C molecules present antigen to CD8+ T cells, the data on CD8+ T cells suggest that genetic linkage studies should be expanded to examine the role of HLA-A, HLA-B, and HLA-C molecules in alopecia areata.

The clinical relevance of CD8+ effector cells is that agents active against CD4+ cells, such as cyclosporine, may have less efficacy against CD8+ cells. If CD8+ helper cells are necessary, as the data suggest, then a CD4+ active agent may be effective alone. However, an ideal agent should also be effective against CD8+ cells. Some of the recombinant fusion proteins and humanized monoclonal antibodies may fit this profile, including antibodies that block antigen presentation or co-stimulation molecules, such as CTLA4Ig, and anti-CD11a. Possible therapies also include agents active against TH1 cytokines (eg, IL-2, interferon γ, and tumor necrosis factor α), or agents that promote TH2 responses (eg, IL-10).

In conclusion, it is possible to transfer alopecia areata to human scalp explants on SCID mice by the injection of scalp T cells. Reproducible, significant hair loss was observed only with a combination of CD4+ and CD8+ T cells, suggesting cooperation between these T cells. An effector role for CD8+ T cells was suggested but not proved. The evidence that CD8+ T cells are important to the pathogenesis of alopecia areata has several important implications. First, it is now necessary to look for HLA-A, HLA-B, and HLA-C associations with disease. Most significantly, therapeutic manipulations that interfere with CD8+ activity should be examined.

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REFERENCE


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News and Notes

The National Registry for Ichthyosis and Related Disorders is seeking enrollment of all patients with inherited disorders of keratinization (except ichthyosis vulgaris). Serum testing for X-linked recessive ichthyosis, as well as molecular diagnosis of selected disorders, is available without charge. We are eager to assist with research efforts, and we welcome proposals. Information and enrollment forms can be downloaded from our Web site. Please contact us to enroll your affected patients or discuss research interests. Phone: (800) 595-1265; e-mail: info@skinregistry.org; Web site: www.skinregistry.org.