Maternal IL-6 can cause T-cell-mediated juvenile alopecia by non-scarring follicular dystrophy in mice

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Abstract: Aiming to decipher immunological mechanisms of the autoimmune disorder alopecia areata (AA), we hypothesized that interleukin-6 (IL-6) might be associated with juvenile-onset AA, for which there is currently no experimental model. Upon intramuscular transgenesis to overexpress IL-6 in pregnant female C57BL/6 (B6) mice, we found that the offspring displayed an initial normal and complete juvenile hair growth cycle, but developed alopecia around postnatal day 18. This alopecia was patchy and reversible (non-scarring) and was associated with upregulation of Ullbp1 expression, the only mouse homolog of the human AA-associated ULBP3 gene. Alopecia was also associated with inflammatory infiltration of hair follicles by lymphocytes, including alpha-beta T cells, which contributed to surface hair loss. Despite these apparently shared traits with AA, lesions were dominated by follicular dystrophy that was atypical of human AA disease, sharing some traits consistent with B6 alopecia and dermatitis. Additionally, juvenile-onset alopecia was followed by complete, spontaneous recovery of surface hair, without recurrence of hair loss. Prolonging exposure to IL-6 prolonged the time to recovery, but once recovered, repeating high-dose IL-6 exposure de novo did not re-induce alopecia. These data suggest that although substantial molecular and cellular pathways may be shared, functionally similar alopecia disorders can occur via distinct pathological mechanisms.

Key words: alopecia areata – B6 alopecia and dermatitis – interleukin-6 – juvenile onset – lymphocyte – maternal immune activation – T cell

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Introduction

Alopecia areata (AA) is among the most common autoimmune disorders, with a lifetime incidence recently reported at 2.1% (1). Mild to moderate AA can be characterized by waxing and waning patchy hair loss, which may progress to severe hair loss (alopecia totalis, affecting the entire head; alopecia universalis, affecting the entire body; Ref. (2)). Mouse models, adoptive transfer experiments and human skin transplanted onto immunodeficient mice suggest that the disease is autoimmune mediated and that T cells play a significant role (3). However, the underlying immunological mechanisms, including the triggering events that lead to breaking tolerance, and subsequent events associated with spontaneous resolution of moderate lesions are incompletely understood.

Because PBMCs from juvenile patients with AA were reported to secrete enhanced levels of IL-6 upon stimulation (4), we considered this cytokine as a candidate factor that might influence AA. IL-6 is a pleiotropic cytokine that participates in diverse immunological, neurological and developmental signalling events (5). IL-6 and its downstream STAT3 signalling pathway are known to play a role in hair follicle cycling and regeneration (6–9). IL-6 is also a critical causal factor in the maternal immune activation model of schizophrenia and autism, where inflammatory cytokine production in pregnant mice leads to permanent changes in utero to brain and immune systems in offspring (10,11). Taken together, these observations suggested that IL-6-mediated maternal immune activation might interfere with normal hair development of progeny via an immune-mediated mechanism.

Here, we present a new example of juvenile alopecia in mice based on maternal IL-6 expression in vivo. This model may provide novel insights into the interactions between the immune system and the follicular microenvironment in C57BL/6 (B6) mice, a strain known to be strongly resistant to AA (12,13).

Methods

Mice

Tcrβ-null (B6.129P2-TerbμMtm1Moa/J), Tcrδ-null (B6.129P2-TerδμMtm1Moa/J) and Rag1-null mice (B6.129S7-Rag1tm1Mom/J) on C57BL/6 (B6) background and B6 wild-type mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed 4–5 mice per cage in Allentown static cages on a 12:12-h light cycle with access to food (Purina LabDiet 5053) and reverse osmosis-filtered tap water, ad libitum. Health screening of sentinel mice was routinely performed by Charles River Laboratories (Tracking Profile and Assessment Plus). Timed matings were performed by placing 2–4 females in a single-housed male’s cage in the late afternoon and by checking for plugs early the next morning. Plugged females were group-housed 3–5 per cage until E12.5, when they were electroeported and single-housed. Pups were raised by the birthing female until P21, when they were weaned. All procedures were performed in accordance with the Mayo Clinic College of Medicine’s Institutional Animal Care and Use Committee (IACUC) regulations.

Plasmids

The mammalian expression vectors pCA-mIL6 (RDB1520) and pCA-LacZ (RDB1870), which use the cytomegalovirus (CMV)
promoter to drive the expression of mouse IL-6 or β-galactosidase, respectively, were obtained from the Riken BRC DNA bank through the national bio-resource project of the MEXT, Japan (14–16). Plasmid DNA was isolated using an Endofree Plasmid Maxi Kit (Qiagen, Valencia, CA, USA) and brought to 1 mg/ml in Tris–EDTA (TE) buffer.

**Electroporation**

The electroporation protocol was modelled after published methods (17,18). Mice were induced under isoflurane anaesthesia and further anesthetized with ketamine/xylazine (50/5 mg/kg) injected intraperitoneally. Sufficient anaesthesia was confirmed by the lack of paw withdrawal reflex upon toe-pinch. The right hind leg was shaved, and 50 μl of DNA was injected into the middle of the tibialis anterior (TA) muscle. Visible swelling of the entire length of the muscle confirmed correct injection. A BTX 5-mm 2-needle array (Harvard Apparatus, Holliston, MA, USA) was then inserted into the TA muscle such that the injection site was at a point equidistant from the two needles, and a BTX 830 electroporator was used to deliver six pulses of 100V, 40 ms/pulse. The mouse was then single-housed, returned to the animal room and monitored daily for the birth of pups and status of pup hair until their weaning at P21.

**Quantification of surface hair loss**

Pups were monitored daily to track the onset of hair growth followed by the time course of hair loss. At weaning, pups were weighed and visually inspected for hair loss by observers blinded to the treatment group. The percentage area of hair loss was subjectively estimated. Hair loss of 100% was defined as total loss of all hair from the dorsal thoracic and lumbar regions and ventral thoracic region; skin from head and abdomen was not included because hair loss was never observed there. All pups had ear-punch identification and were photographed and group-housed by sex. For each litter, the percentage of pups displaying hair loss and the average amount of hair loss per pup were calculated. To minimize litter-size effects, statistical analysis was based on litter averages rather than individual pups.

For more information, see Data S1.

**Results**

**Maternal electroporation produces biologically active IL-6**

Timed-pregnant female wild-type B6 mice were electroporated in the tibialis anterior muscle with the mammalian expression vector pCAGGS encoding mouse IL-6 (pCA-mIL6) or LacZ (pCA-LacZ control) on embryonic day (E) 12.5, as previously described (17). Muscle cells expressed the transgenes, which in pCA-LacZ control mice could be visualized by staining for β-galactosidase activity in muscle fibres (Fig. 1a). In pCA-mIL6-electroporated mice, serum IL-6 rose to levels that peaked at 7 days postelectroporation, declining thereafter as plasmid DNA became inactivated (Fig. 1b).

Notably, even at weaning, 31 days postelectroporation, maternal serum IL-6 remained elevated in pCA-mIL6-electroporated females. Spleen size increased substantially in pCA-mIL6-electroporated mice (Fig 1c), and maternal spleen weight correlated with serum IL-6 at litter weaning (Fig. 1d; \( r^2 = 0.75, P < 0.0001 \)), indicating that transgenic IL-6 was biologically active.

**Maternal IL-6-exposed pups grow hair, then develop alopecia**

Both pCA-LacZ- and pCA-mIL6-electroporated pups initially grew hair normally, beginning at approximately postnatal day (P)7. However, pups from pCA-mIL6-electroporated mothers lost substantial surface hair beginning at approximately P18 ± 1 (Fig. 1e), a phenomenon not observed in the pups from pCA-LacZ-electroporated mothers (Figure S1). Interestingly, while pCA-mIL6-electroporation-induced surface hair loss was variable within and between litters, mothers never lost hair. Among pups displaying alopecia, the extent of surface hair loss ranged from small, patchy areas above the hind legs or shoulders to near-complete loss on the dorsal skin over the thorax sometimes extending to the dorsal lumbar and ventral thoracic areas. Surface hair loss was never observed on the head, or around the ventral abdominal region or base of the tail. Balding areas displayed irregular peripheral
definition and contained sparse hairs throughout, a pattern not
associated with barbering (which usually affects the vibrissae and
other facial hair). Furthermore, hair was not observed in the
mouths or stomachs of affected animals upon sacrifice.

The percentage of pups in each litter that lost surface hair cor-
related significantly with corresponding maternal IL-6 serum levels
measured by ELISA at weaning (Fig. 1f). Litters from mothers
expressing high serum IL-6 displayed severe surface hair loss in
most or all pups, while litters from mothers with moderate serum
IL-6 showed various degrees of surface hair loss, ranging from
none to severe (Fig. 1f). These data demonstrate that
maternal transgenic IL-6 expression causes the development of alopecia in
pups.

Surface hair reappears unless high IL-6 is sustained

At weaning, the serum IL-6 level of pups whose mothers expressed
transgenic IL-6 was below the ELISA detection limit. After wean-
ing, surface hair reappeared in ~7–10 days (Fig. 2a), and no sub-
sequent recurrences of hair loss were noted in up to 6 months of
observation. However, if the pups themselves were electroporated
with pCA-mIL6 at weaning, then surface hair re-growth was sig-
ificantly delayed (Fig. 2b–c; $\chi^2 = 20.49, P < 0.0001$), suggesting
that sustained elevation of IL-6 is required for surface hair sup-
pression.

Timing of IL-6 exposure determines susceptibility/resistance
to surface hair loss

Upon varying the time point of IL-6 transgenesis, we found that
when mothers were electroporated at P0, P3 or P6, 20–80% of
pups in 1–3 litters per time point displayed surface hair loss. In
these experiments, the serum IL-6 level of mothers at weaning
was similar to that of mothers electroporated at E12.5. Thus, high IL-6
exposure starting as late as P6 was sufficient to result in the loss of
surface hair in pups. However, surface hair loss was not
induced in any of the following mice: (i) pCA-mIL6-electro-
porated normal adult animals; (ii) pCA-mIL6-electroporated P21
pups who had never lost their hair before weaning; (iii) pCA-
mIL6-electroporated adult offspring that had previously lost their
hair as pups (due to maternal transgenic IL-6), but then re-grew
their hair after weaning. Thus, resistance to high-IL-6-induced
surface hair loss is acquired by day P21.

Ulbp1 is upregulated in affected skin

ULBP3 encodes an NKG2D ligand that is associated with AA in
humans (19), and the only ULBP encodes an NKG2D ligand. Therefore, we assessed
Ulbp1 expression in affected dorsal thoracic skin, unaffected head
skin from affected animals and unaffected dorsal thoracic skin from
unaffected pups from pCA-mIL6-transfected mothers. Both stan-
dard RT-PCR (Fig. 2d, gel) and real-time RT-PCR (Fig. 2d, graph)
revealed a significant upregulation of Ulbp1 in affected skin, but not
in either source of unaffected skin (ANOVA $F_{2,11} = 8.77; P = 0.008$;
Bonferroni post hoc: affected vs. unaffected $P < 0.05$; affected vs.
affected head $P < 0.05$; affected head versus unaffected, not signifi-
cant). This suggests that Ulbp1 was upregulated specifically in the
affected skin of IL-6-exposed pups.

$\alpha\beta$ T cells and other lymphocytes contribute to IL-6-induced
alopecia

Histologically, affected alopecic skin displayed a significant follicu-
lar dystrophy, along with a mixed inflammatory response consist-
ing of neutrophils, lymphocytes and mast cells not seen in
unaffected, control skin from the pups of pCA-LacZ-transfected
mothers (Figure S2). Immunofluorescence imaging of control skin
from the pups of pCA-LacZ-transfected mothers revealed few
CD3$^+$ γδTCR$^-$ (presumed and later confirmed $\gamma$TCR$^+$) cells,
while CD3$^+$ γδTCR$^+$ dendritic epithelial T cells (DETC) were
regularly spaced along the epithelial surface and lining the hair folli-
cles (Fig. 3a; note, mouse hair was autofluorescent in the green
channel). In contrast, affected alopecic skin from the pups of
pCA-mIL6-transfected mothers contained both CD3$^+$ γδTCR$^+$
and CD3$^+$ γδTCR$^-$ cells (Fig. 3b). Further labelling studies con-
firmed that >95% of γδTCR$^+$ cells were V$\gamma$3+, as expected of
DETCs, and that that >95% of CD3$^+$ γδTCR$^-$ cells were $\gamma$TCR$^+$ (data
not shown). We quantified the number of total CD3$^+$ T cells, $\alpha\beta$ T
cells and γδ T cells by counting DAPI$^+$ nuclei surrounded by
the respective T-cell markers in unmerged, overlapping confocal
image stacks (Fig. 3c; Data S1). Two-way ANOVA revealed a sig-
nificant effect of alopecia ($F_{1,20} = 22.7, P < 0.0001$) and a signifi-
cant effect of cell type ($F_{2,20} = 9.3, P = 0.0014$). Bonferroni post
hoc testing revealed that total CD3$^+$ T cells and $\alpha\beta$ T cells were
significantly elevated in affected animals ($P < 0.01$ and $P < 0.05$
respectively), while the increase in γδ T cells did not reach a
significance.
To determine whether T cells played a pathogenic role in surface hair loss, we performed pCA-mIL6 transgenesis in mice lacking γβ T cells (Tcrb knockout (KO)), or lacking γδ T cells (Tcrd KO), or lacking all T and B cells (Rag1 KO). Importantly, maternal serum IL-6 levels at weaning were similar between genotypes (F3,43 = 0.38, P = 0.77, Fig 4a). In 11 B6 IL-6-electroporated litters, 33/61 pups (54%) showed hair loss. In 8 litters of Rag1-KO mice, 5/49 pups (10%) showed hair loss, which was significantly different from B6 by two-sided Fisher’s exact test (P < 0.0001). In 12 litters of TCRb-KO mice, 11/77 pups (14%) showed hair loss, also significantly different from B6 by two-sided Fisher’s exact test (P < 0.0001). In 9 litters of TCRd-KO mice, 19/53 pups (36%) showed hair loss, which was not significantly different from B6 by two-sided Fisher’s exact test (P = 0.061). No pups in control pCA-LacZ-electroporated litters (3–6 litters per genotype) lost surface hair. In order to control for differences in litter size that could potentially affect statistics, the analysis was re-performed estimating the percentage of surface hair loss per pup and averaged per litter (Fig 4b). Kruskal–Wallis test revealed a significant effect of genotype (P = 0.013), and Dunn’s multiple comparison test post hoc test revealed that both Rag1-KO and Tcrβ-KO litters showed significantly less surface hair loss per pup than B6 litters (P < 0.01 and P < 0.05, respectively; Fig 4b). These data suggest that lymphocytes, and specifically γβ T cells, are involved in IL-6-induced surface hair loss.

Discussion

While there is a well-accepted C3H/HeJ mouse model for adult-onset AA (20), none exists for juvenile-onset AA. Following a clue from the published literature (4), we hypothesized that IL-6 might play a role in juvenile-onset AA. To test this, a model of maternal immune activation was used in which pregnant B6 females were electroporated intramuscularly with an expression vector for temporary transgenic expression of IL-6. Progeny of these dams initially grew hair normally, but around weaning age developed alopecia that was patchy and spontaneously reversible (non-scarring). This alopecia was associated with Ulbp1 upregulation and inflammatory cellular follicular infiltration by a mechanism that involved lymphocytes, specifically γβ T cells. These observations highlight a significant activity in some of the pathways that are dysregulated in human and mouse AA. However, the histologic lesions associated with this alopecia were atypical of human AA, sharing some traits with a B6 strain-specific alopecia and dermatitis that models human central centrifugal alopecia (21). Although that disease in mice is usually seasonal, displays low-frequency incidence and can progress to cicatricial stages, the present alopecia was high incidence but spontaneously reversible, with no recurrence and with long-term subsequent full pelage displayed by all initially affected mice. Thus, the present immune mechanism appears to play a critical role in the pathogenesis of follicular dystrophy, a general feature of both AA and B6 alopecia and dermatitis, but with characteristics that make difficult a simple categorization for this maternal IL-6-induced alopecia.

Figure 3. Immunofluorescence reveals T-cell infiltrates in affected skin. (a) Immunofluorescence labelling revealed CD3+ γδTCR+ DETCs in control sections (arrows) from the pups of pCA-LacZ-transfected mothers. Note the lack of CD3+ γδTCR− cells. (b) Immunofluorescence labelling revealed CD3+ γδTCR+ DETCs (arrows) and CD3+ γδTCR-infiltrating γδ T cells (arrowheads) in the affected skin sections from the pups of pCA-mIL6-transfected mothers. Also note the abnormally oriented and fragmented hair, which autofluoresced in the green channel. (c) Quantification of the number of total T cells, γβ T cells and γδ T cells in control and the affected sections. N = 4–7 mice per group, 4–8 sections per mouse (*P < 0.05; **P < 0.01 by two-way ANOVA followed by Bonferroni’s post hoc test). All scale bars = 100 μm.

Figure 4. Genetic deletion of lymphocytes, specifically γβ T cells, protects against surface hair loss in IL-6-exposed pups. (a) Average level of maternal serum IL-6 measured at weaning did not differ between experimental groups. (b) The average amount of hair loss per pup in each litter was calculated, and the average percentage per litter is shown (N = 8–12 litters per group; *P < 0.05 **P < 0.01 by Dunn’s multiple comparison test).
These observations bear some similarity to the only published example of AA in the B6 mouse strain, in which a specific TCR transgene was shown to target T cells to hair follicles; in that model, while there were histologic lesions similar to the AA observed in C3H/HeJ mice, there were also many histologic lesions that resembled B6 alopecia and dermatitis, which complicated interpretation (22). These studies reveal the complexity of these alopecia diseases and how one strain-specific disease model can complicate another with an overlapping pathogenesis.

The present results fit with prior literature suggesting that IL-6 may play a role in the onset of the hair cycle. The timing of the hair loss in IL-6-exposed pups corresponds with the end of the first postnatal hair cycle and the start of the second cycle (23). Furthermore, the lack of telogen follicles in pups with IL-6-induced alopecia may suggest that IL-6 can contribute to delaying the second hair cycle. In a previous report, juvenile transgenic mice that expressed IL-6 in the skin showed stunted hair growth that resolved by ~5 weeks of age (8). A recent report found that recombinant human IL-6 inhibited hair shaft elongation and suppressed the proliferation of matrix cells in cultured human hair follicles (9). In separate studies, mice with a targeted deletion of STAT3 in keratinocytes had a normal first hair cycle, but disruption of the subsequent anagen, abnormal growth of hair follicles and sparse pelage throughout life (23). Clearly, IL-6 signalling plays a role in hair follicle biology.

Still, there are subtle differences in precise effects of IL-6 on follicle cycling in these various experimental systems. In the present work, we do not know whether IL-6 or a downstream inflammatory mediator is being transmitted to the pups to directly cause hair loss. However, the prolongation of alopecia by IL-6 electroprotonation of affected pups suggests that such a putative downstream mediator must also be available in weaned pups in the absence of further milk. Nevertheless, in a series of studies of ‘inflammatory milk’, Yihong Wan and colleagues demonstrated that resolved by~ inflammatory fatty acids in milk, causing expression of inflammatory cytokines (including IL-6) in pups’ skin and a hair loss phenotype bearing similarities to that reported here (24,25).

The mechanisms by which T cells participate in the IL-6–hair follicle axis are unclear. One wonders whether T cells play a role in the other examples of IL-6-related alopecia in the literature or whether that feature is unique to the present maternal immune activation model. While the infiltrating leukocytes observed in the skin could be a result of inflammation caused by hair penetration into the dermal tissues, the fact that T-cell deletion decreased both incidence and severity of alopecia provides evidence that T cells contribute a causal factor. IL-6 plays several roles in T-cell biology, including driving cells to Th17 instead of Treg differentiation and inducing expression of skin-homing cutaneous lymphocyte antigen (CLA) on both dendritic cells and T cells (26). CLA is upregulated on T cells in AA patients, both in the blood (27) and in punch biopsies from lesions (28), suggesting a potential mechanistic pathway between IL-6 and AA. In mice, AA was also reported to be inhibited via blockade of CD44 (29), another immune cell skin-homing marker contributing to disease. Perhaps upregulation of homing receptors on T cells and upregulation of NKG2D ligands such as Ulbp1 in skin might coordinate to initiate hair follicle attack in immune-mediated alopecia such as in the current model, or in human AA.

In Rag1 null pups, IL-6-induced surface hair loss was rarely observed and was qualitatively different from that seen in other genotypes (Fig. 4). While alopecia in other genotypes ranged from the dorsal skin over the thorax extending to the dorsal lumbar and ventral thoracic areas (Figs. 1e, 2b), Rag1 null pups occasionally showed thinning hair spanning dorsal thorax and lumbar regions, but in a different pattern, with no areas showing near-complete baldness (data not shown). This suggests that there may be two mechanisms through which IL-6 can incite alopecia, one involving lymphocytes with potential to lead to near-complete regional alopecia and one that is lymphocyte independent causing sparse, incomplete surface hair loss.

In conclusion, maternal IL-6 transgenic expression causes juvenile alopecia that shares some descriptive traits with AA, but whose pathologic mechanism involves follicular dystrophy with some apparent similarities to B6 alopecia and dermatitis. The mechanisms by which T cells contribute to this disorder, how affected mice recover following initial lesions and what protective factors prevent this alopecia from evolving into a more elaborate autoimmune response remain to be elucidated.

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Author contributions
SEPS, RLGM, TRD and JPS performed experiments; SEPS, JPS and AGS wrote the manuscript.

Conflict of interest
The authors declare no conflict of interest.

Supporting Information
Additional supporting data may be found in the supplementary information of this article.

Data S1. Methods.
Figure S1. Normal pelage in offspring of pcA-LacZ-transfected mothers.
Figure S2. Histological analysis of maternal/IL-6-induced juvenile alopecic skin reveals inflammatory infiltrates and follicular dystrophy.

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