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T-Cell Reconstitution after Thymus Xenotransplantation Induces Hair Depigmentation and Loss

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Here we present a mouse model for T-cell targeting of hair follicles, linking the pathogenesis of alopecia to that of depigmentation disorders. Clinically, thymus transplantation has been successfully used to treat T-cell immunodeficiency in congenital athymia, but is associated with autoimmunity. We established a mouse model of thymus transplantation by subcutaneously implanting human thymus tissue into athymic C57BL/6 nude mice. These xenografts supported mouse T-cell development. Surprisingly, we did not detect multiorgan autoimmune disease. However, in all transplanted mice, we noted a striking depigmentation and loss of hair follicles. Transfer of T cells from transplanted nudes to syngeneic black-coated RAG$^{-/-}$ recipients caused progressive, persistent coat-hair whitening, which preceded patchy hair loss in depigmented areas. Further transfer experiments revealed that these phenomena could be induced by CD4+ T cells alone. Immunofluorescent analysis suggested that Trp2+ melanocyte-lineage cells were decreased in depigmented hair follicles, and pathogenic T cells upregulated activation markers when exposed to C57BL/6 melanocytes *in vitro*, suggesting that these T cells are not tolerant to self-melanocyte antigens. Our data raise interesting questions about the mechanisms underlying tissue-specific tolerance to skin antigens.

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INTRODUCTION

Alopecia is a disfiguring form of hair loss, which may be reversible, or irreversible, as in scarring alopecias. There are several subtypes of immune-mediated hair loss, ranging in severity from small isolated patches of reversible alopecia (alopecia areata) to extensive or total loss of body hair (alopecia totalis, AT; alopecia universalis), which in some cases is permanent (Lew *et al.*, 2009). Disease is thought to occur when autoreactive T cells attack hair follicles, which are normally immune privileged (Gilhar *et al.*, 1999, 2007). Follicular melanocytes are frequently damaged in early alopecia lesions (Tobin *et al.*, 1990). Clinically, it has been noted that hair regrowth is often white, and that pigmented hairs may be preferentially targeted during alopecia (Gilhar *et al.*, 2007). Autoimmune vitiligo in humans typically presents as patchy skin depigmentation resulting from a T-cell response against self-melanocytes (Ongenae *et al.*, 2003). The etiology of autoimmune subtypes of vitiligo and alopecia is not fully understood, but is likely to involve the loss of tolerance to skin antigens.

Here we used the athymic nude mouse (Pantelouris, 1968; Nehls *et al.*, 1994) as a recipient for human thymus grafts, to establish a model of thymus transplantation for studying tolerance induction and autoimmunity. Complete DiGeorge Syndrome, a rare, fatal congenital athymia, is treated by unmatched thymus transplantation, which reconstitutes naive T-cell output, although not to normal levels (Markert *et al.*, 2007). Autoimmunity has been observed in one-third of thymus transplant recipients (Markert *et al.*, 2007; Levy *et al.*, 2012), presumably due to major histocompatibility complex (MHC) mismatching.

Here we show that *de novo* mouse T-cell development is induced by transplanting human thymus epithelium into T cell–deficient nude mice, and that autoimmunity, consistently and predominantly directed at hair follicles, resulted from the presence of this thymus graft. We characterize the cellular mechanisms underlying this skin-specific immune response after thymus xenotransplantation. We present an inducible mouse model for depigmentation and hair loss, and suggest that there are as yet uncharacterized mechanisms driving induction of tolerance to skin self-antigens.

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Abbreviations: AT, alopecia totalis; MHC, major histocompatibility complex; NK, natural killer; PBL, peripheral blood lymphocytes; WT, wild type

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RESULTS

Human thymus tissue supports murine T-cell development in athymic nude mice

To investigate mechanisms driving autoimmunity *in vivo*, we established a mouse model of thymus transplantation. We implanted fragments of fresh or cultured (Supplementary Table S1 online) human thymus subcutaneously into the scruff of nude mice, providing a thymic epithelial niche, which expressed non-self-MHC and species-inappropriate antigens. We used subcutaneous implantation because clinical transplantations are performed in the thigh fascia (Markert *et al.*, 1997).

Nude mice lack conventional T cells because of congenital athymia. Other lymphocytes are present, including B cells, which are functionally limited by the lack of T-cell help (Mongini *et al.*, 1981), natural killer (NK) cells, some NKT and γδ-T cells, and gut intra-epithelial lymphocytes (De Geus *et al.*, 1990). Therefore, to screen blood for conventional T cells, we used a stringent gating strategy to remove B cells, CD3-expressing NK cells, or γδ-T cells/intra-epithelial lymphocytes, which may express CD8, and dendritic cells that may express CD4/CD8 (Figure 1a). Nude mice transplanted with human thymus tissue (Nu-Tp) accepted xenografts and displayed mature T cells in peripheral blood

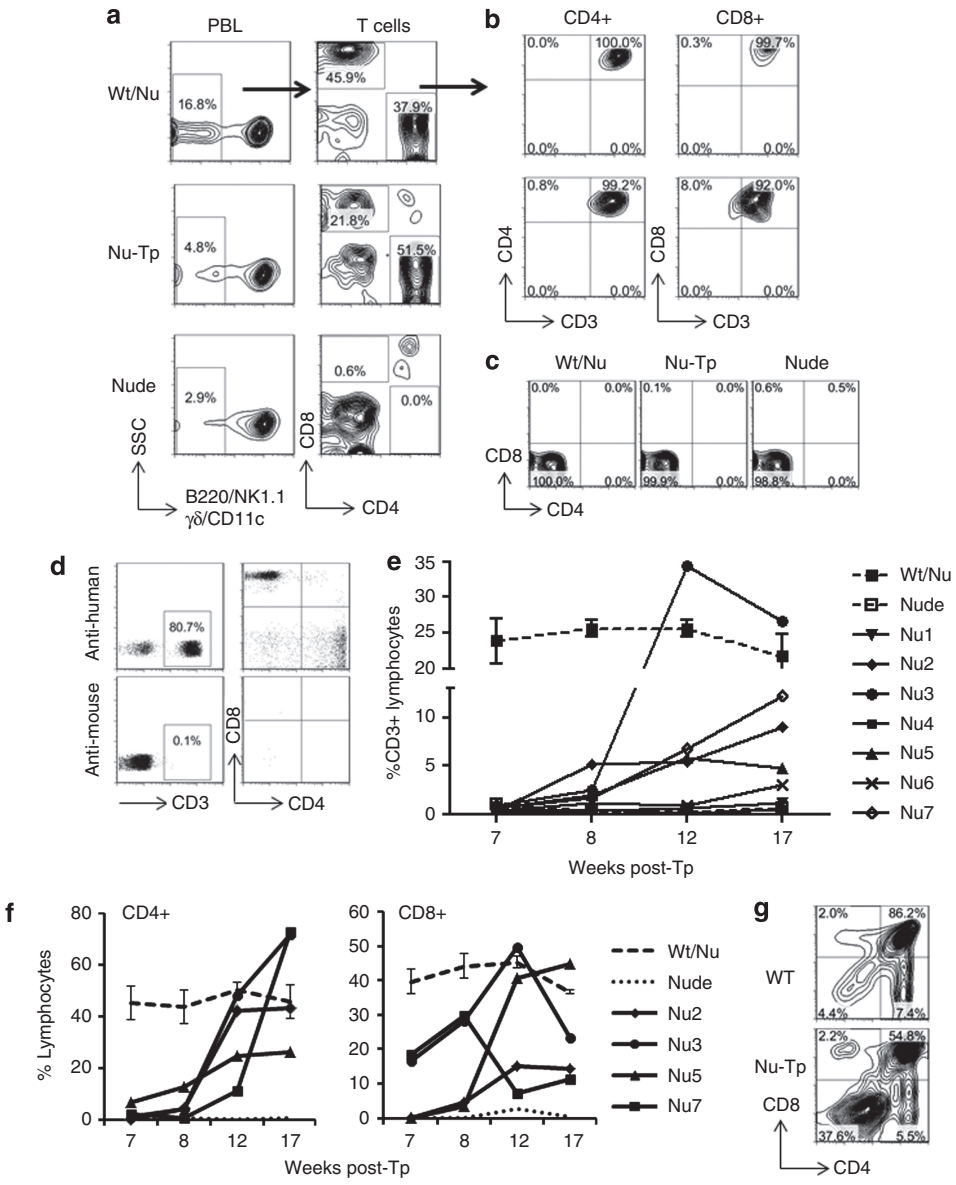

Figure 1. *De novo* T-cell development in nude mice with human thymus xenografts. T-cell output following transplantation was assessed by flow cytometric analysis of peripheral blood lymphocytes (PBL). Control nudes received no graft; Wt/Nu are immunocompetent. (**a**) CD4+/CD8+ T cells identified by gating on PBL (B, NK, γδT, and dendritic cells were excluded). Example staining at 18 weeks post transplant is shown. (**b**) CD3 expression on CD4+ and CD8+ PBL. Example staining of (**c**) mouse PBL with anti-human antibodies, and of (**d**) human PBL with anti-human and anti-mouse antibodies. (**e**) Time-course analysis of % CD3+ T cells in blood. (**f**) % CD4+ and CD8+ cells as shown in **a** for four Nu-Tp mice. (**g**) Anti-mouse staining of normal mouse thymus and tissue retrieved from Nu-Tp transplantation site.

lymphocytes (PBL) 12 weeks post transplant (Figure 1a and b). These T cells were of mouse origin, staining only with anti-mouse antibodies. No T cells of human origin were detected in transplanted mice (Figure 1c), indicating that thymus-resident human T cells did not persist. Anti-mouse antibodies did not bind human lymphocytes (Figure 1d). Most transplanted mice showed variable but lower proportions of CD3+ cells than Wt/Nu immunocompetent control animals. Wt/Nu mice had on average 23.6% ± 5.7 CD3+ PBL, whereas Nu-Tp displayed 3.0% ± 1.9 at 8 weeks, 6.1% ± 0.7 at 12 weeks, and 8.5% ± 3.9 at 17 weeks post transplant. A small number of transplants did not graft, likely because of failed implantation, with <1% T cells in the PBL gate (Figure 1a and e, Supplementary Table S1 online).

Reconstitution of T-cell subsets showed different kinetics, with CD4+ cells appearing in PBL 2–4 weeks later than CD8+ cells (Figure 1f). Postmortem, we detected immature mouse T cells (CD4+CD8+CD3−) in disaggregated subcutaneous scruff tissue (Figure 1g), demonstrating that T-cell development occurred in Nu-Tp animals.

To confirm that resident nude bone marrow–derived precursors were the source of *de novo* T cells, we performed transplants into Nude-RAG2$^{-/-}$IL2R$\gamma^{-/-}$ mice, which lack a thymus and functional murine T precursors. As expected, these mice were unable to generate T cells after human thymus grafting (data not shown), remaining profoundly lymphopaenic.

Nude mice given human thymus xenografts develop depigmentation and hair loss

C57BL/6 nude mice lack fur because of abortive hair growth, but are not strictly hairless. The presence of hair follicles, which produce hairs that are unable to fully penetrate the epidermis, give the skin a black color (Militzer, 2001). Striping is observed as these hairs progress through murine hair cycling, and sparse patches of short black hair are occasionally seen (Eaton, 1976). After appearance of PBL in Nu-Tp mice, we observed macroscopic changes in body coloration, where skin appeared smooth and pink compared with the gray/black striping of untransplanted control nude mice (Figure 2a). We noted sparse areas of white hair, particularly on the face (Figure 2a), which were ultimately lost. We only observed this in mice that developed T cells after thymus grafting (Supplementary Table S1 online). We performed 11 grafts using tissue from five independent human donors. Three of these transplants failed. Of the remaining mice, all eight nude recipients displayed this characteristic skin phenotype.

Postmortem skin from Nu-Tp mice was thin, liable to tear, and had lost its black/gray coloration (Figure 2b). Skin histology revealed hair follicle dystrophy and loss in Nu-Tp (Supplementary Figure S2 online, Figure 2c–f) compared with control nude samples (Supplementary Figure S2 online, Figure 2g–j). Hair follicle disruption was widespread in Nu-Tp skin, although we were able to identify occasional follicles in anagen (Figure 2c–f), most of which were not pigmented. Cellular infiltrates were seen around the hair follicles, frequently in the upper region (Figure 2c and d). We did not observe epidermal hyperplasia, cysts, trichogranulomas, fibrotic lesions or external rashes, scarring, scaling, ulceration/blistering, redness, swelling, or dermatitis (Sundberg *et al.*, 2011) in Nu-Tp mice. Nu-Tp hypodermis was severely disrupted, in several cases showing almost total loss of hair follicles, sebaceous glands, and subcutaneous fat (Supplementary Figure S2 online). Occasionally, hair follicle remnants were observed, although we did not observe hair regrowth in any animal during our experiments (6 months post transplant). Histomorphometry revealed that Nu-Tp skin showed significantly fewer follicles than non-transplanted controls, and most remaining hairs observed in Nu-Tp samples were white (Figure 2k).

This phenotype occurred after *de novo* T-cell output, suggesting that depigmentation and hair loss were mediated by a T-cell response against hair follicles, resembling autoimmune alopecia. We therefore examined the lymphocyte composition of skin from transplanted animals, observing CD4^{hi}CD3^{hi} T cells in Nu-Tp skin (Figure 2l), which were largely absent in nude controls. Interestingly, the proportion of CD4+ cells in digests was higher in Nu-Tp than in immunocompetent wild-type (WT) mice, typically 6.7% of live cells compared with 2.4%, respectively. Increased proportions of CD3+ T cells suggested enhanced infiltration or expansion of CD4+ T cells in Nu-Tp mice. Transplanted mice showed lower proportions of PBL than control immunocompetent animals (Figure 1e), and low but detectable populations of T cells in the spleen (not shown). Therefore, the presence of skin-resident T cells in these animals is likely to be functionally significant.

Nude mice are widely used for xenotransplantation, accepting many grafts, including human tissues, with no reports of adverse ''unconventional'' host immune responses or pathological dermatological effects (Manning *et al.*, 1973; Gershwin *et al.*, 1977; Drago *et al.*, 1979). Therefore, in our model, it seems likely that the human tissue provides a niche in which host murine T-cell precursors can develop. Mouse TCR can bind and be selected on human MHC (Kievits *et al.*, 1987), and the development and thymic selection of mouse TCR on HLA can occur without a human coreceptor (Altmann *et al.*, 1995). In this case, an MHC mismatch would exist between the T cells' specificity and the host tissues, which express ''foreign'' murine MHC molecules. A widespread autoimmune syndrome might therefore be expected. However, we noted no other pathology in Nu-Tp mice displaying hair loss. To formally rule out chronic inflammation and autoimmunity, we screened several solid organs. We saw no histological abnormalities in Nu-Tp specimens, indicating that species mismatch between thymus and host tissues did not provoke multiorgan autoimmune disease (Supplementary Figure S1 online).

Adoptive transfer of transplanted nude lymphocytes to immunodeficient mice causes coat depigmentation

Hair biology in nude mice is abnormal, and our observations may relate to an underlying defect in nude skin. To test this and investigate the mechanism of hair follicle targeting, we collected lymphocytes from Nu-Tp mice with/without alopecia and adoptively transferred them into black-coated

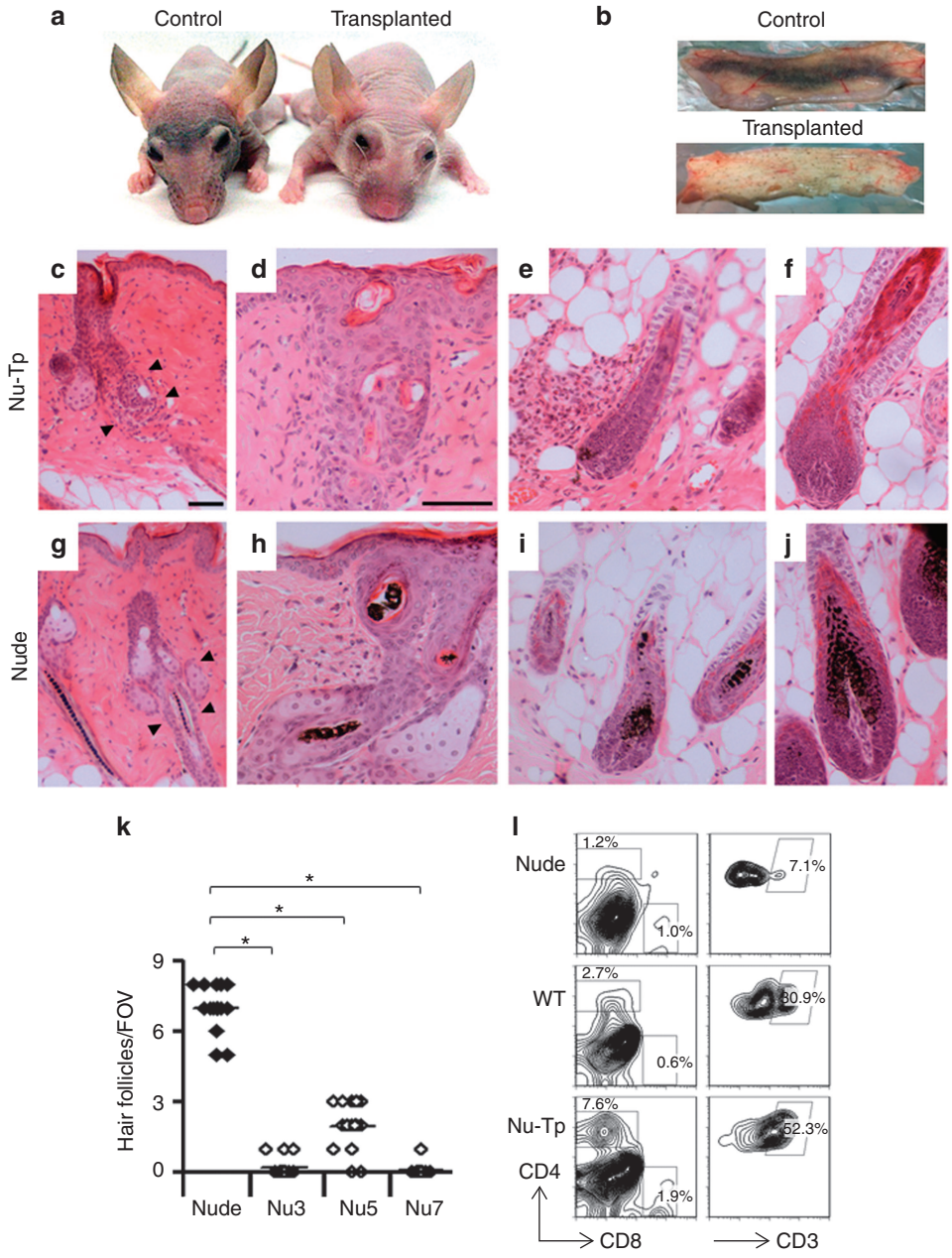

Figure 2. Nude mice with functional thymus grafts develop profound hair loss. (**a**) Gross anatomy of control (left, pigmented) or Nu-Tp (right, depigmented) mice. (**b**) Loss of hair and thinning of Nu-Tp skin. Equivalent regions in Nu-Tp (**c**–**f**) and nude control (**g**–**j**) skin by hematoxylin and eosin histology. Pigmented hairs are seen in control nude skin but not in Nu-Tp skin. Arrows show immune infiltrates around (**c**) Nu-Tp and (**g**) control follicles. (**k**) Intact black/white hair follicles per field of view (FOV, $n = 15$/sample at intervals through specimen, $^*P < 0.0001$) were quantified under low-power microscopy (see Supplementary Figure S2 online). Nude mean: 7.0 ± 1.0, median: 7; Nu3 mean: 0.2 ± 0.4, median: 0; Nu5 mean: 1.9 ± 1.0, median: 2; Nu7 mean: 0.07 ± 0.3, median: 0. (**l**) Digested dorsal skin samples analyzed by flow cytometry for skin-resident T cells. WT, wild type.

lymphocyte-deficient C57BL/6 RAG$^{-/-}$ mice (Figure 3a, AT1). We detected CD4+ and CD8+ PBL in mice given Nu-Tp alopecia lymphocytes, indicating successful transfer. No T cells were seen in RAG$^{-/-}$ hosts receiving no cells, or cells from lymph node of control/non-alopecia nude mice (not shown). Remarkably, at 8 weeks post transfer, mice that received cells from Nu-Tp with alopecia developed a striking coat depigmentation, manifesting initially in a white dorsal stripe (Figure 3b). At 15 weeks post transfer, this depigmentation had spread (Figure 3c), and eventually white areas showed hair loss (Figure 3d and e). This experiment (Figure 3a) was repeated with independent human thymus, nude host, and RAG$^{-/-}$ recipients. The same results were seen, except that depigmentation began over the cranium and extended down the body (Figure 3f). Interestingly, our phenotype resembled vitiligo-like autoimmunity seen in mice with a transgenic TCR specific for Trp-1, a melanocyte antigen (Muranski *et al.*, 2008).

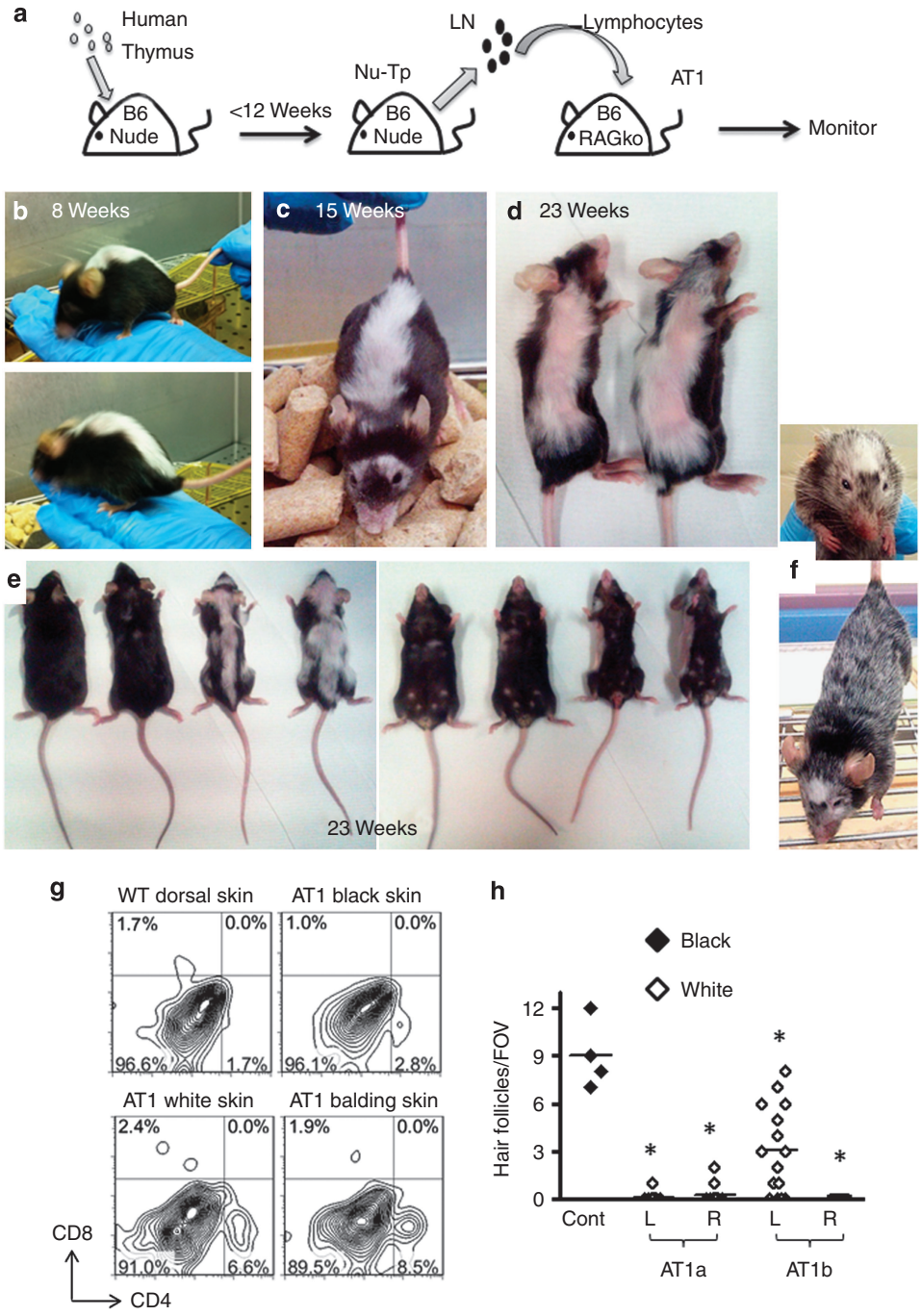

Figure 3. Adoptive transfer of lymphocytes from Nu-Tp into hairy immunocompromized mice causes striking coat depigmentation. (**a**) Lymph node (LN) cells were injected intravenously into RAG$^{-/-}$ recipients (AT1). (**b**–**e**) Progressive coat depigmentation in AT1 mice. (**f**) Independent repeat of **a**. (**g**) Flow cytometric analysis of skin from wild type (WT) (immunocompetent) and black, white, and balding areas of AT1 RAG$^{-/-}$ mouse. Non-transferred and sham-transferred RAG$^{-/-}$ mice showed no skin phenotype (first/second mice from left in **e**) or T cells in blood (not shown). (**h**) Quantification of intact hair follicles per field of view (FOV) (*$P<0.001$, see Supplementary Figure S2 online). Control mean: 9.0 ± 2.2, median: 8.5; AT1a left flank mean: 0.1 ± 0.3, median: 0; AT1a right flank mean: 0.3 ± 0.6, median: 0; AT1b left flank mean: 3.1 ± 2.8, median: 3; AT1b right flank mean: 0, median: 0.

We found T cells in skin from depigmented mice (Figure 3g), a greater proportion of which expressed CD4 and CD3 compared with WT controls. Significantly fewer intact follicles were observed in AT1 skin compared with WT animals (Supplementary Figure S2b online, Figure 3h). AT1 hair follicles appeared microscopically to lack pigment, even in sections from areas of black fur, suggesting an active process of depigmentation before hair loss.

Histological analysis of AT1 skin showed hair follicle dystrophy compared with control skin (Supplementary Figure S2b online). Depigmented hairs (Figure 4a–d), or hairs with little pigment compared with controls (Figure 4e–g), were

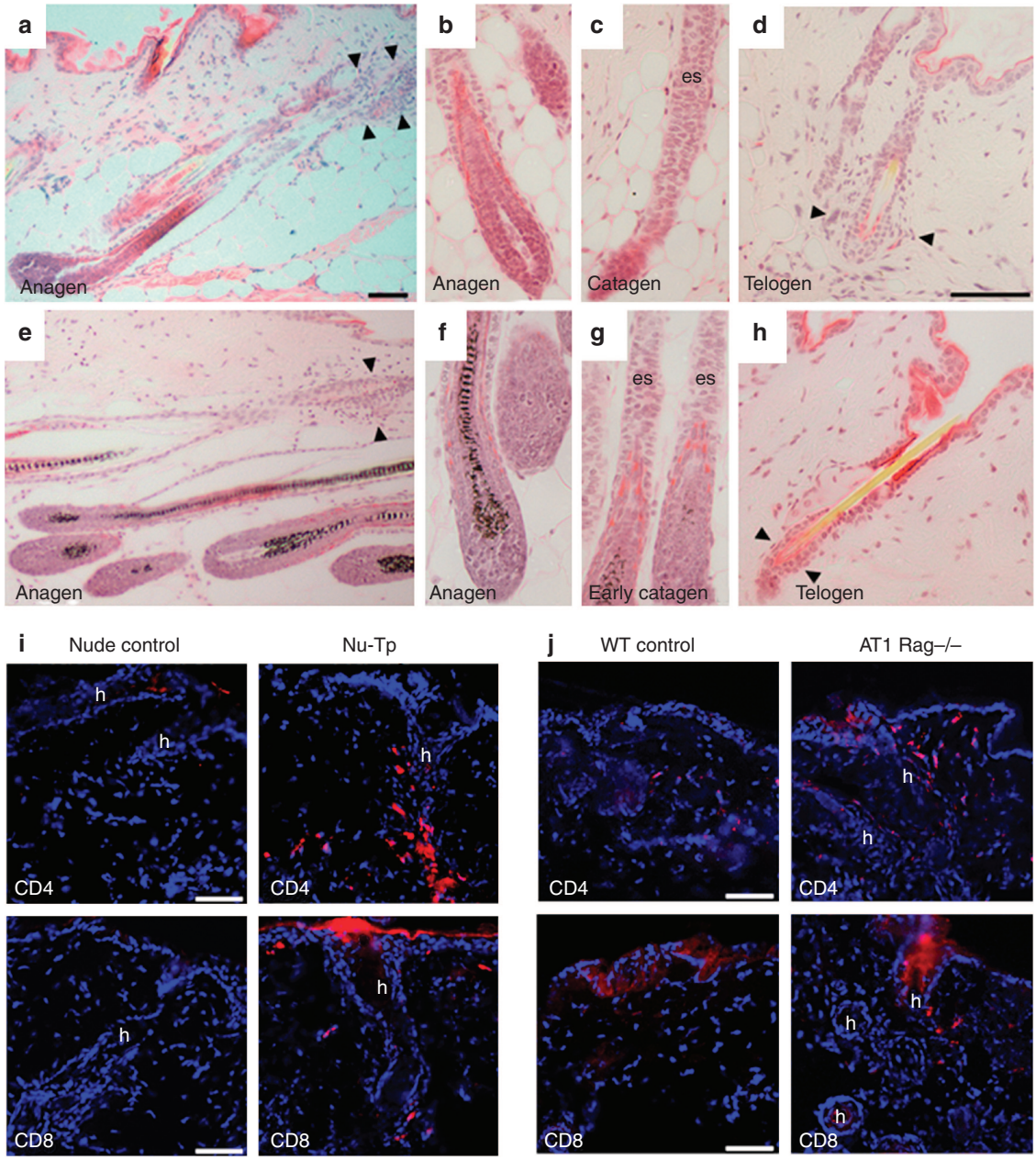

Figure 4. T cells were observed in skin of RAG$^{-/-}$ AT1 mice with depigmentation. (**a**–**d**) Hematoxylin and eosin stain and histology of skin from AT1 RAG$^{-/-}$ mice and (**e**–**h**) controls; bars = 100 μm. Analysis of equivalent regions in AT1 and control skin during (**a**, **b**, **e**, **f**) anagen, (**c**, **g**) catagen, (**d**, **h**) and telogen. Arrows show cellular infiltrates around the follicular bulge region in (**a**) anagen and bulb in (**d**) telogen and equivalent locations in (**e**, **h**) control skin. (**c**, **g**: es) Catagen epithelial strands appear normal. (**i**, **j**) Immunofluorescent identification of CD4+ and CD8+ cells in skin of (**i**) Nu-Tp and nude control, and (**j**) AT1 Rag$^{-/-}$ and wild-type (WT) control mice. Control sections (no primary antibody) did not show staining (not shown). ''h'' on images denotes hair follicles.

seen. We noted occasional swarms of cells around follicles, particularly in the infundibular region of anagen follicles, and around the receding bulb of telogen hairs (Muller-Rover *et al.*, 2001) (Figure 4a and d).

More infiltrating CD4+ cells were observed around follicles and in the dermis of Nu-Tp (Figure 4i) and AT1 (Figure 4j) skin than in control sections. CD8+ cells were present in skin from experimental animals, although at lower frequencies than CD4+ cells (Figure 4), suggesting that in our model CD4+ cells are active in skin.

CD4+ T cells cause depigmentation and hair loss in thymus xenotransplantation–induced autoimmunity, possibly by targeting melanocytes

To dissect the cellular mechanism and identify the reactive T-cell subset/s, we performed further adoptive transfer experiments (Figure 5a). Lymphocytes were collected from lymph node of AT1 experimental animals (mice in Figure 3b–e), and T cells were fractionated by fluorescence-activated cell sorting. CD3+CD4+, CD3+CD8+, and CD3+γδTCR+ cells were purified (>98% pure, Figure 5b) and transferred into

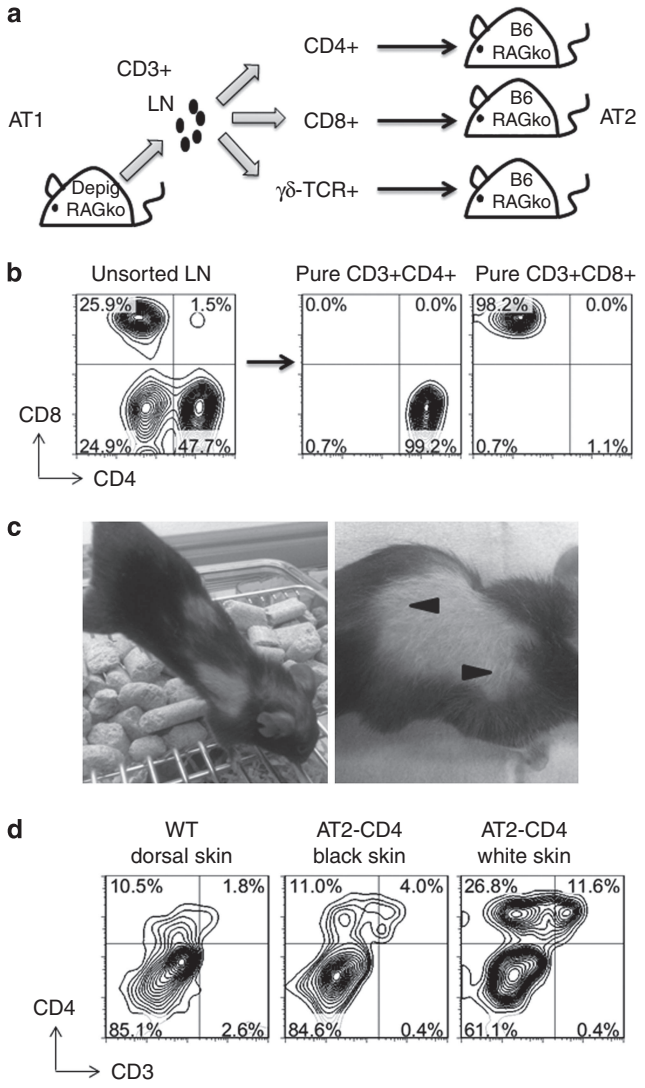

Figure 5. CD4+ cells alone from depigmented mice caused depigmentation and hair loss in further immunocompromised animals. (**a**) CD4+, CD8+, and γδTCR+ LN (lymph node) lymphocytes were sorted from AT1 RAG$^{-/-}$ depigmented mice by FACS and adoptively transferred into further RAG$^{-/-}$ recipients (AT2). (**b**) Purity of sorted populations confirmed by flow cytometry. (**c**) Receipt of CD4+ cells only caused areas of fur thinning, where the remaining hair was downy and depigmented. (**d**) The proportion of CD4+CD3hi cells was assessed in white and black areas of AT2-CD4 skin in comparison with wild-type (WT) control skin by flow cytometry.

new RAG$^{-/-}$ recipients. At 16 weeks post transfer, a patch of localized alopecia surrounded by downy white hairs was observed on the shoulder of an animal that had received CD4+ cells (Figure 5c). CD3+CD4+ T cells were identified in skin from this area (Figure 5d). Mice that received CD8+ and γδ-T cells were kept for 21 weeks post transfer, but did not show any skin phenotype (not shown). Thus, CD4+ cells in isolation can cause pathology.

Given the hair whitening, loss of follicles in Nu-Tp mice, and subsequent widespread depigmentation of furred mice in adoptive transfer experiments, we hypothesized that melanocytes were the target-cell population for T cell–mediated autoimmune attack. Supporting this, we observed decreased levels of Trp2 staining in Nu-Tp hair follicles compared with controls, suggesting that there are fewer melanocyte-lineage cells in Nu-Tp skin (Figure 6a). We therefore tested responsiveness of Nu-Tp T cells to murine melanocytes. Splenocytes (including host antigen-presenting cells) from Nu-Tp and WT mice were cultured with C57BL/6 melan-a cells, a transformed melanocyte cell line, which we irradiated to prevent rapid proliferation. The proportion of T cells in Nu-Tp splenocyte cultures was lower than WT, reflecting suboptimal T-cell reconstitution (Figure 1e). However, despite the low frequency of T cells, after 48 hours in culture, a higher proportion of CD4+ T cells from Nu-Tp spleen expressed the T-cell activation marker CD25, compared with WT splenocyte cultures, indicating that a larger proportion of T cells in the mixed culture were activated by the presence of melanocytes (Figure 6b). A higher proportion of T cells in melan-a+Nu-Tp spleen cocultures were of memory phenotype (CD44^{hi}CD62L^{lo}) compared with melan-a+WT spleen cultures (Figure 6c). The proportion of naive T cells (CD44^{lo}CD62L^{+}) was low in the Nu-Tp culture, whereas naive T cells were observed in melan-a+WT spleen cocultures (Figure 6c).

DISCUSSION

Here we showed that subcutaneous grafting of T cell–deficient nude mice with human thymus fragments restored murine T-cell development, despite the absence of species-appropriate signals that might be required for grafting, vascularization and colonization of human tissue. We observed murine T cells in Nu-Tp blood for >6 months post transplant, suggesting that the graft continues to function, and/or that human MHC is not required to support peripheral T-cell survival. The proportion of T cells in blood and lymphoid organs was low, as observed after thymus allografting in other systems (Yan *et al.*, 2003; Markert *et al.*, 2007); thus, tissue mismatching may impair positive selection and peripheral T-cell homeostasis.

We did not observe multiorgan autoimmunity in the Nu-Tp mice, suggesting either that graft-educated cells were unreactive, low-affinity clones or that some degree of self-tolerance was induced, centrally or in peripheral tissues. We cannot exclude subclinical autoimmunity, but some transplanted mice were kept for over 35 weeks post transplant without visible non-dermatological disease. All transplanted animals developed an alopecia-like disorder, resulting in severe hair follicle disruption and hair loss, which was not due to companion barbering. This occurred irrespective of human thymus donor. *In vitro* assays indicated that unprimed T cells from Nu-Tp mice activated in response to syngeneic melanocytes, and transfer of pathogenic Nu-Tp T cells to black-coated animals, led to significant depigmentation and subsequent localized hair loss. We therefore propose that a proportion of graft-educated mouse antigen–intolerant T cells attack follicular melanocytes, causing depigmentation. Loss of melanocytes then compromises follicle integrity/stability, leading to hair loss. Further, targeting of melanocyte-lineage cells in the infundibular region may disrupt the bulge stem-cell niche, which could prevent hair regrowth. This requires future investigation, as follicles may remain dormant for some time. In addition, as we were able to identify some remaining

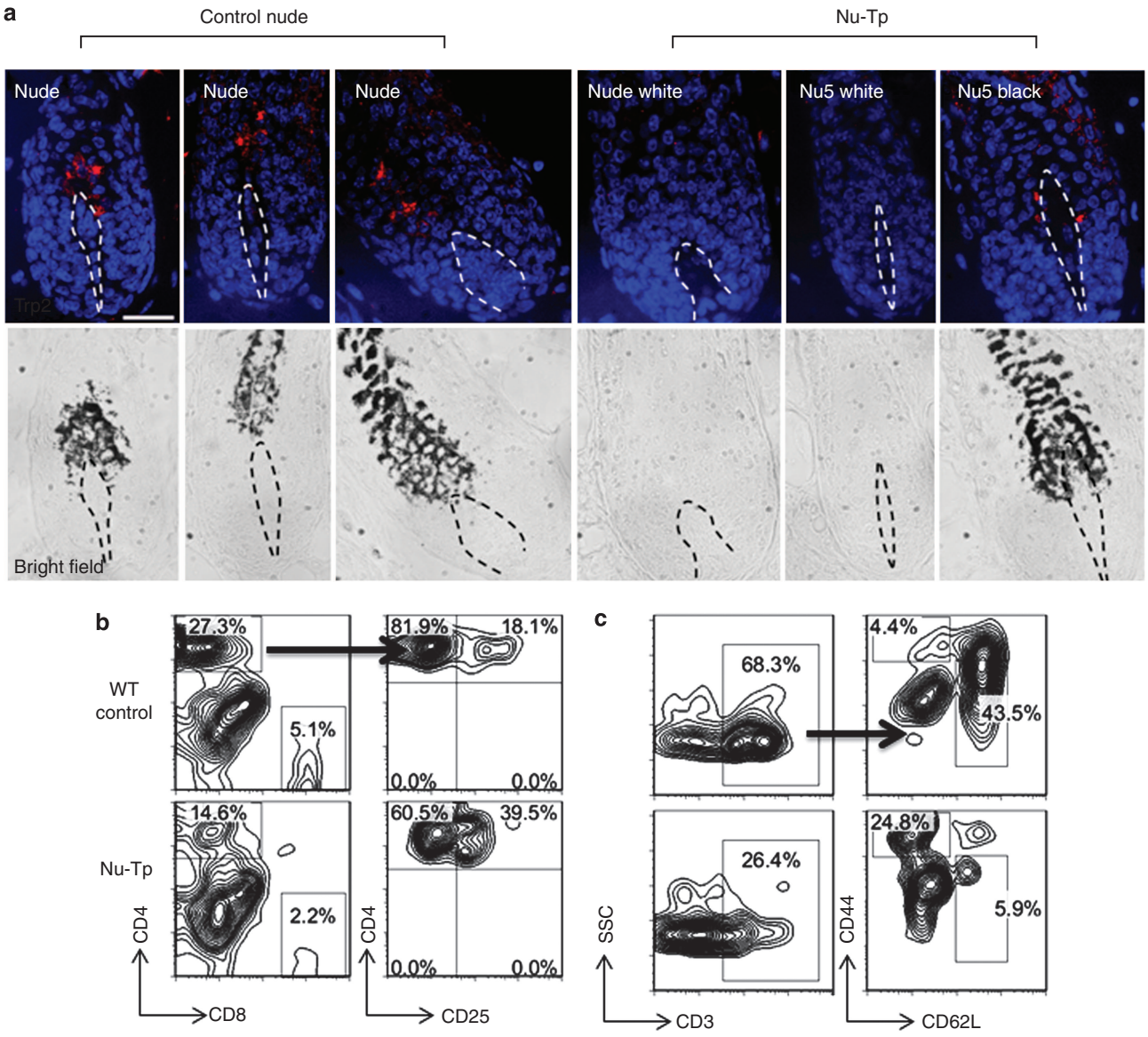

Figure 6. Melanocyte-lineage cells are decreased in Nu-Tp skin, and Nu-Tp T cells activate in response to syngeneic melanocytes. (**a**) Trp2 staining in hair bulbs of control and Nu-Tp (Nu5), coincident with regions containing melanocytes/melanosomes, as shown in the companion bright-field view. Note the reduction of Trp2 staining in the remaining black hairs in the Nu5-transplanted mouse. Dotted lines denote the dermal papilla–matrix boundary. Bar = 50 μm. (**b**) The expression of CD25 (T-cell activation marker) on CD4 + cells from Nu-Tp spleen or CD4 + B6 splenocytes after 48 hours of exposure to syngeneic B6 melanocytes (irradiated melan-a). (**c**) The proportion of effector memory CD4 + cells (CD4 + $CD44^{hi}CD62L^{lo}$) and naive (CD4 + CD62L + CD44lo) cells in Nu-Tp spleen + B6 melanocyte cocultures compared with B6 spleen + B6 melanocyte cocultures. WT, wild type.

follicles in nude mice (Nu5), follicular destruction was not necessarily complete or irreversible during the time frame of our experiments.

Spontaneous rodent models of alopecia and vitiligo are available, but incidence of disease is low and unpredictable (Lerner *et al.*, 1986; Sun *et al.*, 2008). The C3H/HeJ skin grafting mouse model has provided insight into the pathogenesis of murine alopecia areata (McElwee *et al.*, 1998, 2002, 2005; Sun *et al.*, 2008), but this elegant model requires appearance of initial disease and surgical expertise. Recently, models of hair follicle autoimmunity have been engineered by transgenic expression of melanocyte antigen-specific TCR (Lambe *et al.*, 2006; Gregg *et al.*, 2010; Alli *et al.*, 2012; Harris *et al.*, 2012). These sophisticated tools are valuable, but suffer the disadvantage that autoimmunity is driven by a non-physiological, single TCR specificity. Our model, either of thymus grafting to C57BL/6 nudes or transfer of Nu-Tp T cells to $RAG^{-/-}$ recipients, can be considered inducible and therefore useful for studying disease onset and kinetics. Indeed, we were able to observe the active disease process (Figure 4). In addition, our model will aid investigation of the etiology of autoimmunity, as it relies on the failure of self-tolerance during the development of the endogenous T-cell repertoire.

Our data support the established hypothesis that autoimmune hair loss and depigmentation are T cell–mediated and

suggest that these disorders can share a common pathogenesis, as previously reported in mice (Nagai *et al.*, 2006) and chickens (Smyth and McNeil, 1999). Although there are clinical reports of coincidence of alopecia areata and vitiligo (Dhar and Kanwar, 1994; Adams and Lucky, 1999; Tan *et al.*, 2002; Yadav *et al.*, 2009; Akay *et al.*, 2010; Ramot *et al.*, 2010), the link between the two diseases is controversial, with some studies finding no association (Majumder *et al.*, 1993).

CD4+ and CD8+ T cells have been detected in skin from alopecia areata and vitiligo sufferers (Gilhar *et al.*, 2007). In our model, transfer of purified CD4+ T cells alone caused depigmentation and hair loss in furred RAG$^{-/-}$ mice. This indicates that CD4+ cells can mediate disease in isolation, without B cells or CD8+ T cells. CD4+ T cells generally function to activate other immune cell types. We did not observe large-scale inflammation in the skin of Nu-Tp or adoptively transferred mice, or an increase in the proportion of macrophages or neutrophils in skin (not shown). Melanocytes can express MHC Class II (Lu *et al.*, 2002) and process antigen for presentation to T cells (Le Poole *et al.*, 1993). Therefore, CD4+ T cells may directly recognize antigens presented by melanocytes (Rivoltini *et al.*, 1998). There are several reports of CD4+ T cells functioning atypically to act as cytotoxic effectors (Marshall and Swain, 2011). In the future, it will be important to investigate this in our model, to determine whether CD4+ cell depletion strategies or directed immunotherapy can ameliorate pathology.

The fact that skin was predominantly and consistently targeted by autoimmune T cells in our system suggests that there are specific requirements for induction of tolerance to skin antigens, and is consistent with the observation that skin is a frequent site of autoimmunity.

In summary, we demonstrate a link between hair loss and depigmentation and show that these disorders can be caused by CD4+ T cells, in the absence of other lymphocyte populations. This study therefore provides an inducible mouse model to investigate the etiology, induction, and pathology of T cell–driven hair-follicle disorders.

MATERIALS AND METHODS

Human thymus tissue

Tissue was obtained during elective cardiac surgery at Great Ormond Street Hospital, London, fragmented by dissection (1 mm^3 explants), or cut into <1-mm-thick slices and cultured for 14 days (Markert *et al.*, 2010) before fragmentation. This study was conducted with institutional ethical approval, with written informed consent, and according to the Declaration of Helsinki principles.

Mice and procedures

C57BL/6 (B6) WT/nude (B6.Cg-Foxn1nu/J heterozygotes) and RAG1$^{-/-}$ (B6.127S7-Rag1tm1Mom/J) mice were from Jackson Labs (Bar Harbour, ME). Animals were housed in individually ventilated cages and underwent procedures in sterile conditions under the UK Home Office regulations.

Xenografting: Human thymus tissue (<100 mg) was subcutaneously injected in phosphate-buffered saline into the scruff under light inhaled anesthetic.

Adoptive transfer: 5×10^5–5×10^6 lymphocytes were injected intravenously in 200 µl of phosphate-buffered saline. Mice were tail-bled at regular intervals.

Skin digests

Skin samples were collected from anatomically matched locations, minced with scissors, digested with Liberase (0.15 mg ml^{-1}, Roche, Burgess Hill, UK) and DNAse (0.5 mg ml^{-1}, Roche) for 30 minutes at 37 °C, and filtered to obtain a single-cell suspension.

Flow cytometry

Cells were stained with fluorochrome-conjugated antibody (eBiosciences, Hatfield, UK or BD Pharmingen, Oxford, UK) in phosphate-buffered saline + 5% fetal calf serum + 0.01% azide for 10 minutes at room temperature, and washed and analyzed by flow cytometry (instrument: FACScan, BD; software: Cell Quest, BD, and FlowJo, TreeStar, Ashland, OR).

Histology, immunohistochemistry and immunofluorescence

Tissues were fixed in Bouin's solution, embedded in wax, sectioned, deparaffinized, and examined by hematoxylin and eosin staining. Unfixed frozen sections (7 µm) were stained with rat anti-mouse CD4 and CD8 antibodies (eBiosciences, UK), followed by Alexa-Fluor594 anti-rat antibody (Invitrogen, Paisley, UK). Deparaffinized wax sections (5 µm) were stained with goat-anti Trp2 (Santa Cruz, Insight Biotechnology, London, UK), anti-goat Biotin (Alpha Diagnostic Intl, Source BioScience Life Sciences, Nottingham, UK), and Alexa-Fluor555 Streptavidin (Invitrogen). Sections were viewed by light/fluorescence microscopy (Leica DMLB, Milton Keynes, UK); representative examples are shown.

Melanocyte stimulation assay

C57BL/6-transformed melanocyte cell line, melan-a, was obtained from the Wellcome Trust Functional Genomics Cell Bank, St George's University, London. Melan-a cells were grown to 60% confluence (Sviderskaya *et al.*, 1997) and irradiated (60 Gy, gamma-source). Splenocytes were cultured at a density of 1×10^6 per ml at a 1:1 ratio with irradiated melan-a or B6 control splenocytes for 48 hours, before analysis by flow cytometry.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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