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A Mouse Model of Clonal CD8+ T Lymphocyte-Mediated Alopecia Areata Progressing to Alopecia Universalis

Rajshshekar Alli,* Phuong Nguyen,* Kelli Boyd,*† John P. Sundberg,‡ and Terrence L. Geiger*

Alopecia areata (AA) presents as a typically patchy, non-scarring hair loss, although it may progress to total hair loss (alopecia universalis) (1). Lifetime incidence is greater than 1%. Murine models and human skin transplanted into NOD/SCID mice support an autoimmune, T cell-dependent cause in which a breakdown of immune privilege is followed by destruction of hair follicles (2, 3). Indeed, a case report has described the cure of a person with AA by allogeneic hematopoietic stem cell transplantation, supporting an immunologic origin (4). During AA, class I and class II MHC are upregulated, and CD8+ cell transplantation, supporting an immunologic origin (4). During the cure of a person with AA by allogeneic hematopoietic stem cell transplantation, supporting an immunologic origin (4). During AA, class I and class II MHC are upregulated, and CD8+ and CD4+ lymphocytes infiltrate and penetrate affected follicles (5). CD8+ T cells predominate within the follicular epithelium during active disease (6–8). It has been suggested, although not proved, that CD8+ T lymphocytes independently mediate the pathologic response (1). The Ag(s) responsible for AA are unknown. Preservation of amelanotic hairs in AA and a report that damage to hair bulb melanocytes preceded damage to hair follicle keratinocytes led to the suggestion that melanocytes are initial targets of autoreactive lymphocytes (1, 9). The absence of widespread melanocyte destruction during AA, however, suggests a more complex specificity relationship. Indeed, although AA is in rare cases associated with vitiligo, it is also associated with other autoimmune diseases and most commonly presents as an isolated condition (10, 11). Furthermore, in the C3H/HeJ mouse model of disease, white hairs, created by localized freeze branding, were not spared (12).

The C3H/HeJ mouse is the primary animal model of spontaneous AA (2). Female mice greater than 6 mo of age have a low frequency (<1%) of a spontaneous AA-like disease, though this can reach 20% by 12 mo of age (13, 14). Older DEBR rats similarly develop AA, although at a higher frequency (15). In each case, CD4+ and CD8+ T cells are required for disease, although the ultimate effectors of follicular damage have not been conclusively identified. Low frequency and late disease onset in these systems are significant impediments to studies of disease cause and pathogenesis.

We describe a new model for AA in which clonal C57BL/6J-derived CD8+ T lymphocytes independently mediate follicular destruction. While evaluating the TCR expressed by a myelin-specific T cell, we identified a dual TCR, one of which guides specificity against a myelin Ag. Surprisingly, the second directed CD8+ T cells not against myelin, but selectively against hair follicles. Retroviral transgenic (retrogenic) mice on a Rag1tm1Mom (Rag1−/−) background, in which T cells exclusively express this TCR, develop alopecia at ∼6–7 wk, with a nearly 100% incidence by 4 mo. Alopecia mimics that seen in patients with reticular AA and ultimately progresses to alopecia universalis.

Materials and Methods

Mice

C57BL/6J (B6), B6.129P2-R2m1Wmc/J (B2m−/−), and B6.129S7-Rag1tm1Mom/J (Rag1−/−) mice were obtained from The Jackson Laboratory...
and bred under specific pathogen-free conditions, including all detectable *Helicobacter* spp. B6.129S-H2*Abd-a1* (Ab−/−) mice were provided by Dr. P. Doherty (St. Jude Children’s Hospital, Memphis, TN). Experiments were performed in accordance with institutional animal care and use procedures.

**Media, reagents, Abs, and flow cytometry**

Cells were cultured in Eagle’s–Hank’s Amino Acid Medium (BioSource) supplemented with 10% heat-inactivated Premium FCS (BioWhittaker), penicillin G (100 U/ml), streptomycin (100 μg/ml), L-glutamine 292 μg/ml (Invitrogen Life Technologies), and 50 μg/ml 2-mercaptoethanol (Fisher Biotech). mAbs specific for mouse CD4 (clone L3T4), CD8 (clone 53-6.7), CD25 (clone 7D4), TCRB (clone H57-597), CD69 (clone H1.2F3), CD45RB (clone 16A), CD44 (clone IM7), CD3 (clone 145-2c11), and CD28 (clone 37.51) were obtained from BD Biosciences. mAbs against mouse IL-10R (clone 1B1.3A), IFNG (clone XMG1.2), TNFA (clone XT3.11), and polyclonal rat IgG (cat. no. BE0094) were purchased from BioXcell (West Lebanon, NH). Anti-mouse IL-17 (clone 50104) was purchased from R&D Systems (Minneapolis, MN). Flow cytometry was performed on a FACSCalibur (BD Biosciences) and flow cytometric sorting on a MoFlo high-speed sorter (DakoCyto- mation).

**TCR cloning**

The 1MOG244.1 TCR was cloned from the 1MOG244 hybridoma as described (16). TCRα and TCRβ cDNA separated by a 2A sequence was cloned into a variant of the murine stem cell virus (MSCV)-driven MSCV-I-GFP retroviral vector that replaces GFP with cyan fluorescent protein (CFP) (17). The OT-1 TCR was provided by Dr. D. Vignali (St. Jude Children’s Research Hospital, Memphis, TN).

**Retroviral transduction of TCR**

Retrovirus was produced as described (16, 18). Briefly, 10 μg each of retroviral receptor and helper DNA constructs were cotransfected into 293 T cells using calcium phosphate precipitation, and the cells were incubated in DMEM with 10% FCS for 48 h. Supernatant was then collected twice daily and used to infect GP+E86 retroviral producer cells in the presence of 8 μg/ml polybrene. Transduced GP+E86 cells were flow-cytometrically sorted for the presence of CFP and cell-free supernatant used for transduction.

**Generation of retrogenic mice**

Retrogenic mice were generated as described (18). Briefly, bone marrow cells were harvested from the femurs of Rag1−/− mice 48 h after the administration of 0.15 mg 5-fluorouracil per gram of body weight. The pooled cells were cultured in complex Eagle’s–Hank’s Amino Acid Medium containing 20% FCS supplemented with IL-3 (20 ng/ml), IL-6 (50 ng/ml), and stem cell factor (50 ng/ml) for 48 h at 37°C in 5% CO2. The cells were then cocultured for an additional 48 h with 1200 rad irradiated HPCs, then transplanted into 450 rad-irradiated B6 mice. Hair loss was monitored and scored as follows: 0, <5% hair loss from total body surface area; 1, 5–20%; 2, 20–40%; 3, 40–60%; 4, 60–80%; 5, >80%. For adoptive transfer disease, CD8+ T cells were isolated by magnetic bead selection (MACS; Miltenyi Biotec), and the indicated concentrations of anti-CD3 and 1 μg/ml anti-CD28, pulsed with 1 μCi [3H]thymidine after 72 h, and then harvested for scintillation counting. Samples were analyzed in triplicate.

**Cytokine analysis**

Primary CD8+ T cells (5 × 10^6) were isolated and stimulated as described earlier. Culture supernatants were collected at 24 or 48 h and analyzed for IL-2, IL-4, IFNG, and IL-17 by Bio-Plex assay (Bio-Rad).

**Real-time PCR**

Skin specimens were taken from 1MOG244.1 mice with alopecia or control B6 mice. Hair was shaved prior to specimen removal. For 1MOG244.1 mice without alopecia universalis, specimens were taken at the edges of active lesions. Skin samples were flash frozen in liquid nitrogen and then homogenized in TRIZol (Invitrogen) using a 19-gauge needle. RNA was extracted using Phase Lock gel (Eppendorf) and the RNAeasy Mini Kit (Qiagen) following the manufacturers’ protocols. First-strand cDNA was synthesized using the Superscript III RT-PCR kit (Invitrogen). IL-2, IL-10, IL-17, IFNG, and TNFA were quantified as described after 45 cycles of PCR (19). As a positive control, RNA was isolated from B6 spleenocytes activated with ConA and 10 U/ml rhIL-2 for 48 h. Cytokine Ct data were compared with GAPDH as an internal control, and this was normalized to Ct data from simultaneously analyzed activated spleenocytes using the comparative Ct approach (2−ΔΔCt).

**Ab treatment**

Abs (250 μg/mouse) against mouse (m)IL-10R, mIFNG, mTNFA, mIL-17, and polyclonal rat IgG were administered i.p. to five to eight mice per group beginning 7 wk after HPC transplantation and prior to the onset of disease symptoms. Administration was biweekly for 5 wk. The treatment conditions with mIFNG and mTNFA Abs were validated by demonstrating that serum from treated mice (3 d postdosing) could fully deplete the respective cytokine in vitro after protein G clearance. For mIL-10R Ab, dosing was validated by demonstrating that serum acquired from similarly treated mice could specifically stain mouse macrophage at a 1:50 dilution. The mIL-17 Ab was dosed based on conditions previously described (20–22).

**Results**

**Production of 1MOG244.1 retrogenic mice**

We identified two productively rearranged TCRA chains within the 1MOG244 T cell hybridoma. One TCRA, when transduced with the second TCRA chain, we generated retrogenic mice selecting for tolerance (23). To analyze functionally TCR expression, we used two monoclonal antibodies recognizing the different chains of the 1MOG244 T cell hybridoma. One TCRA, when transduced with the second TCRA chain, we generated retrogenic mice selecting for tolerance (23). To analyze functionally TCR expression, we used two monoclonal antibodies recognizing the different chains of the 1MOG244 T cell hybridoma.

**Alopecia areata**

Cohorts of retrogenic mice were followed for 6 mo or as indicated, and clinical signs of alopecia and body weight were measured three times per week. Hair loss was monitored and scored as follows: 0, <5% hair loss from total body surface area; 1, 5–20%; 2, 20–40%; 3, 40–60%; 4, 60–80%; 5, >80%. For adoptive transfer disease, CD8+ T cells were isolated by magnetic bead selection (MACS; Miltenyi Biotec), and the indicated number of cells were transferred intravenously into unmanipulated Rag1−/− mice, 450 rad-irradiated B6 mice, or unirradiated B6 mice. Alternatively, 10 × 10^6 total spleenocytes (~1.5 × 10^7 T lymphocytes) were transferred into 450 rad-irradiated B6 β2m−/− or Aβ−/− mice.

**Histology**

Tissues were harvested and fixed overnight in Formalin’s solution, processed routinely, embedded in paraffin, and sectioned at 4 μm. Gram stains and periodic acid–Schiff stains were performed to rule out bacterial and fungal infection, respectively. Immunohistochemical labeling was performed on a Lab Vision autostainer. Complete necropsies were performed on two mice from each group to identify concurrent lesions.

**Cell proliferation**

Spleen or lymph node (LN) cells were isolated from retrogenic or B6 mice, and CD8+ T cells were purified using PE anti-CD8 Ab (clone 53-6.7) and anti-PE–coated microbeads (MACS; Miltenyi Biotec). Cells were cultured at 5 × 10^5 per well in 96-well plates with 3 × 10^5 irradiated syngeneic APCs and the indicated concentrations of anti-CD3 and 1 μg/ml anti-CD28, pulsed with 1 μCi [3H]thymidine after 72 h, and then harvested for scintillation counting. Samples were analyzed in triplicate.

**Ab treatment**

Abs (250 μg/mouse) against mouse (m)IL-10R, mIFNG, mTNFA, mIL-17, and polyclonal rat IgG were administered i.p. to five to eight mice per group beginning 7 wk after HPC transplantation and prior to the onset of disease symptoms. Administration was biweekly for 5 wk. The treatment conditions with mIFNG and mTNFA Abs were validated by demonstrating that serum from treated mice (3 d postdosing) could fully deplete the respective cytokine in vitro after protein G clearance. For mIL-10R Ab, dosing was validated by demonstrating that serum acquired from similarly treated mice could specifically stain mouse macrophage at a 1:50 dilution. The mIL-17 Ab was dosed based on conditions previously described (20–22).

**Results**

**Production of 1MOG244.1 retrogenic mice**

We identified two productively rearranged TCRA chains within the 1MOG244 T cell hybridoma. One TCRA, when transduced with the single rearranged TRBV13-2∗ TRBI2-7∗ TCRβ into T cells, conferred specificity for the immunizing Ag, myelin oligodendrocyte glycoprotein (16). The second, a TRAV8D-2∗ TRAJ37∗ TCRA, did not. TCRA chain allelic exclusion is not robust, and dual TCR expression has been hypothesized to facilitate T cell escape from tolerance (23). To analyze functionally TCR expressing the second TCRA chain, we generated retrogenic mice selectively expressing it. The TCRA and B chains, denoted as 1MOG244.1, were introduced into a retroviral vector separated by the *Thosea asigna* virus 2A sequence (Fig. 1). The 2A allows for stoichiometric translation of both TCR chains from a single cistron (24). The construct was transduced into B6 Rag1−/− HPCs, which were then transplanted into Rag1−/− mice.

**Development of alopecia in 1MOG244.1 mice**

1MOG244.1 mice showed no gross abnormalities until ∼6–7 wk after HPC transplant, when individuals began experiencing hair loss. At 2 mo, greater than 50% of mice developed alopecia, and by 4 mo virtually all had developed alopecia (Fig. 2A). Hair loss did not result from barbering, as a similar incidence of disease was observed in mice housed individually (data not shown). Neither was the alopecia a nonspecific consequence of the retrogenesis.

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This phenotype has not been observed with other class I- or class II-restricted TCRs produced in our or other laboratories (16, 25–27). Further, mice retrogenic for the OA-specific, class I MHC-restricted OT-1 TCR (28) produced simultaneously and co-housed with a cohort of 1MOG244.1 mice did not develop alopecia (Fig. 2A). The hair loss additionally did not accompany other clinical manifestations of illness. The weight of 1MOG244.1 and control OT-1 mice did not significantly differ over a 120-d observation period (Fig. 2B), and no abnormalities were detected other than those affecting the skin in 1MOG244.1 mice by necropsy and histologic analysis (data not shown). Therefore, the 1MOG244.1 TCR transgene provokes alopecia in the normally AA-resistant B6 mouse strain. Disease is spontaneous in that it does not require any intervention in the 1MOG244.1 mice for its initiation.

1MOG244.1 retrogenic hair loss was patchy, most typically with a clear delineation of alopecic and normal areas. Alopecia was not linearly progressive; measurements of the extent of hair loss in individual mice demonstrated a waxing and waning pattern of disease in most animals (Fig. 3A). Furthermore, similar to the reticular pattern of AA in humans, different hair patches experienced hair loss and regrowth independently (Fig. 3B); simultaneous loss and regrowth in different regions was often apparent. This suggests that local skin factors, rather than a more generalized state of immune responsiveness, was responsible for the regional periodicity. All mice in a cohort of 20, followed until they developed complete hair loss or died, progressed to alopecia universalis. A median of three cycles of hair loss and regrowth, as defined by a change in overall disease score $\geq 1$, was seen (Table I). Therefore, 1MOG244.1 retrogenic mice develop a disease that resembles reticular AA progressing to alopecia universalis.

**Spontaneous T cell activation in 1MOG244.1 mice**

We next examined T lymphocyte engraftment in 1MOG244.1 mice. T cells were predominantly CD8+, with few CD4+ or coreceptor negative cells (Fig. 4A). Good splenic engraftment was seen in mice prior to the onset of disease, and T cell numbers were increased in age-matched (18.7 ± 1.6 wk posttransplant, score 0–1; 18.5 ± 1.6 wk, score 3–5) alopecic mice with more advanced disease (Fig. 4B; 3.8 ± 1.5 $\times 10^6$ cells, score 0–1; 6.9 ± 2.2 $\times 10^6$ cells, score 3–5). An increase in T cell blasts, determined as the percent of cells with increased forward and side scatter measurements, was also seen in 1MOG244.1 mice with more advanced disease compared with those with no or early disease and with simultaneously produced OT-1 mice (Fig. 4C; 16.5 ± 5.9%, score 0–1; 50.1 ± 9.7%, score 3–5; Supplemental Fig. 1A). 1MOG244.1 AA was associated with manifest adenopathy and splenomegaly compared with control OT-1 or C57BL/6 mice (Fig. 5).
metrically determined forward and side scatter, are plotted. These cells that are blasted (athy and splenomegaly in diseased 1MOG244.1 animals. 3), age-matched OT-1 mouse, and C57BL/6 mouse indicates adenopathy and splenomegaly in diseased 1MOG244.1 animals. 4D), and moderately diminished TCR expression was frequently apparent on T cells from diseased animals (data not shown). Cumulatively, these results indicate spontaneous activation of T cells in 1MOG244.1 mice with the development of AA. The association of T cell activation with disease development was confirmed by immunophenotyping. A dramatic increase of splenic T cells expressing the CD25 and CD69 activation markers was seen in concert with the progression of alopecia (Fig. 5). Disease was likewise associated with markedly increased proportions of cells with a memory/activated phenotype as defined by diminished levels of CD45Rb and CD62L and increased CD44 compared with C57BL/6 T cells. The percentage of memory, CD69+, and CD25+

### Table I. Development of AA in 1MOG244.1 mice

<table>
<thead>
<tr>
<th>Retrogenic TCR</th>
<th>Mice (n)</th>
<th>Mean</th>
<th>Median</th>
<th>Range</th>
<th>Mean</th>
<th>Median</th>
<th>Range</th>
<th>Mean</th>
<th>Median</th>
<th>Range</th>
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<td>1MOG244.1</td>
<td>20</td>
<td>58 ± 16</td>
<td>53</td>
<td>38–118</td>
<td>139 ± 4</td>
<td>140</td>
<td>132–143</td>
<td>2.7 ± 1.1</td>
<td>3</td>
<td>0–5</td>
<td>5 ± 0</td>
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<tr>
<td>OT-1</td>
<td>10</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<td>NA</td>
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</tr>
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The indicated numbers of 1MOG244.1 or control OT-1 TCR retrogenic mice were monitored for clinical signs of alopecia. Time to initial disease, to alopecia universalis, and the number of disease relapses, indicated by a change in disease score ≥1, are listed. AA was not observed in the OT-1 mice.

NA, not applicable.

The association of T cell activation with disease development was confirmed by immunophenotyping. A dramatic increase of splenic T cells expressing the CD25 and CD69 activation markers was seen in concert with the progression of alopecia (Supplemental Fig. 1B, 1C, p < 0.01 for each). Notably, in individual mice, T cell activation measured by CD69 expression was elevated in skin-draining LN compared with that in paired spleens, implying that T cell priming is more pronounced at these locations (Fig. 6, p < 0.05). Therefore, the 1MOG244.1 TCR guides T cells toward the CD8 lineage. These T cells are spontaneously and specifically activated in retrogenic mice and differentiate into memory cells in association with the development of alopecia.

To determine whether the AA was associated with elevated expression of skin-homing markers, we analyzed expression of CD162 and CCR10. However, CD162 expression was not altered relative to that observed on B6 splenocytes, and CCR10 was similar or modestly diminished (data not shown). This suggests that the spontaneous activation of 1MOG244.1 T cells is not associated with the acquisition of a phenotype biasing the cells toward skin-specific homing.

### Histopathology of 1MOG244.1 mice

Skin sections from mice affected by the cyclical alopecia had classical features of AA. Characteristics of cicatricial alopecia that can normally be found in B6 mice were also seen (29), although the microscopic lesions of AA predominated. Lymphocyte infiltrates were observed in and around anagen and late-catagen stage hair follicles, extending from the bulge downward to just above the hair bulb (Fig. 7A and not shown). Analysis of diseased skin, however, failed to correlate disease presence or magnitude with the hair cycle phase of residual follicles (data not shown). Intrafollicular mononuclear cells expressing CD3 were readily detected, demonstrating that retrogenic T cells had infiltrated the follicles (Fig. 7B). In the dermis, just adjacent to the hair follicles, lymphocytic infiltrates were mixed with macrophages and a few neutrophils (Fig. 7 and data not shown). Small, fragmented, condensed, and membrane-bound nuclear material consistent with apoptosis was a common finding in the affected follicular epithelium. Apoptosis was confirmed in these cells by positive labeling with a cleaved caspase 3 Ab (data not shown). Special stains for bacteria and fungi were consistently negative in all sections of skin examined, indicating that the response did not result from secondary infections (data not shown).

The progression through multiple hair cycles from patchy alopecia to alopecia universalis was correlated to the microscopic findings. In early hair cycles in haired skin adjacent to alopecic areas, swarms of lymphocytes were present in and around intact hair follicles as described earlier. As the disease and follicular destruction progressed over time, the density of hair follicles declined, and the absence of intact follicles became more prominent. In mice with alopecia universalis, the alopecic areas were devoid of follicles with only remnant clusters of hair bulbs in the deep dermis and few remaining lymphocytes in the dermal tissue.

With lesion progression, superficial fibrous connective tissue organized parallel to the epidermis at the dermal–epidermal junc-
tion, a sequela of the B6 alopecia and dermatitis complicating this model. In areas interpreted as containing long-standing injury, there were stretches of dermal tissue in which hair follicles were absent. Remnant follicular structures consisted of mild linear fibrosis, dermal papilla, and small clusters of melanocytes. In summary, histopathology was consistent with severe AA together with the sequelae of concurrent cicatricial alopecia and dermatitis previously described in B6 mice (29).

Class I MHC dependence of T cell response
To establish better the independent role of T cells in alopecia development, we determined whether they could transfer disease to unaffected mice. Splenic CD8+ T cells were isolated from mice with intermediate hair loss (disease score 3), and doses of $7.5 \times 10^5$ or $1.5 \times 10^6$ T cells were transferred i.v. into otherwise unmanipulated Rag1-/- mice (Fig. 8A). All mice developed AA, and time to initial disease was dose dependent (low dose, 63 ± 6 d; high dose, 43 ± 2 d; $p = 0.001$). All mice also eventually progressed to alopecia universalis, and time to this was likewise dose dependent (low dose, 133 ± 17 d; high dose, 108 ± 2 d; $p = 0.03$).

Lymphocytopenia may promote T cell survival and homeostatic expansion. To examine whether the lymphocytopenia in the Rag1-/- recipients played a role in disease transfer, isolated CD8+ T cells were transferred into either unirradiated (1 x $10^5$ or 10 x $10^6$ doses) or control sublethally irradiated (1 x $10^5$ dose) C57BL/6 recipients. Whereas the irradiated recipients showed a disease incidence similar to that observed after transfer into the Rag1-/- mice, the unirradiated recipients did not achieve a minimal score of 1. However, by 20–40 d after transfer, these mice manifested substantial hair shedding with routine manipulation, something not observed in control mice. The incidence and time course of this shedding was T-cell dose dependent (Supplemental Fig. 2).

Therefore, CD8+ T cells can transfer AA into lymphopenic recipients. However, disease penetrance and severity is markedly attenuated.

Considering that the T cells in 1MOG244.1 mice were predominantly CD8+, we hypothesized that the alopecia would be class I MHC dependent. To test this, we transferred ~1.5 x $10^6$ 1MOG244.1 T cells into sublethally irradiated class I MHC-deficient b2m-/- or class II MHC-deficient IAb-/- mice. Whereas 7 of 8 IAb-/- mice developed alopecia, 0 of 8 b2m-/- recipients did (Fig. 8B). Therefore, an exclusively class I MHC-restricted response is adequate for alopecia development after transfer; class II MHC is not required. Notably, in humans and C3H/HeJ mice, the T cell infiltrate colocalizes with aberrant upregulation of class I MHC (30, 31), consistent with these observations.

Promiscuous cytokine production by 1MOG244.1 T cells
To better delineate the effector potential of the spontaneously activated 1MOG244.1 T cells, we analyzed their proliferative response to stimulation with CD3- and CD28-specific Abs. Similar or only mildly diminished proliferative responses compared with those of control B6 CD8+ T cells were seen regardless of disease
Enhancements in IL-17 (score = 5) were associated with additional dramatic cytokine production. Increased cytokine production was observed. An effector response is detected in 1MOG244.1 and control mice. Therefore, even prior to clinical disease development, different T cells produced detectable quantities of IL-4 or IL-10 (data not shown). However, moderate quantities of IL-17 were produced by cells from mice with the indicated disease scores. Representative data from a total of 24 analyzed 1MOG244.1 skin sections is shown.

In contrast to the proliferative responses, 1MOG244.1 cytokine production differed substantially from that of control CD8+ B6 T cells. IL-2 and IFNG generation by 1MOG244.1 T cells from mice without disease was modestly elevated compared with that from control cells (Fig. 10A). Neither 1MOG244.1 nor control cells produced detectable quantities of IL-4 or IL-10 (data not shown). However, moderate quantities of IL-17 were produced by 1MOG244.1 T cells, a cytokine not detected from the B6 T cells. Therefore, even prior to clinical disease development, different T effector responses are detected in 1MOG244.1 and control mice.

In mice with intermediate disease (score = 2), dramatically increased cytokine production was observed. An ~3-fold increase in IL-2 production was accompanied by an ~7-fold increase in IL-17 and ~20-fold increase in IFNG production. Further, low but detectable levels of IL-4 and IL-10 were seen. Progression to severe disease (score = 5) was associated with additional dramatic enhancements in IL-17 (>10-fold), IL-4 (>100-fold), and IL-10 (>10-fold) production compared with T cells from mice with score 2 disease. The enhanced and promiscuous cytokine production of the 1MOG244.1 mice was not a consequence of retrogenesis. Direct comparison of T cells from score 3 1MOG244.1 and simultaneously produced OT-1 mice demonstrated markedly increased production of all cytokines by the 1MOG244.1 T cells (Supplemental Fig. 3). Therefore, the cytokine profile of 1MOG244.1 T cells varies both quantitatively and qualitatively with advancing disease.

To assess for cytokine production in target tissue, RNA was isolated from skin specimens of 1MOG244.1 mice with alopecia or from control B6 mice. TNFA, IFNG, IL-17, and IL-10 were analyzed by quantitative PCR and normalized to levels present in ConA-stimulated B6 splenocytes (Fig. 10B). Production of single cytokines varied substantially between individual specimens, with often several log differences in quantities.

With the exception of positive testing for IFNG in 1 of 4 B6 skin samples, none of the cytokines were detectable in the control specimens. Significant quantities of TNFA, IL-17, and IL-10 were observed in mice with disease scores of 1–3. IL-17 and IL-10 production remained elevated in mice with alopecia universalis (score = 5), although TNFA production was diminished, with 2 of 4 mice showing no detectable cytokine. This is consistent with the presence of active inflammation and a regulatory response in the skin of mice with active disease.

To determine the role of individual cytokines, a single large cohort of 1MOG244.1 retrogenic mice was segregated into subgroups that were treated with neutralizing Abs against TNFA (n = 7), IL-17 (n = 5), or IFNG (n = 7), blocking IL-10R Ab (n = 7), or an Ab control (n = 8). Treatment began at 7 wk, at which time alopecia was first detected in the population, and continued for 5

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**FIGURE 7.** Clinical appearance and histopathology of 1MOG244.1 mice. A, Skin sections (boxes) were isolated from mice with the indicated alopecia scores and a clinically unaffected control B6 mouse with follicular dystrophy unrelated to alopecia areata. H&E-stained paraffin sections of representative fields at low magnification (x20) and high magnification (x50; enlargement of boxed area) are shown. B, Immunohistochemistry with T-specific CD3 Ab (brown) of skin sections from mice with the indicated disease scores. Representative data from a total of 24 analyzed 1MOG244.1 skin sections is shown.

**FIGURE 8.** Adoptive transfer of AA by 1MOG244.1 T cells. A, The indicated numbers of CD8+ 1MOG244.1 T cells from mice with alopecia were adoptively transferred i.v. into unmanipulated Rag1−/− mice and recipients monitored for the development of alopecia. A Kaplan–Meier analysis of disease-free survival is shown. n = 5 per group. Data are representative of two experiments. B, Disease-free survival of semilethally irradiated (450 rad) B2m−/− and IAb−/− mice after transfer of splenocytes including ~1.5 × 10^6 T cells from 1MOG244.1 mice with alopecia. Data are pooled from two experiments (n = 8 per group).
wk. The index diseased mice used to identify first symptoms in the population were excluded from treatments and further analyses. Mice were monitored for disease incidence and severity in a blinded manner, and mice from the different treatment regimens were co-housed in individual pens to minimize environmental effects. Kaplan–Meier and area-under-the-curve analyses failed to find significant differences between any of the groups (data not shown). This suggests that either these cytokines primarily exert their effects prior to alopecia development or that they do not play strong independent roles in alopecia progression and that redundant pathways are available.

Discussion

AA is a chronic, highly prevalent disfiguring condition, which an abundance of data now indicates is primarily mediated by auto-reactive T lymphocytes. Dissecting disease pathogenesis has been hampered by the limitations in the animal model systems available. Both the C3H/HeJ mouse and DEBR rat model systems are characterized by low-incidence spontaneous disease first developing late in life. Although adoptive transfer of lymphocytes or skin grafting can transfer disease, understanding the pathogenesis of incipient spontaneous alopecia requires analysis of de novo disease development. We characterize a new high-incidence retrogenic model for AA. It is to our knowledge the first described TCR transgenic AA model system and the first in the B6 mouse strain.

In both humans and rodents, AA is associated with the infiltration of both CD4+ and CD8+ T cells in the hair follicle, though CD8+ cells more specifically localize to the intrafollicular region (6–8). The relative roles of these T cell subsets are not fully elucidated. McElwee and colleagues (33) found that s.c. injected CD8+ T cells from alopecic mice promoted local disease at the injection site at high incidence, whereas CD4+ T cells could provoke a more generalized alopecia although at lower incidence. This study differed from ours in mouse strain (C3H versus B6) and

FIGURE 9. Proliferative response of 1MOG244.1 T cells. CD8+ T lymphocytes were purified by magnetic bead selection from 1MOG244.1 mice or control B6 mice and identical numbers stimulated with varied concentrations of anti-CD3 and 1 μg/ml anti-CD28 in the presence or absence of 10 U/ml rhIL-2. Proliferation was measured by [3H]thymidine incorporation at 72 h. Data shown are representative from separate experiments assessing mice with disease scores of 0 (A), 3 (B), or 5 (C).

FIGURE 10. Cytokine production by 1MOG244.1 T cells and in 1MOG244.1 skin. A, CD8+ T lymphocytes from 1MOG244.1 or control B6 mice were purified and stimulated as in Fig. 9. At 48 h, cell-free cytokine was collected and concentrations of the indicated cytokines measured. B, RT-PCR was performed on skin specimens from 1MOG244.1 mice with alopecia (scores 1–3 or 5) or B6 controls. Cytokine levels were compared with simultaneous quantitative PCR performed on B6 splenocytes stimulated with ConA for 48 h, and relative quantity was calculated using the comparative Ct approach. Circles indicate data from individual mice, and bars indicate group averages. *p < 0.05 (by Kruskal–Wallis test using Dunn’s multiple comparison test).
route of cell administration (s.c. versus i.v.), potentially explaining differences in outcome. The localized reaction in that study may have resulted from immune exhaustion in the transferred cell population or a failure to migrate from the skin, effects obviated by our transfers to lymphopenic mice, which will promote cell survival and homeostatic expansion, and i.v. transfer, which will promote dissemination of the transferred cells. In contrast, in studies of human scalp explants, cooperation between CD4+ and CD8+ T cells was required for reproducible hair loss (34). Likewise, depletion of CD4+ or CD8+ T cells with mAb proved transiently therapeutic in DEBR rats (35, 36). With development of alopecia, MHC class I and II is upregulated on follicular epithelium, potentially supporting both CD4+ and CD8+ T cell responses (3).

One possibility for CD4+ T cells is to provide help for the formation of pathologic CD8+ T cells (37). A key finding of our analysis is that class I MHC-restricted T cells are capable of independently mediating alopecia development and progression. Few CD4+ T cells are present in 1MOG244.1 retrogenic mice (Fig. 4A). Further, purified adoptively transferred CD8+ T cells mediated disease after transfer into Rag1−/− mice but failed to induce alopecia in class I MHC-deficient B2m−/− recipients. In contrast, class II MHC was not required for disease development after adoptive transfer. This implies that class I MHC-restricted 1MOG244.1 CD8+ T cells are capable of independently producing effector responses necessary for AA development and full progression. An alternative possibility is that the CD4+ T cells provided help to the CD8+ cells prior to transfer. We consider this improbable, as it would seem unlikely that the very small number of these cells present would be effectively stimulated by a class I-restricted autoantigen in the absence of a CD8 coreceptor. More likely, the relatively high numbers of monoclonal 1MOG244.1 CD8+ T cells bypasses requirements for CD4+ T cell help, as has been observed elsewhere (38). Indeed, alopecia was only weakly induced after transfer into wild-type recipients, contrasting with the more severe disease after transfers into Rag1−/− or sublethally irradiated mice. Disease in these wild-type recipients was regional and associated with infiltrates of CD3+ cells. Potentially, the homeostatic stimulus of the lymphodeplete microenvironment helps sustain the 1MOG244.1 T cells and compensates for the absence of CD4+ T cell help. The development of model systems assessing monospecific CD4+ T cell responses, similar to that in this study with CD8+ cells, will assist in fully evaluating the role of Th cells. Notably, unlike CD8+ T cells, both regulatory and effector functions have been ascribed to CD4+ T cells in AA (39, 40).

The effector pathways used by the 1MOG244.1 T cells remain to be defined. Several cytokines have been implicated in AA pathogenesis. TNFA affects skin in multiple ways, and polymorphisms in it have been associated with AA (41). Notably, despite being generally proinflammatory, blockade of TNFA is not only ineffective in AA but also has been associated in case reports with AA development (42–45). Biologically, TNFA has been shown to block the IFN-mediated MHC upregulation in hair follicles that promotes AA (3, 46). A pathologic role for IFNG is more established in model systems, and the effector T cell response in AA appears primarily Th1/Tc1 (32, 47). Ifng−/− C3H/HeJ mice were found to be resistant to AA (48). IFNG treatment was also associated with enhanced disease in one study (49) and not a second (50). It was therefore curious that relatively small amounts of IFNG were observed in skin sections in 1MOG244.1 mice (Fig. 10B). However, this may reflect the limited number of T cells present within the skin mass, which may serve as the primary source of the cytokine. Indeed, similar findings were present in a cross-sectional transcriptome study done at 2-wk intervals of the C3H/HeJ skin graft model (32) and confirmed in a more recent transcriptome study at 5-wk intervals (J.P. Sundberg, unpublished data). IL-17 is prominently induced in the 1MOG 244.1 T cells during alopecia development, and though not well studied in the context of AA, its significance in other inflammatory autoimmune conditions is well recognized. IL-10 is widely considered to play a regulatory role in AA and was found to be upregulated in C3H/HeJ mice that were resistant to AA through grafting of alopecic skin (40). However, C3H AA lesional skin grafts placed on IL10−/− mice paradoxically also showed decreased transfer of disease compared with wild-type controls (51). Therefore, these cytokines have complex roles in AA, with seemingly mixed pro- and anti-inflammatory activities.

mAb inhibition of TNFA, IFNG, IL-17, and IL-10 in 1MOG244.1 mice did not lead to significant differences in disease incidence or severity compared with controls, suggesting that they do not play essential roles in the CD8+ T cell AA response. This interpretation must be tempered, however, by the fact that mAb inhibition was performed for a period of just 5 wk beginning at the time of anticipated clinical disease development, and it is possible that critical cytokine actions occur earlier than this. It is also possible that functional redundancy among cytokines in this model permits normal disease development despite blockade of individual cytokines. The generation of 1MOG244.1 retrogens in Rag2−/− mice bred for select cytokine deficiencies will be important to evaluate further the role of individual cytokines.

Importantly, no Ag has been identified that is recognized by AA-associated T cells. The 1MOG244.1 T cell clone independently mediates AA and may therefore serve as a tool for Ag screening. The loss of immune privilege in the hair follicle in association with AA suggests that follicle-associated Ags stimulate autoreactivity. Melanocytes have also been hypothesized to be a target, and decreased numbers of follicle-associated melanocytes are observed in cases of AA (52). Likewise, stimulation of T cells with melanocyte-derived peptides enhanced AA in an experimental model, and immunotherapy of a mouse model of melanoma led to the development of alopecia (9, 53). However, AA is not intrinsically associated with melanocyte loss, and though graying was seen in hairs in the 1MOG244.1 model (Fig. 7), this was typically observed only after several rounds of hair loss and regrowth and not uniformly seen, particularly in early disease states. We therefore consider it more probable that the 1MOG244.1 T cells are specific for Ags that are not melanocyte associated, and that bystander loss of follicular melanocytes with progressive cycles of alopecia leads to amelanism. Keratins have also been implicated as immunologic targets in AA (54) and may serve as good candidates for screening with the 1MOG244.1 TCR.

Although alopecia universalis ultimately developed in all mice, disease was cyclical and highly regional. Alopecic regions were not defined by dermatomes or other obvious borders and were often irregular in shape and with distinct borders. Histologic studies were unable to associate the follicular loss with specific hair follicle phases. Further, cyclical disease was regional, and at a single time point distinct patches showed hair loss or regrowth. Patchy baldness is characteristic of the most common forms of alopecia areata in people, too, and the simultaneous growth and loss of hair is apparent in the reticular variant of the disease. This seemingly arbitrary regionalism may indicate that disease is unassociated with either global cycles of T cell functional potential or with hair cycle changes. Alternatively, studies of hair cycle in mice, including B6 mice, have demonstrated complex hair cycle domains in individual animals (55). Indeed, some mutant mice possess a cyclical alopecia where hair shafts dissociate from the follicles during specific hair cycle stages, and patchiness is observed in these circumstances.
In summary, we describe a novel mouse model for AA on the B6 background with similarities to the reticular variant through progressing to alopecia universalis. Our findings indicate that monocolonal CD8+ T cells can independently mediate spontaneous AA in a class I MHC-dependent manner and that progressive disease is associated with systemic T cell expansion and degenerate cytokine production. The 1MOG244.1 T cells further provide an opportunity to better define Ags responsible for AA and disease pathophysiology.

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Disclosures

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References