Epidermal Cadm1 Expression Promotes Autoimmune Alopecia via Enhanced T Cell Adhesion and Cytotoxicity

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Autoimmune alopecia is characterized by an extensive epidermal T cell infiltrate that mediates hair follicle destruction. We have investigated the role of cell adhesion molecule 1 (Cadm1; Necl2) in this disease. Cadm1 is expressed by epidermal cells and mediates heterotypic adhesion to lymphocytes expressing class 1-restricted T cell-associated molecule (CRTAM). Using a murine autoimmune alopecia model, we observed an increase in early-activated cytotoxic (CD8-restricted, CRTAM-expressing) T cells, which preferentially associated with hair follicle keratinocytes expressing Cadm1. Coculture with Cadm1-transduced MHC-matched APCs stimulated alopecic lymph node cells to release IL-2 and IFN-γ. Overexpression of Cadm1 in cultured human keratinocytes did not promote cytokine secretion, but led to increased adhesion of alopecic cytotoxic T cells and enhanced T cell cytotoxicity in an MHC-independent manner. Epidermal overexpression of Cadm1 in transgenic mice led to increased autoimmune alopecia susceptibility relative to nontransgenic littermate controls. Our findings reveal that Cadm1 expression in the hair follicle plays a role in autoimmune alopecia. The Journal of Immunology, 2012, 188: 000–000.

Autoimmune alopecia is a common skin disorder affecting up to 2% of people worldwide. Despite a lack of clinical morbidity, the disease causes significant economic and health burdens because of its negative psychological effects. It is characterized by an extensive skin inflammatory T cell infiltrate, hair follicle destruction and focal, extensive or complete baldness in males and females (1, 2). Inflammation in autoimmune alopecia involves both CD4+ and CD8+ T cells and is most commonly observed in association with dystrophic anagen (growing) hair follicles (3). Alopecia also occurs in other autoimmune diseases, including lupus erythematosus, raising the possibility of common mechanisms (4, 5). The genetic basis of autoimmune alopecia is currently unknown, and recent genome-wide association studies implicate both innate and adaptive immunity (6).

The C3H/HeJ inbred mouse strain is a widely accepted experimental model of autoimmune alopecia initiation and progression, as up to 20% of aged C3H/HeJ mice spontaneously develop the disease (7, 8). Mice exhibit a characteristic nonscarring, inflammatory cell-dependent hair loss that closely resembles human autoimmune alopecia. Mononuclear infiltrates occur within the hair follicles, which fail to enter anagen and subsequently degenerate (7, 8). Alopecia can be induced in unaffected mice by full-thickness skin grafts or peripheral lymph node-derived cell transfer from affected individuals (9, 10). CD8-expressing T cells are the principal cell type responsible for inducing alopecia (9). Reducing or eliminating the procytotoxic Th1 cell-derived cytokines IL-2 and IFN-γ inhibits alopecia susceptibility (11, 12). Thus, class 1-restricted, CD8-expressing T cells are the primary effector cells for alopecia in this model (13).

Genetic linkage studies of murine autoimmune alopecia have previously identified several T cell regulatory genes that may mediate disease susceptibility (14). These genes include class-1 restricted T cell-associated molecule (CRTAM), a molecule known to modulate T cell polarity as well as CD8 and NK T cell cytotoxicity (15–17). CRTAM is functionally regulated via nonredundant, heterotypic adhesion to cell adhesion molecule 1 (Cadm1); also known as tumor suppressor of lung cancer 1 [Tslc1], nectin-like molecule 2 [Necl2], Ig-like superfamily member 4 [Igsf4], and synaptic cell adhesion molecule [Syncam]) (18, 19). Cadm1–CRTAM interactions promote elevated MHC-indepenendent lymphocyte cytotoxicity (20, 21), enhanced Cadm1-expressing tumor cell immunosurveillance (22), increase T cell maturation and retention within the thymus and lymph nodes (15, 17, 23, 24), and promote T cell autoimmunity in a murine model of diabetes (15).

We previously demonstrated that Cadm1 expression is enriched in the bulge reservoir of hair follicle stem cells, and modulates epidermal cell proliferation, motility, and wound healing by promoting homotypic intercellular adhesion (25). We have investigated whether Cadm1-mediated heterotypic adhesion to CRTAM T lymphocytes plays a role in autoimmune alopecia.

Materials and Methods

Mouse husbandry and transgenesis

Spontaneously alopecic, retired female breeder C3H/HeJ mice were obtained from Harlan Laboratories and used for initial peripheral lymph node isolation to establish a colony of wild type alopecic C3H/HeJ mice via s.c. lymphocyte transfer. All mice were housed in individually ventilated cages on a 12-h light-dark cycle and allowed access to food and water ad libitum. Sentinel screenings for common murine pathogens were routinely
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In vitro culture assays

Primary human neonatal foreskin epidermal keratinocytes were isolated and cultured on a J2 3T3 feeder layer in FAD medium (one part Ham’s F12 medium, three parts DMEM, and 1.8 \times 10^{-4} \text{M} \text{adenine}) supplemented with 10% FCS and HICE mixture (0.5 \mu\text{g/ml} \text{hydrocortisone}, 5 \mu\text{g/ml} \text{insulin}, 10^{-8} \text{M} \text{cholera enterotoxin}, and 10 \text{ng/ml} \text{epidermal growth factor}) as described previously (25). Murine LK35.3 cells (LK cells, also termed HB-98) were cultured in standard tissue culture plates in RPMI 1640 as described previously (27). Full-length Cadm1-FLAG (28) was cloned into pBabe-puromycin (pBp) and sequence verified. pBp-Cadm1 and pBp-empty vector (EV) retroviral vectors were used to infect primary keratinocytes or murine LK cells via supernatant transfer from AM12 amphotropic retrovirus-producing cells, as described previously (25).

IL-2 and IFN-\gamma ELISAs were performed according to the manufacturer’s protocol (BD Pharmingen) using lysed supernatants derived from cocultures of \(10^5\) transduced primary human keratinocytes or murine LK cells (target cells) with \(10^6\) or \(10^7\) freshly isolated alopecic or normal lymph node-derived cells (killer cells). Cocultures were grown in RPMI 1640 medium for 12–72 h in round-bottom 96-well plates (LK cells) or flat-bottom 96-well plates (keratinocytes). Data are representative of two separate experiments with a minimum \(n = 8\) samples per target-killer ratio and per cell combination.

Mitochondrial lactate dehydrogenase (LDH) release was measured according to the manufacturer’s protocol (Roche) following overnight coculture of \(10^5\) control or Cadm1-transduced primary human keratinocytes with \(10^6\) freshly isolated murine alopecic lymphocytes or in flat-bottom 96-well plates. The assay was performed three times with a minimum \(n = 6\) wells per cell combination.

Leukocyte rosette assays were performed by plating \(10^6\) control or Cadm1-transduced keratinocytes on Permanox chamber slides, allowing cells to adhere for 24 h in standard keratinocyte medium, and subsequently coculturing cells overnight in RPMI 1640 with \(10^6\) freshly isolated normal or alopecic murine lymph node cells that were either unsorted or FACS-selected based on CD3 reactivity. Chamber slides were then fixed, gently washed, and stained with Diff-Quick or primary Abs. Keratinocyte-leukocyte aggregates were classed as rosettes if at least two leukocytes were in contact with one keratinocyte. Quantitation of relative rosette abundance was scored double-blinded for \(n = 4\) leukocyte-keratinocyte combinations. The assay was performed three times.
Flow and imaging cytometry

Single-cell suspensions were derived from mouse epidermis following overnight trypsin digestion at 4°C or 2 h at 37°C for flow cytometric analysis and stained for epithelial and immune cell markers (CD45, CD3, CD4, CD8, CD25, CD69, CD49b, CTRAM, y6-TCR, and Igα6), as described previously (26). Dead cells were identified on the basis of DAPI positivity, and epithelial cells were excluded on the basis of high surface Igα6 expression. Data were collected using an LSR II flow cytometer (BD Biosciences). Flow cytometric data were analyzed using FlowJo 7.6.1. software.

In vitro keratinocyte-lymphocyte aggregation was measured using an Amnis ImageStream imaging cytometer as described previously (25). Cadm1 or EV-transduced human keratinocytes were isolated (29), stained with anti–α6-integrin-FITC (BD Biosciences), and leukocytes obtained from peripheral lymph nodes of normal or alopelic mice were isolated and stained with anti-mouse-CD45-RPE (BD Biosciences). Equal numbers of CD45-labeled lymphocytes and Igα6-labeled keratinocytes were allowed to adhere in suspension for 6 h at 37°C and subsequently labeled with the cell-penetrating dye Drag5 (Biostatus, U.K.). Single images representing 20,000 or 25,000 individual nucleated cells were analyzed per experiment. Three independent experiments were performed.

Sample preparation and Ab staining

Frozen tissue sections were fixed briefly in 1% paraformaldehyde before staining. Mouse tail epidermal whole mounts and sections of paraffin-embedded tissue were prepared as described previously (29). H&E staining was performed using an automated staining system (Tissue Tek). Chamber slides were fixed overnight in 1% paraformaldehyde before staining with either Diff-Quick (Sigma) or cell type-specific primary Abs. Cells and sections were blocked in 5% FCS, 0.2% Tween-20, and 0.2% fish skin gelatin for 1 h at room temperature, then incubated overnight in primary Ab at 4°C. After washing, secondary Ab was applied in blocking solution for 3 h at room temperature. The following Abs were used: rat anti-Cadm1 (generated by YT), chicken anti-Cadm1 (MBL), rabbit anti-keratin 14 (ABR Biosystems), mouse anti-keratin 14 (LL002) (29), rabbit anti-ki67 (Novoceastra), mouse anti-keratin 15 (LHK15) (29), goat-anti-CRTAM (Santa Cruz), rat anti–α6 integrin, CD3, CD4, CD8, y6-TCR, and CD45 (all BD Pharmingen). Species and isotype-specific secondary Abs conjugated to Alexa Fluor 488, 555, or 633 or Streptavidin-HRP were purchased from Invitrogen. Streptavidin-HRP staining was detected using a standard DAB histochemical kit (Vector laboratories). Nuclei were counterstained with either DAPI (fluorescent Ab staining) or hematoxylin (DAB histochemical staining). Fluorescent microscopy images were acquired using a Leica TCS Tandem confocal with ×10 or ×20 objectives. H&E- and DAB-stained sections were imaged using an Olympus upright microscope with a 20× objective. All images were postprocessed using Adobe Photoshop (rotate, crop, brightness, and contrast adjustments only).

Quantitative RT-PCR

Total RNA was isolated from whole skin and lymph nodes using standard phenol:chloroform extraction followed by mRNA purification (polyATtract; Promega). RNA from cultured cells was obtained using SV RNA isolation kit (Promega). Quantitative PCR was performed using qScript cDNA Supermix (Quanta biosciences). PCR Master mix was purchased from Applied Biosystems.

Statistical analysis

Pairwise t tests with Welch’s correction to account for unequal sample variance were used for all statistical comparisons. Kaplan-Meier survival curves of alopecic disease incidence between transgenic and nontransgenic mice were analyzed using a log-rank test. Statistical significance was accepted at p < 0.05 for all analyses, and all error bars represent SEM. All statistical analyses were performed using GraphPad Prism and Microsoft Excel software.

Results

Immunophenotyping of alopecic epidermis

We obtained peripheral cervical, brachial, and inguinal lymph node single cell suspensions from spontaneously alopelic, reticulated female breeder C3H/HeJ mice and transferred them to healthy recipients via s.c. injection. Five to 13 wk after transfer, ~50% of recipients exhibited patchy, nonscarring hair loss, beginning at the injection site and subsequently expanding across the entire skin surface, as reported previously (9).

We used flow cytometry to determine the abundance and phenotype of immune cells in the epidermis of alopelic mice compared with nonalopecic controls (Fig. 1A, 1B). Epidermal keratinocytes were gated out on the basis of Igα6 expression. There was an almost 3-fold increase in CD45-expressing cells in alopelic compared with control epidermis (<5% to >12%; Fig. 1C). Greater than 60% of immune cells in normal epidermis were CD3+ y6TCR+ dendritic epidermal T cells. In contrast, these cells represented only 30% of alopelic immune cells (Fig. 1D). In alopelic epidermis, there was a significant increase in CD3+CD8+ cytotoxic T cells but not in CD3+CD4+ cells (Fig. 1D).

Expression of cell surface markers of T cell activation was analyzed by staining for CD69 (early activation) and CD25 (late activation). The average percentage of CD3+CD8+ cytotoxic T cells that were in the early activation state (CD69+CD25−) was 35.65 ± 2.23% in alopelic epidermis versus 26.3 ± 0.34% in normal epidermis (n = 4 mice per group; p = 0.006; Supplemental Fig. 1A).

Alopelic cytotoxic T cells are associated with Cadm1+ epidermal stem cells

To characterize the distribution of T cells in normal and alopelic epidermis, we stained tail skin sections (Fig. 2A–D) and tail epidermal XYZ, site and subsequently expanding across the entire skin surface, as reported previously (9).

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dermal whole mounts (Fig. 2E–H) with Abs to Cadm1 and T cell markers. Cadm1 was highly expressed in the hair follicle bulge of both normal and alopecic follicles (Fig. 2A–F). There was a modest increase in CD4-expressing T cells in alopecic skin compared with controls; however, these cells were localized to the dermis and were not in direct contact with Cadm1-expressing keratinocytes (Fig. 2A, 2B). There was a dramatic increase in CD8-expressing cells in alopecic skin, primarily in the epidermis (Fig. 2C, 2D), which was consistent with the flow cytometry results (Fig. 1).

Dual labeling of whole mounts with Abs to CD3 and Cadm1 provided more information about the location of T cells in alopecic epidermis. In normal epidermis, T cells were predominantly located in the interfollicular epidermis and their morphology was consistent with γδ TCR+ dendritic epidermal T cells (Fig. 2E). In alopecic mice there was a substantial increase in CD3-expressing cells throughout the interfollicular epidermis and within hair follicles (Fig. 2F), which was consistent with the increase in epidermal T cells observed by flow cytometry (Fig. 1D). Cadm1 staining revealed abnormal swelling of the hair follicle bulge in alopecic skin, correlating with the large numbers of CD3-expressing cells infiltrating the bulge (Fig. 2F).

The intimate association of CD3-expressing T cells with Cadm1-expressing hair follicle stem cells was further visualized using cross-sectional (XZ) confocal microscopic imaging of whole mounts (Fig. 2G, 2H). Direct contact between CD3+ cells and Cadm1-expressing keratinocytes was observed in alopecic, but not control, follicles (Fig. 2H, arrowheads). These data are in agreement with the flow cytometry results and indicate that cytotoxic (i.e., CD8-expressing) alopecic T cells are associated with Cadm1-expressing keratinocytes in the hair follicle bulge.

*Cadm1-associated alopecic leukocytes exhibit increased CRTAM expression*

We next assessed whether alopecic cytotoxic T cells exhibited elevated expression of the heterotypic Cadm1 binding protein CRTAM. We isolated mRNA from whole lymph nodes and epidermis of individual normal (n = 4) and alopecic (n = 4) mice, and

**FIGURE 3.** Alopecic lymph node and epidermal T cells exhibit elevated CRTAM expression. A, Relative CRTAM mRNA expression in lymph nodes and epidermis from normal or alopecic wild type mice (black and red bars, respectively). B and C, Cadm1 (green) and CRTAM (red) immunostaining of normal (B) and alopecic (C) tail epidermal whole mounts. Arrowheads in C denote bulge-infiltrating CRTAM-expressing cells. D and E, Normal (D) and alopecic (E) epidermal whole mounts labeled with Abs to Ki67 (red) and Cadm1 (green). F and G, Whole mounts of normal (F) and alopecic epidermis (G) labeled with Abs to Cadm1 (green) and keratin 15 (Krt15, red). DAPI (blue) was used as a nuclear counterstain. Scale bars, 100 μm. *p < 0.05.
determined relative CRTAM gene expression using Taqman quantitative RT-PCR (Fig. 3A). CRTAM expression was increased in alopecic lymph nodes and epidermis relative to nonalopecic controls ($p < 0.05$; Fig. 3A).

To further characterize the expression and distribution of CRTAM-expressing lymphocytes, we performed dual whole-mount immunostaining using Abs directed against CRTAM (Fig. 3B, red) and Cadm1 (Fig. 3B, 3C, green). CRTAM$^+$ cells were located adjacent to Cadm1-expressing hair follicle bulge stem cells in alopecic but not in normal epidermis. In normal epidermis, the cells of the bulge proliferate infrequently, and most Ki67 positive cells are found at the bottom (bulb) of anagen follicles (Fig. 3D) (29). In contrast, Ki67-positive, Cadm1-positive cells were frequent in the bulge of alopecic follicles, which was consistent with the abnormal swelling of the bulge (Fig. 3E). Despite the swelling and increased proliferation of the bulge, expression of the bulge markers keratin 15 and CD34 was normal (Fig. 3F, 3G, and data not shown). In some alopecic follicles, abnormal upregulation of Cadm1 outside the bulge, in the sebaceous glands, outer root sheath, and infundibulum was observed (Fig. 3F, 3G).

We also performed flow cytometry of single-cell preparations from peripheral lymph nodes and epidermis of alopecic and control mice. The average percentage of CD8$^+$ T cells that coexpressed CRTAM in alopecic lymph nodes was $8.28 \pm 0.79\%$, whereas in nonalopecic lymphocytes $7.16 \pm 0.38\%$ of the CTLs were CRTAM positive; this difference was not statistically significant (Supplemental Fig. 1B). However, a higher number of NK cells were positive for CRTAM in alopecic epidermis ($n = 4$) relative to controls ($n = 4$; Supplemental Fig. 1C). The average percentage of CD49b$^+$CRTAM$^+$ cells was $14.00 \pm 1.90\%$ in alopecic versus $4.95 \pm 0.58\%$ in wild type epidermis ($p = 0.002$). There was no significant difference in the proportion of CD49b$^+$CRTAM$^+$CD3$^+$ cells in alopecic and wild type epidermis ($\sim50\%$ of CD49b$^+$CRTAM$^+$ cells; data not shown).

**Epithelial Cadm1 expression stimulates adhesion of alopecic lymphocytes**

To determine whether epidermal Cadm1 expression could stimulate adhesion of cytotoxic, CD8$^+$, and CRTAM$^+$ lymphocytes, we monitored intercellular adhesion using in vitro imaging cytometry. We transduced primary human epidermal cells with full-length, FLAG-tagged murine Cadm1 or EV (control) and confirmed Cadm1 overexpression as described previously (25). Cells were disaggregated into single-cell suspensions and labeled with FITC-conjugated Abs to the $\alpha 6$-integrin subunit (Itga6), a basal keratinocyte marker. Separately, we isolated single lymphocytes from peripheral lymph nodes of normal or alopecic mice and labeled them with CD45-PE.

Equal numbers of FITC- and RPE-labeled cells were combined and incubated in suspension at 37°C for 6 h. At the end of the incubation period, cells were labeled with Draq5, and imaging cytometry was used to distinguish keratinocyte singlets, keratinocyte-keratinocyte aggregates, and lymphocyte-keratinocyte aggregates, based on cell surface and nuclear labeling (Fig. 4A–C). Dual RPE+FITC-labeled aggregates were then quantified (Fig. 4D). When alopecic CD45-labeled lymph node cells were combined with Cadm1-transduced primary human keratinocytes, they formed significantly more aggregates than did CD45$^+$ cells obtained from nonalopecic mice incubated with either control or Cadm1-transduced keratinocytes or alopecic lymphocytes incubated with control keratinocytes. These results demonstrate that Cadm1 expression promotes heterotypic adhesion between keratinocytes and alopecic lymphocytes.

**FIGURE 4.** Cadm1 promotes enhanced alopecic leukocyte-keratinocyte aggregation. A, Positive selection for images containing epithelial cells based on Itga6-FITC pixel area and intensity (red gate). Representative images of gated epithelial and nonepithelial cell populations are shown (red and black boxes, respectively). $B$, Further identification of epithelial cell images containing more than one cell based on Itga6-FITC plus Draq5 nuclear area/intensity aspect ratio (red gate and representative image). $C$, Discrimination between true lymphocyte-keratinocyte cell aggregates (red gate) and coincidental images of two or more cells (black gate) based on Itga6-FITC plus CD45-PE $\Delta$Centroid XY versus Draq5 nuclear Centroid Y parameters. Images show a true lymphocyte-keratinocyte doublet (red box) and a nonadherent pair of cells (black box). $D$, Quantitation of lymphocyte-keratinocyte doublet abundance following admixture of FITC-labeled control (open boxes) or Necl2-transduced keratinocytes (solid boxes) with PE-labeled normal or alopecic lymphocytes (black and red, respectively). Relative percentage of total images analyzed are shown for each selected population (A–C). **$p = 0.009$.**
Cadm1-dependent adhesion increases alopecic T cell cytotoxicity

To determine whether Cadm1-dependent heterotypic adhesion between keratinocytes and alopecic lymphocytes promoted increased T cell activation or enhanced T cell-mediated killing, we performed in vitro cytokine secretion and cytotoxicity assays. We analyzed IL-2 and IFN-γ release as markers of T cell cytokine secretion using commercially available ELISA kits. We assessed T cell cytotoxicity by measuring mitochondrial LDH release and lymphocyte–keratinocyte rosette formation upon T cell–target cell coculture.

Coculture of alopecic lymphocytes with Cadm1-transduced primary human keratinocytes or transgenic K14Cadm1 C3H/HeJ strain murine keratinocytes did not stimulate release of IL-2 or IFN-γ (Supplemental Fig. 2A, 2B). Coculture of MHC-matched professional APCs (LK cells; derived the C3H/HeJ strain of mice that exhibits autoimmune alopecia) with a 10-fold excess alopecic lymph node cells did not stimulate IL-2 and IFN-γ secretion either (data not shown). However, there was a significant increase in IL-2 and IFN-γ release when a 100-fold excess of alopecic lymph node cells was cocultured with Cadm1-transduced LK cells (Fig. 5A; Supplemental Fig. 2C). Although Cadm1 was capable of pro-

**FIGURE 5.** Epithelial Cadm1 expression increases alopecic leukocyte cytotoxicity. A, Leukocyte IL-2 release from 10⁷ normal (black bars) or alopecic leukocytes (red bars) cultured alone or with 10⁵ control or Cadm1-transduced APCs (open and solid bars, respectively). B, Cytotoxicity (LDH release) of alopecic lymph node cells cultured with control or Cadm1-transduced keratinocytes (black and red bars, respectively). C and D, Keratin 14 (red) and CD45 (green) immunostaining of control (C) and Cadm1-transduced keratinocytes (D) cultured overnight with alopecic lymph node-derived cells. (E) Abundance of rosettes formed in cocultures of control (open bars) or Cadm1-transduced (solid bars) alopecic lymph node cells cultured with control or Cadm1-transduced keratinocytes (black and red bars, respectively). F, Keratin 14 (gray), CD45 (green), and CD3 (red) immunostaining of Cadm1-transduced keratinocytes cocultured with alopecic lymph node-derived cells. G, Abundance of rosettes in cocultures of control (black bars) or Cadm1-transduced keratinocytes (red bars) with unsorted (solid bar), CD3+ (vertical striped), and CD3− (shaded) alopecic lymph node cells. H, Abundance of rosettes in cocultures of control (black bar) or Cadm1-transduced keratinocytes (red bars) in the absence (shaded) or presence of CRTAM-Fc (open bar). Scale bars, 10 μm. Error bars represent SEM. *p < 0.05, **p < 0.005.
moting enhanced T cell cytokine secretion, this process required coordinated MHC engagement. These findings are consistent with previous reports of Cadm1–CRTAM interactions in lymph nodes and other tissues (15, 23).

Although Cadm1 expression in non-MHC matched cocultures did not promote cytokine secretion, we hypothesized that it might increase T cell-mediated cytotoxicity. To characterize this, we performed overnight lymphocyte–keratinocyte cocultures by mixing normal and alopecic C3H/HeJ peripheral lymph node cells with control and Cadm1-transduced primary human keratinocytes. We studied keratinocyte killing by measuring intracellular mitochondrial LDH release. Cadm1-transduced cells exhibited significantly greater LDH release than EV controls when cocultured with alopecic lymphocytes (Fig. 5B).

We also studied keratinocyte:lymphocyte rosette formation as an indicator of T cell cytotoxicity. In the absence of added lymphocytes, both Cadm1 and control keratinocytes were healthy. In contrast, numerous rosettes were present following overnight coculture of keratinocytes with lymphocytes (Fig. 5C–E); these were significantly increased when alopecic lymphocytes were mixed with Cadm1-transduced keratinocytes (Fig. 5D, 5E). We confirmed that rosettes represented interactions between keratinocytes and CD3+ T cells by performing immunolabeling for keratin 14, CD45, and CD3 (Fig. 5F). Anti-keratin 14 labeling detected the keratin filament network in control and Cadm1-transduced keratinocytes, while CD45-expressing hemopoietic cells coexpressed the lymphocyte marker CD3 (Fig. 5C, 5D, 5F).

We also sorted CD3+ T cells and CD3− (non T) cells from alopecic lymph nodes and compared their ability to form rosettes. CD3+ T cells were both necessary and sufficient to promote killing of Cadm1-transduced keratinocytes (Fig. 5G). Preincubation of keratinocytes with soluble human CRTAM-Fc abolished alopecic T cell mediated cytotoxicity (Fig. 5H). This finding provides strong evidence that direct Cadm1–CRTAM interactions mediate T cell-keratinocyte rosette formation and cytotoxicity.

**Epithelial Cadm1 expression promotes alopecia and skin inflammation in vivo**

We next investigated whether epithelial Cadm1 expression influenced autoimmune alopecic susceptibility or severity in vivo. We did not investigate this using existing CRTAM and Cadm1 knockout mice, as previous studies have demonstrated that Cadm1–CRTAM interactions within lymph nodes can regulate systemic immune cells (15, 17, 24). To evaluate local effects in the epidermis, we generated C3H/HeJ strain Keratin 14 (K14) Cadm1 transgenic mice. Keratin 14 is specifically expressed by epithelial cells and not by lymphocytes (30).

The expression of Cadm1 in C3H/HeJ strain K14-Cadm1 transgenic mice was comparable to that reported previously for FVB/n strain K14-Cadm1 mice (line 7248A.5; see 25; Fig. 6A). C3H/HeJ strain K14-Cadm1 transgenics exhibited normal growth and development and there were no overt skin abnormalities. There were no differences in the incidence of spontaneous autoimmune alopecia between K14-Cadm1 and wild type C3H/HeJ strain mice. However, flow cytometric analysis revealed a moderate increase in the number of cells expressing the bulge stem cell marker CD34 (Fig. 6B, 6C).

To determine whether K14-Cadm1 transgenic mice exhibited altered autoimmune alopecic susceptibility or severity, we injected wild type and transgenic mice with alopecic mouse-derived cervical, brachial, and inguinal lymph node cells from alopecic mice. K14-Cadm1 transgenic mice exhibited significantly increased disease incidence relative to wild type littermates (Fig. 6D; 76.5% versus 41.7%; p = 0.0452). Representative wild type and transgenic recipients 12 wk after cell transfer are shown in Fig. 6E and 6F.

To assess disease severity, we examined H&E-stained sections of skin from affected individuals. Alopecic wild type mice exhibited lymphocyte accumulation at hair follicle bulbs and sebaceous glands (Fig. 7A, A′) (8). In contrast, lymphocytes were found in K14-Cadm1 alopecic skin along the full length of the hair follicles, including the bulge and extending to the underlying muscle layer (Fig. 7B, B′, arrowheads). Ki67 immunostaining revealed extensive lymphocyte proliferation in both transgene positive and transgene negative skin (Fig. 6C, 6D). Thus the primary effect of

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**FIGURE 6.** Cadm1 expression, epithelial stem cell abundance, and alopecia incidence are increased in K14-Cadm1 transgenic mice. A, Flow cytometric analysis of Cadm1 expression in CD34+Itga6+ stem cells of wild type (black line) and transgenic mice (red line). B and C, Flow cytometric analysis of viable, CD45−o6−CD34+ (red boxes), and CD34−o6− (black boxes) cells in wild type (B) and K14-Cadm1 transgenic (C) mouse epidermal cells. The abundance of each cell population is indicated in parentheses. D, Kaplan-Meier plot of autoimmune alopecia incidence in wild type and K14-Cadm1 transgenic mice (black and red lines, respectively). E and F, Representative images of wild type (E) and K14-Cadm1 (F) mice 12 wk after lymphocyte transfer. *p < 0.05, log-rank test.
Cadm-1 overexpression was to direct the lymphocyte infiltrate to associate more widely with the hair follicles.

**Discussion**

Our data reveal a previously unknown link between epithelial and immune cell adhesiveness and in vivo autoimmune alopecia susceptibility. We have demonstrated that the cell adhesion molecule Cadm1, which is highly expressed by hair follicle bulge stem cells (25), promotes heterotypic adhesion of CRTAM-expressing, activated, alopecic CD8-expressing cytotoxic T lymphocytes. The Cadm1–CRTAM interaction promoted enhanced T cell-keratinocyte adhesion and lymphocyte-dependent keratinocyte cytotoxicity. Epidermal overexpression of Cadm1 was not sufficient to cause alopecia, but did increase the incidence and severity of the disease. In alopecic epidermis, more cytotoxic T lymphocytes were in an early activation state, and there were increased numbers of CRTAM+ NK cells compared with control epidermis, which is consistent with increased cytotoxicity toward keratinocytes. CRTAM can be transiently expressed in the early stage of T cell activation (21), but it can also be expressed by mature activated CD8+ and NK cells, where it mediates late-stage activation (15).

Based on our results, we propose a model for the role of the hair follicle in autoimmune alopecia (Fig. 7E). In this model, injury or infection disrupts normal epidermal homeostasis, resulting in a wound-healing response as well as inflammatory and immune cell infiltrates (Fig. 7E, step 1). In susceptible individuals, activated CD4+ and cytotoxic T lymphocytes recognize as-yet unidentified, follicular keratinocyte-specific self-antigens, resulting in initiation of tissue autoimmunity (Fig. 7E, step 2). Subsequently, activated cytotoxic T lymphocytes adhere to the hair follicles via heterotypic CRTAM-Cadm1 adhesion. This results in keratinocyte killing and hair follicle destruction (Fig. 7E, step 3). Our model predicts that disruption of CRTAM-Cadm1 adhesion could be used to treat autoimmune alopecia.

**FIGURE 7.** Transgenic Cadm1 overexpression promotes alopecia-dependent skin inflammation and lymphocyte proliferation. A and B, H&E-stained skin sections from alopecic wild type (A) and K14-Cadm1 transgenic mice (B). C and D, Ki67 immunostaining (brown) of alopecic wild type (C) and K14-Cadm1 (D) skin. Tissue sections in C and D are adjacent serial sections to A and B, respectively. Boxed areas in A, B, C, and D are shown at higher magnification in A’, B’, C’, and D’. Arrowheads in A and B denote regions of robust lymphocyte accumulation. E, A model of the role of CRTAM–Cadm1 adhesive interactions in autoimmune alopecia initiation and progression. Scale bars, 100 μm.
We reported previously that Cadm1 expression in the hair follicle bulge promotes intercellular adhesion and stem cell quiescence (25). Enforced overexpression of Cadm1 inhibits keratinocyte motility and delays epidermal wound healing. Cadm1 expression is transiently downregulated during normal wound healing, and this could facilitate epidermal cell proliferation and migration (25). Our new data suggest that decreased Cadm1 expression also minimizes potential CRTAM-mediated T cell-dependent tissue cytotoxicity during wound repair. The increase in proliferation of bulge stem cells in alopoeic follicles also suggests that CRTAM engagement can override Cadm1-mediated stem cell quiescence.

Cadm1–CRTAM interactions regulate T cell function in an MHC-independent manner in several different contexts (20, 21). Cadm1–CRTAM adhesion promotes Cadm1-expressing tumor cell immunosurveillance and tumor cell clearance (22), as well as late-stage T cell maturation within secondary lymphoid tissues (15, 17, 23, 24). Cadm1 has also been shown recently to interact with the $\zeta$-chain of the TCR, enhancing TCR activation and promoting T cell interactions with APCs (31). Taken together with our observations, these findings suggest that CRTAM–Cadm1 interactions in both lymph nodes and target tissues are involved in a variety of different autoimmune disorders.

In conclusion, Cadm1, like other cell adhesion molecules (integrins, p120, CD200), regulates intrinsic cellular effector functions (e.g., adhesion, migration, cellular quiescence) (32) and mediates interactions with bone marrow-derived cells to regulate skin inflammation and immunity (33–35). It will now be of interest to investigate whether Cadm1–CRTAM interactions are associated with other skin disorders involving T cell-mediated cytotoxicity, such as lichen planus, graft-versus-host disease, dermatomyositis, and systemic lupus erythematosus.

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Disclosures
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