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[Continued on next page]

(54) Title: METHODS FOR TREATING HAIR LOSS DISORDERS

(57) Abstract: The invention provides for methods for treating a hair loss disorder in a subject by administering a NKG2D inhibitor, IL-15 inhibitor, or a combination thereof.

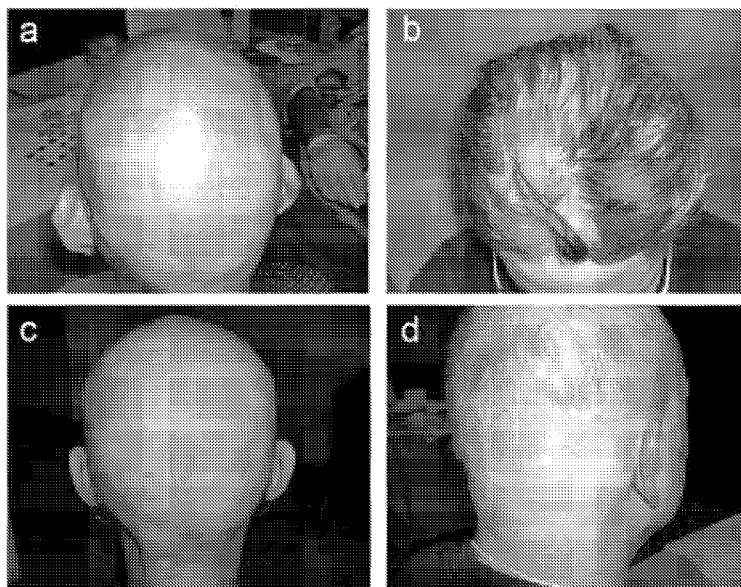


FIG. 1



RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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METHODS FOR TREATING HAIR LOSS DISORDERS

[0001] This application claims priority to United States Provisional Application No. 61/409,514, which was filed November 2, 2010, and which is herein incorporated by reference in its entirety.

[0002] All patents, patent applications and publications cited herein are hereby incorporated by reference in their entirety. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

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GOVERNMENT INTERESTS

[0004] This invention was made with government support under RO1 AR56016 awarded by the National Institutes of Health/ National Institute of Arthritis and Musculoskeletal and Skin Diseases. The United States Government has certain rights to the invention.

BACKGROUND OF THE INVENTION

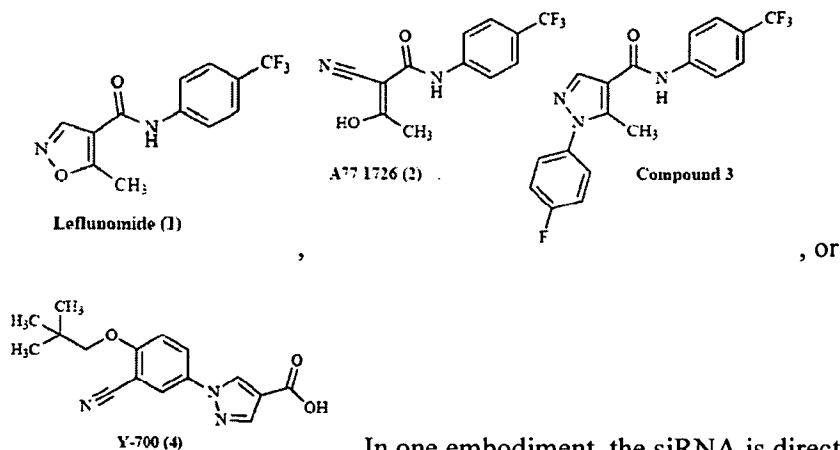
[0005] Alopecia Areata (AA) is one of the most highly prevalent autoimmune diseases, leading to hair loss due to the collapse of immune privilege of the hair follicle and subsequent autoimmune destruction. AA is a skin disease which leads to hair loss on the scalp and elsewhere. In some severe cases, it can progress to complete loss of hair on the head or body. Although Alopecia Areata is believed to be caused by autoimmunity, the gene level diagnosis and treatment are seldom reported. The genetic basis of AA is largely unknown.

SUMMARY OF THE INVENTION

[0006] An aspect of the invention encompasses a method of treating a hair-loss disorder in a mammalian subject in need thereof, the method comprising administering to the subject an IL-15 inhibitor, an NKG2D inhibitor, or a combination thereof.

[0007] An aspect of the invention encompasses a method of treating a hair-loss disorder in a mammalian subject in need thereof, the method comprising administering to the subject

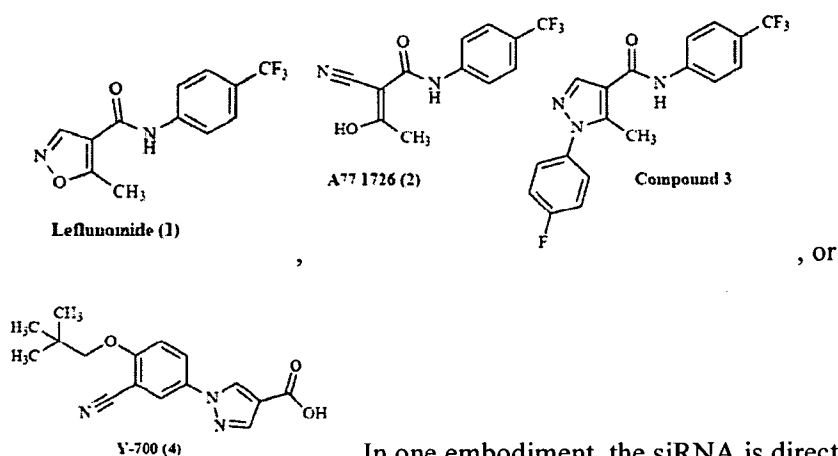
an IL-15 inhibitor. In one embodiment, the inhibitor is an antibody or antibody fragment that is directed to SEQ ID NO: 1, 5, or 7. In another embodiment, the hair-loss disorder comprises androgenetic alopecia, telogen effluvium, alopecia areata, telogen effluvium, tinea capitis, alopecia totalis, hypotrichosis, hereditary hypotrichosis simplex, or alopecia universalis. In one embodiment, the method further comprises the step (b) determining whether the inhibitor administered induced hair growth in the subject afflicted with a hair loss disorder as compared to the subject's hair growth prior to treatment with the inhibitor. In one embodiment, the inhibitor is an antisense RNA that specifically inhibits expression of the gene that encodes the IL-15 protein; a siRNA that specifically targets the gene that encodes the IL-15 protein; or a phenylpyazoleanilide small molecule. In one embodiment, the phenylpyazoleanilide small molecule is



. In one embodiment, the siRNA is directed to a human nucleic acid sequence comprising SEQ ID NO: 2, 6, or 8. In another embodiment, an siRNA directed to a human nucleic acid sequence comprising a IL-15 gene can comprise any one of the sequences listed in Table 11. In another embodiment, an siRNA directed to a human nucleic acid sequence comprising a IL-15RA gene can comprise any one of the sequences listed in Table 12.

[0008] An aspect of the invention provides for a method for inducing hair growth in a subject where the method comprises administering to the subject an effective amount of an IL-15 inhibitor, thereby controlling hair growth in the subject. In one embodiment, the inhibitor comprises an antibody that specifically binds to a protein comprising SEQ ID NO: 1, 5, or 7. In some embodiments, the subject is afflicted with a hair-loss disorder. In other embodiments, the hair-loss disorder comprises androgenetic alopecia, telogen effluvium, alopecia areata, telogen effluvium, tinea capitis, alopecia totalis, hypotrichosis, hereditary

hypotrichosis simplex, or alopecia universalis. In some embodiments, the modulating compound can also inhibit hair growth, thus it can be used for treatment of hair growth disorders, such as hypertrichosis. In one embodiment, the method further comprises the step (b) determining whether the inhibitor administered induced hair growth in the subject afflicted with a hair loss disorder as compared to the subject's hair growth prior to treatment with the inhibitor. In one embodiment, the inhibitor is an antisense RNA that specifically inhibits expression of the gene that encodes the IL-15 protein; a siRNA that specifically targets the gene that encodes the IL-15 protein; or a phenylpyazoleanilide small molecule. In one embodiment, the phenylpyazoleanilide small molecule is



. In one embodiment, the siRNA is directed to a human nucleic acid sequence comprising SEQ ID NO: 2, 6, or 8. In another embodiment, an siRNA directed to a human nucleic acid sequence comprising a IL-15 gene can comprise any one of the sequences listed in Table 11. In another embodiment, an siRNA directed to a human nucleic acid sequence comprising a IL-15RA gene can comprise any one of the sequences listed in Table 12.

[0009] An aspect of the invention encompasses a method of treating a hair-loss disorder in a mammalian subject in need thereof, the method comprising administering to the subject a NKG2D inhibitor. In one embodiment, the inhibitor is an antibody or antibody fragment that is directed to SEQ ID NO: 3. In another embodiment, the hair-loss disorder comprises androgenetic alopecia, telogen effluvium, alopecia areata, telogen effluvium, tinea capitis, alopecia totalis, hypotrichosis, hereditary hypotrichosis simplex, or alopecia universalis. In one embodiment, the method further comprises the step (b) determining whether the inhibitor administered induced hair growth in the subject afflicted with a hair loss disorder as compared to the subject's hair growth prior to treatment with the inhibitor. In one

embodiment, the inhibitor is an antisense RNA that specifically inhibits expression of the gene that encodes the NKG2D protein; a siRNA that specifically targets the gene that encodes the NKG2D protein. In one embodiment, the siRNA is directed to a human nucleic acid sequence comprising SEQ ID NO: 4. In another embodiment, an siRNA directed to a human nucleic acid sequence comprising a NKG2D gene can comprise any one of the sequences listed in Table 13.

[0010] An aspect of the invention provides for a method for inducing hair growth in a subject where the method comprises administering to the subject an effective amount of a NKG2D inhibitor, thereby controlling hair growth in the subject. In one embodiment, the inhibitor comprises an antibody that specifically binds to a protein comprising SEQ ID NO: 3. In some embodiments, the subject is afflicted with a hair-loss disorder. In other embodiments, the hair-loss disorder comprises androgenetic alopecia, telogen effluvium, alopecia areata, telogen effluvium, tinea capitis, alopecia totalis, hypotrichosis, hereditary hypotrichosis simplex, or alopecia universalis. In some embodiments, the modulating compound can also inhibit hair growth, thus it can be used for treatment of hair growth disorders, such as hypertrichosis. In one embodiment, the method further comprises the step (b) determining whether the inhibitor administered induced hair growth in the subject afflicted with a hair loss disorder as compared to the subject's hair growth prior to treatment with the inhibitor. In one embodiment, the inhibitor is an antisense RNA that specifically inhibits expression of the gene that encodes the NKG2D protein; a siRNA that specifically targets the gene that encodes the NKG2D protein. In one embodiment, the siRNA is directed to a human nucleic acid sequence comprising SEQ ID NO: 4. In another embodiment, an siRNA directed to a human nucleic acid sequence comprising a NKG2D gene can comprise any one of the sequences listed in Table 13.

[0011] In a further embodiment, the administering comprises a subcutaneous, intramuscular, intra-peritoneal, or intravenous injection; an infusion; oral, nasal, or topical delivery; or a combination thereof. In some embodiments, the administering occurs daily, weekly, twice weekly, monthly, twice monthly, or yearly.

BRIEF DESCRIPTION OF THE FIGURES

[0012] FIG. 1 are photographic images of clinical manifestations of AA. In the upper panels (FIGS. 1A-B), patients with AA multiplex. In FIG. 1B, the patient is in regrowth

phase. For patients with alopecia universalis, there is a complete lack of body hair and scalp hair (**FIG. 1C**), while patients with alopecia totalis only lack scalp hair (**FIG. 1D**). In **FIG. 1D**, hair regrowth is observed in the parietal region, while no regrowth in either occipital or temporal regions is evident.

[0013] **FIG. 2** show a diagram showing the inflammatory response underlying Alopecia Areata (AA).

[0014] **FIGS. 3A-C** are immunofluorescent photomicrographs showing that IL-15 is produced in AA hair follicles and can drive the NKG2D axis.

[0015] **FIGS. 4A-B** show that NKG2D-bearing CD8 NKT cells are the “killer bees.” CD8+NKG2D+ T cells in alopecic C3H mice are expanded in the skin of alopecic C3H mice.

[0016] **FIG. 5A** is a photograph of a subject afflicted with alopecia areata (AA).

[0017] **FIG. 5B** is a photomicrograph of immunofluorescent staining that shows that NKG2D-bearing CD8 T cells infiltrate the Hair Follicle (HF) in human and murine AA ((m) staining of CD8; (n) staining of NKG2D; and (o) colocalization of NKG2D and CD8).

[0018] **FIG. 5C** shows mice with Focal (left), scalp (middle) and Universal (right) AA.

[0019] **FIG 5D** is a photomicrograph of immunofluorescent staining that shows NKG2D-bearing CD8 T cells infiltrate the Hair Follicle (HF) in AA.

[0020] **FIG. 6** shows a diagram showing the inflammatory response underlying Alopecia Areata (AA).

[0021] **FIG. 7A** is a photograph of lymph nodes and spleen isolated from C3H mice afflicted with AA (top) and control mice (bottom).

[0022] **FIG. 7B** show the results from FACS analysis that NKG2D bearing CD8 and Th1 cells are expanded in murine AA cutaneous lymph nodes.

[0023] **FIG. 7C** is a bar graph that shows a 6-fold increase in the number of cytokine producing cells in C3H mice afflicted with AA as compared to control mice.

[0024] **FIG. 8A** is a RNA signature of alopecic skin in C3H mice. The heat map shows that 16 of the top 20 unregulated genes are Interferon-Response Genes. 16 of top 20

upregulated genes are Interferon Responsive Genes (IFN- γ but not Type I IFNs are elevated)

[0025] **FIG. 8B** shows transcriptional profiling of AA skin. qPCR verification of single genes picked from the list generated shows an average fold change over 3 experiments.

[0026] **FIGS. 9A-D.** **FIG. 9A** show that interferon γ , TNF and TLRs upregulate NKG2DLs in the HF. **FIG. 9B** shows human peripheral blood mononuclear cells (PBMC) that were cultured in high dose IL-2 (1000/ml) to generate lymphokine-activated killer (LAK) cells. The CFSE green fluorescence labeled LAK cells were incubated with human hair follicle organ culture. **FIG. 9C** shows murine splenocytes that were cultured in high dose IL-2 (1000/ml) to generate lymphokine-activated killer (LAK) cells. The CFSE green fluorescence labeled LAK cells were incubated mouse hair follicle culture from non-lesion region (top left two) or lesion. **FIG. 9D** shows the promotion of CD8 killer cell engagement.

[0027] **FIG. 10** shows a diagram showing the targets in AA.

[0028] **FIG. 11** shows a diagram showing the interferon γ pathway.

[0029] **FIG. 12** shows a diagram that shows the inflammatory response underlying AA.

[0030] **FIGS. 13A-B** shows two gene mapping approaches. **FIG 13A** shows linkage analysis in families. **FIG. 13B** shows association analysis in a population cohort.

[0031] **FIG. 14** shows a Genomewide Association Study (GWAS) in AA.

[0032] **FIGS. 15A-B** show danger signals: ULBP3 expression in the hair follicle in both control individuals (**FIG. 15A**) and AA patients (**FIG. 15B**).

[0033] **FIGS. 16A-B** show that AA is caused by a “swarm of bees”: Killer CD8 T cells attracted by NKG2DL. **FIG. 16A** shows “pre-2010” swarm of bees in AA. **FIG. 16B** shows “2012” identification of bees ((m) staining of CD8; (n) staining of NKG2D; and (o) colocalization of NKG2D and CD8).

[0034] **FIG. 17** shows a diagram that shows the inflammatory response underlying AA.

[0035] **FIG. 18** is a schematic that shows the pre-clinical strategies to interrupt AA pathogenesis are aimed at interruption of the induction and effector function of CD8 T cells by blocking IL-15/NKG2D and IFN- γ signals.

[0036] **FIG. 19** shows that CD8⁺ NKG2D⁺ T cells infiltrate AA hair follicles in both mouse and human. *Top panel*: Human Alopecia Areata (Pethukova *et al.*, 2010, Nature, 466(7302):113-7); *bottom panel*- C3H/HeJ Alopecia Areata.

[0037] **FIG. 20** shows CD8⁺NKG2D⁺ T cells in alopecic C3H mice CD8 $\alpha\beta$ ⁺NKG2D⁺ T cells are massively expanded in the skin, lymph node and blood of alopecic C3H mice. (NKG2D⁺ $\gamma\delta$ T cells are present in both affected and unaffected skin).

[0038] **FIG. 21.** shows CD8⁺NKG2D⁺ T cells in alopecic C3H mice. Immunophenotype of gated LN CD8 α ⁺NKG2D⁺ populations shows that they are uniformly CD8 $\alpha\beta$ ⁺CD44⁺CXCR3⁺DX5⁺NKG2A/C/E⁺ IFN- γ producing memory T cells.

[0039] **FIG. 22** shows spectratype analysis of C3H T cells in skin and LN: Similar TCR repertoires are shared by total skin and sorted CD8⁺NKG2D⁺ LN T cells identifying these as the relevant effectors.

[0040] **FIGS. 23A-B.** **FIG. 23A.** shows that IL-15R β ⁺ CD8 T cells infiltrate IL-15/IL15R α expressing HF⁺s in AA mice. **FIG. 23B** shows enhanced IL-15 and IL-15R α expression on HF⁺s in AA patients.

[0041] **FIG. 24** shows that alopecia is present 10 weeks after grafting in controls but not in mice treated with anti-IL-15R β .

[0042] **FIG. 25A** shows that human dermal sheath cells are attacked by human LAK cells *in vitro*. Human dermal sheath cells were expanded and then treated or not with IFN γ /LPS for 24 hours. Human LAK cells were added to the culture and cytolysis measured by LDH release. The cytolysis is blocked by either anti-NKG2D antibody but not isotype-matched IgG.

[0043] **FIG. 25B** shows a CTL assay (C3H-HeJ mouse). Targets include CFSE labeled dermal sheath cells (DS). Effector T cells include cutaneous lymph node CD8⁺ NKG2D⁺ T cells.

[0044] **FIG. 25C.** shows that the lysis of TNF-primed dermal papillae (DP) cells by lymphokine activated killer cells (LAKs) requires NKG2D. C3H/HeJ DP cells were treated with or without TNF for three days and then incubated with IL-2 induced LAKs in the presence or absence of neutralizing anti-NKG2D or anti-MHC-1 antibodies (* $p < 0.05$).

[0045] **FIGS. 26A-B** show that IFN- γ producing CD8 T cells dominate human and mouse AA. **FIG. 26A:** Human: T cells obtained from T cell crawl-outs from AA skin biopsies are uniformly CD8+NKG2D+ and produce IFN- γ upon PMA/Ionomycin stimulation. **FIG. 26B:** Mouse: C3H/HeJ stimulated lymph node cells from alopecic mice contain 3 times more IFN- γ producing CD8 and CD4 T cells than unaffected mice.

[0046] **FIG. 27A** shows that circulating “NK-type” CD8 T cells are eliminated by anti-IL-15R β treatment. After 6 weeks of anti-IL-15 treatment grafted C3H mice remained healthy and whole blood flow shows elimination of both NK cells and CD8⁺ NKG2A/C/E⁺ T cells.

[0047] **FIG. 27B** are graphs that show elevated serum chemokines and cytokines in Human AA. Interferon- γ and IFN-induced chemokines (e.g., IP-10/CXCL10) are elevated in the serum of human AA, in some cases correlating with disease severity, i.e., patchy disease (AAP) vs. universalis (AU).

[0048] **FIG. 28** shows that interferon- γ induced genes are eliminated by anti-IL-15R β treatment. RNA obtained from skin samples obtained 10 weeks after grafting shows IFN-induced genes in AA-grafted mice, but not after anti-IL-15 treatment nor in “sham” surgery mice receiving autologous non-diseased skin. In anti-IL-15 treated mice, dramatic changes in gene expression are observed in skin biopsies of grafted mice. IFN response genes were down regulated in IL-15 treated vs. untreated mice. CD3 γ , CD3 δ , CD8 and NK immunoreceptor genes were also downregulated consistent with reduced skin infiltration by “NK-type” T cells in responding mice.

[0049] **FIG. 29** shows elevated IL-15 in the serum of Human AA subjects, in some cases correlating with disease severity, i.e., patchy disease (AAP) vs. universalis (AU).

[0050] **FIG. 30** is a luminex analysis that shows elevated IL-13 in the serum of Human AA subjects, in some cases correlating with disease severity, i.e., patchy disease (AAP) vs. universalis (AU).

[0051] **FIG. 31** shows flow cytometry data that were gathered on a LSRII using the following mAbs in one tube to stain the entire population of C3H dermal cells liberated from a 0.5 cm by 2 cm patch of alopecic skin after 30 minutes collagenase digestion: (MHC II, CD11b, CD11c, CD19, 120G8, CD3, CD4, CD8, DX5, NKG2D). $\gamma\delta$ NKG2D positive cells and CD8+DX5+NKG2D+ population are identified. These cells were also CD3+, with $\gamma\delta$ CD3 expression 100x higher than that seen in CD8+ NK+ T cells. >95% of the total CD3 positive cells were accounted.

[0052] **FIG. 32** is a graph that shows the evaluation of PBMCs from four AA subjects. NKG2D expression was increased in subsets of lymphocytes.

[0053] **FIG. 33** shows spectratyping in a C3H-HeJ mouse. RNA was Isolated from skin biopsies, and from sorted NKG2D- and NKG2D+ CD8+ T cells from cutaneous lymph nodes of C3H-HeJ mice. Spectratyping analysis shows CDR3 length skewing in several Vbeta families. Some of the skewed Vbeta families are shared between skin and NKG2D+ lymph node CD8+ T cell populations (e.g., Vbeta2, Vbeta5.2, Vbeta20) whereas additional Vbeta families are skewed in skin (reflecting the CD4+ T cell population).

[0054] **FIG. 34** is a schematic showing that human scalp skin procured from control or AA affected individuals can be used to establish primary cultures of individual cellular populations within the skin and hair follicle.

[0055] **FIG. 35** shows a T cell 'crawl-out' assay, in which cultured human dermis obtained from punch skin biopsies are used as source material for flow cytometric analysis. Trisected skin punch biopsies of a 6mm skin biopsy is obtained from alopecic lesional and non-lesional areas. After removing the fat, the finely minced skin is gently pressed in a Cellfoam matrix and then transferred, skin side-up, to a well of a 24-well plate containing 2 mls of Skin T medium with IL-2 (6ng/ml) and IL-15 (10 ng/ml). T cells crawl outs are shown in **FIG. 35A** after the matrix is removed 3 days later. Flow cytometry (**FIGS. 35B-C**) and spectratyping (**FIG. 35D**) of these cells are also shown.

[0056] **FIG. 36** shows that human peripheral blood mononuclear cells (PBMC), top panels or murine splenocytes were cultured in high dose IL-2 (1000/ml) to generate lymphokine-activated killer (LAK) cells. The CFSE green fluorescence labeled LAK cells were incubated with human hair follicle organ culture (top panels), or mouse hair follicle culture (bottom panels) from non-lesion region (bottom left two) or lesion.

[0057] FIG. 37 shows immunohistochemical (IHC) staining of unaffected and affected AA skin in C3H-HeJ mice.

[0058] FIG. 38 shows upregulation of NKG2DL mRNA in lesional AA skin. FIG. 38A shows a bar graph of qRT-PCR analysis of the expression of MICA, ULBP1, ULBP3 and ULBP6 in normal skin and three lesional AA skin biopsies. FIG. 38B shows IF microscopy of MICA, ULBP1, and ULBP3 detection in AA and control, stained with anti-ULBP3 and with DAPI.

DETAILED DESCRIPTION OF THE INVENTION

[0059] The invention provides for methods of treating a hair loss disorder (e.g., Alopecia Areata (AA), a common autoimmune form of hair loss) with an inhibitor of NKG2D and IL-15. The invention also provides for a method of treating a hair loss disorder (e.g., Alopecia Areata (AA)) with an Shh agonist. Clinical research in AA has lagged behind its more heavily investigated “sister” autoimmune diseases in which this gene has been implicated (e.g., rheumatoid arthritis (RA), type 1 diabetes mellitus (T1D), multiple sclerosis (MS)). The invention provides for therapeutics previously untested in AA, that can inform one about the clinical relevance of this pathways in AA and related diseases.

Overview of the Integument and Hair Cells

[0060] The integument (or skin) is the largest organ of the body and is a highly complex organ covering the external surface of the body. It merges, at various body openings, with the mucous membranes of the alimentary and other canals. The integument performs a number of essential functions such as maintaining a constant internal environment via regulating body temperature and water loss; excretion by the sweat glands; but predominantly acts as a protective barrier against the action of physical, chemical and biologic agents on deeper tissues. Skin is elastic and except for a few areas such as the soles, palms, and ears, it is loosely attached to the underlying tissue. It also varies in thickness from 0.5 mm (0.02 inches) on the eyelids (“thin skin”) to 4 mm (0.17 inches) or more on the palms and soles (“thick skin”) (Ross MH, Histology: A text and atlas, 3rd edition, Williams and Wilkins, 1995: Chapter 14; Burkitt HG, *et al*, Wheater’s Functional Histology, 3rd Edition, Churchill Livingstone, 1996: Chapter 9).

[0061] The skin is composed of two layers: a) the epidermis and b) the dermis. The epidermis is the outer layer, which is comparatively thin (0.1 mm). It is several cells thick and is composed of 5 layers: the stratum germinativum, stratum spinosum, stratum granulosum, stratum lucidum (which is limited to thick skin), and the stratum corneum. The outermost epidermal layer (the stratum corneum) consists of dead cells that are constantly shed from the surface and replaced from below by a single, basal layer of cells, called the stratum germinativum. The epidermis is composed predominantly of keratinocytes, which make up over 95% of the cell population. Keratinocytes of the basal layer (stratum germinativum) are constantly dividing, and daughter cells subsequently move upwards and outwards, where they undergo a period of differentiation, and are eventually sloughed off from the surface. The remaining cell population of the epidermis includes dendritic cells such as Langerhans cells and melanocytes. The epidermis is essentially cellular and non-vascular, containing little extracellular matrix except for the layer of collagen and other proteins beneath the basal layer of keratinocytes (Ross MH, Histology: A text and atlas, 3rd edition, Williams and Wilkins, 1995: Chapter 14; Burkitt HG, *et al*, Wheater's Functional Histology, 3rd Edition, Churchill Livingstone, 1996: Chapter 9).

[0062] The dermis is the inner layer of the skin and is composed of a network of collagenous extracellular material, blood vessels, nerves, and elastic fibers. Within the dermis are hair follicles with their associated sebaceous glands (collectively known as the pilosebaceous unit) and sweat glands. The interface between the epidermis and the dermis is extremely irregular and uneven, except in thin skin. Beneath the basal epidermal cells along the epidermal-dermal interface, the specialized extracellular matrix is organized into a distinct structure called the basement membrane (Ross MH, Histology: A text and atlas, 3rd edition, Williams and Wilkins, 1995: Chapter 14; Burkitt HG, *et al*, Wheater's Functional Histology, 3rd Edition, Churchill Livingstone, 1996: Chapter 9).

[0063] The mammalian hair fiber is composed of keratinized cells and develops from the hair follicle. The hair follicle is a peg of tissue derived from a downgrowth of the epidermis, which lies immediately underneath the skin's surface. The distal part of the hair follicle is in direct continuation with the external, cutaneous epidermis. Although a small structure, the hair follicle comprises a highly organized system of recognizably different layers arranged in concentric series. Active hair follicles extend down through the dermis, the hypodermis (which is a loose layer of connective tissue), and into the fat or adipose layer (Ross MH,

Histology: A text and atlas, 3rd edition, Williams and Wilkins, 1995: Chapter 14; Burkitt HG, *et al*, Wheater's Functional Histology, 3rd Edition, Churchill Livingstone, 1996: Chapter 9).

[0064] At the base of an active hair follicle lies the hair bulb. The bulb consists of a body of dermal cells, known as the dermal papilla, contained in an inverted cup of epidermal cells known as the epidermal matrix. Irrespective of follicle type, the germinative epidermal cells at the very base of this epidermal matrix produce the hair fiber, together with several supportive epidermal layers. The lowermost dermal sheath is contiguous with the papilla basal stalk, from where the sheath curves externally around all of the hair matrix epidermal layers as a thin covering of tissue. The lowermost portion of the dermal sheath then continues as a sleeve or tube for the length of the follicle (Ross MH, Histology: A text and atlas, 3rd edition, Williams and Wilkins, 1995: Chapter 14; Burkitt HG, *et al*, Wheater's Functional Histology, 3rd Edition, Churchill Livingstone, 1996: Chapter 9).

[0065] Developing skin appendages, such as hair and feather follicles, rely on the interaction between the epidermis and the dermis, the two layers of the skin. In embryonic development, a sequential exchange of information between these two layers supports a complex series of morphogenetic processes, which results in the formation of adult follicle structures. However, in contrast to general skin dermal and epidermal cells, certain hair follicle cell populations, following maturity, retain their embryonic-type interactive, inductive, and biosynthetic behaviors. These properties can be derived from the very dynamic nature of the cyclical productive follicle, wherein repeated tissue remodeling necessitates a high level of dermal-epidermal interactive communication, which is vital for embryonic development and would be desirable in other forms of tissue reconstruction.

[0066] The hair fiber is produced at the base of an active follicle at a very rapid rate. For example, follicles produce hair fibers at a rate 0.4 mm per day in the human scalp and up to 1.5 mm per day in the rat vibrissa or whiskers, which means that cell proliferation in the follicle epidermis ranks amongst the fastest in adult tissues (Malkinson FD and JT Kearn, *Int J Dermatol* 1978, 17:536-551). Hair grows in cycles. The anagen phase is the growth phase, wherein up to 90% of the hair follicles said to be in anagen; catagen is the involuting or regressing phase which accounts for about 1-2% of the hair follicles; and telogen is the resting or quiescent phase of the cycle, which accounts for about 10-14% of the hair follicles. The cycle's length varies on different parts of the body.

[0067] Hair follicle formation and cycling is controlled by a balance of inhibitory and stimulatory signals. The signaling cues are potentiated by growth factors that are members of the TGF β -BMP family. A prominent antagonist of the members of the TGF β -BMP family is follistatin. Follistatin is a secreted protein that inhibits the action of various BMPs (such as BMP-2, -4, -7, and -11) and activins by binding to said proteins, and purportedly plays a role in the development of the hair follicle (Nakamura M, et al., *FASEB J*, 2003, 17(3):497-9; Patel K *Intl J Biochem Cell Bio*, 1998, 30:1087-93; Ueno N, et al., *PNAS*, 1987, 84:8282-86; Nakamura T, et al., *Nature*, 1990, 247:836-8; Iemura S, et al., *PNAS*, 1998, 77:649-52; Fainsod A, et al., *Mech Dev*, 1997, 63:39-50; Gamer LW, et al., *Dev Biol*, 1999, 208:222-32).

[0068] The deeply embedded end bulb, where local dermal-epidermal interactions drive active fiber growth, is the signaling center of the hair follicle comprising a cluster of mesenchymal cells, called the dermal papilla (DP). This same region is also central to the tissue remodeling and developmental changes involved in the hair fiber's or appendage's precise alternation between growth and regression phases. The DP, a key player in these activities, appears to orchestrate the complex program of differentiation that characterizes hair fiber formation from the primitive germinative epidermal cell source (Oliver RF, *J Soc Cosmet Chem*, 1971, 22:741-755; Oliver RF and CA Jahoda, *Biology of Wool and Hair* (eds Roger et al.), 1971, Cambridge University Press:51-67; Reynolds AJ and CA Jahoda, *Development*, 1992, 115:587-593; Reynolds AJ, et al., *J Invest Dermatol*, 1993, 101:634-38).

[0069] The lowermost dermal sheath (DS) arises below the basal stalk of the papilla, from where it curves outwards and upwards. This dermal sheath then externally encases the layers of the epidermal hair matrix as a thin layer of tissue and continues upward for the length of the follicle. The epidermally-derived outer root sheath (ORS) also continues for the length of the follicle, which lies immediately internal to the dermal sheath in between the two layers, and forms a specialized basement membrane termed the glassy membrane. The outer root sheath constitutes little more than an epidermal monolayer in the lower follicle, but becomes increasingly thickened as it approaches the surface. The inner root sheath (IRS) forms a mold for the developing hair shaft. It comprises three parts: the Henley layer, the Huxley layer, and the cuticle, with the cuticle being the innermost portion that touches the hair shaft. The IRS cuticle layer is a single cell thick and is located adjacent to the hair fiber. It closely interdigitates with the hair fiber cuticle layer. The Huxley layer can comprise up to four cell layers. The IRS Henley layer is the single cell layer that runs adjacent to the ORS

layer (Ross MH, Histology: A text and atlas, 3rd edition, Williams and Wilkins, 1995: Chapter 14; Burkitt HG, *et al*, Wheater's Functional Histology, 3rd Edition, Churchill Livingstone, 1996: Chapter 9).

Alopecia areata

[0070] Alopecia areata (AA) is one of the most prevalent autoimmune diseases, affecting approximately 4.6 million people in the US alone, including males and females across all ethnic groups, with a lifetime risk of 1.7% (1) In AA, autoimmunity develops against the hair follicle, resulting in non-scarring hair loss that can begin as patches, which can coalesce and progress to cover the entire scalp (alopecia totalis, AT) or eventually the entire body (alopecia universalis, AU) (**FIG. 1**). AA was first described by Cornelius Celsus in 30 A.D., using the term "ophiasis", which means "snake", due to the sinuous path of hair loss as it spread slowly across the scalp. Hippocrates first used the Greek word 'alopekia' (fox mange), the modern day term "alopecia areata" was first used by Sauvages in his *Nosologica Medica*, published in 1760 in Lyons, France.

[0071] Curiously, AA preferentially affects pigmented hair follicles in the anagen (growth) phase of the hair cycle, and when the hair regrows in patches of AA, it frequently grows back white or colorless. The phenomenon of 'sudden whitening of the hair' is therefore ascribed to AA with an acute onset, and has been documented throughout history as having affected several prominent individuals at times of profound grief, stress or fear (2). Examples include Shahjahan, who upon the death of his wife in 1631 experienced acute whitening of his hair, and in his grief built the Taj Mahal in her honor. Sir Thomas More, author of *Utopia*, who on the eve of his execution in 1535 was said to have become 'white in both beard and hair'. The sudden whitening of the hair is believed to result from an acute attack upon the pigmented hair follicles, leaving behind the white hairs unscathed.

[0072] Several clinical aspects of AA remain unexplained but can hold important clues toward understanding pathogenesis. AA attacks hairs only around the base of the hair follicles, which are surrounded by dense clusters of lymphocytes, resulting in the pathognomic 'swarm of bees' appearance on histology. Based on these observations, it is postulated that a signal(s) in the pigmented, anagen hair follicle is emitted which invokes an acute or chronic immune response against the lower end of the hair follicle, leading to hair cycle perturbation, acute hair shedding, hair shaft anomalies and hair breakage. Despite these

dramatic perturbations in the hair follicle, there is no permanent organ destruction and the possibility of hair regrowth remains if immune privilege can be restored.

[0073] Throughout history, AA has been considered at times to be a neurological disease brought on by stress or anxiety, or as a result of an infectious agent, or even hormonal dysfunction. The concept of a genetically-determined autoimmune mechanism as the basis for AA emerged during the 20th century from multiple lines of evidence. AA hair follicles exhibit an immune infiltrate with activated Th, Tc and NK cells (3, 4) and there is a shift from a suppressive (Th2) to an autoimmune (Th1) cytokine response. The humanized model of AA, which involves transfer of AA patient scalp onto immune-deficient SCID mice illustrates the autoimmune nature of the disease, since transfer of donor T-cells causes hair loss only when co-cultured with hair follicle or human melanoma homogenate (5, 6). Regulatory T cells which serve to maintain immune tolerance are observed in lower numbers in AA tissue (7), and transfer of these cells to C3H/HeJ mice leads to resistance to AA (8). Although AA has long been considered exclusively as a T-cell mediated disease, in recent years, an additional mechanism of disease has been postulated. The hair follicle is defined as one of a select few immune privileged sites in the body, characterized by the presence of extracellular matrix barriers to impede immune cell trafficking, lack of antigen presenting cells, and inhibition of NK cell activity via the local production of immunosuppressive factors and reduced levels of MHC class I expression (9). Thus, the notion of a 'collapse of immune privilege' has also been invoked as part of the mechanism by which AA can arise. Support for a genetic basis for AA comes from multiple lines of evidence, including the observed heritability in first degree relatives (10, 11), twin studies (12), and most recently, from the results of family-based linkage studies (13).

Treatment of Hair Loss Disorders

[0074] This invention provides for the discovery that a known therapeutic, for example an inhibitor of a NKG2D protein (e.g., an anti-NKG2D antibody), can be used for the the treatment of hair loss disorders. This invention provides for the discovery that a known therapeutic, for example an inhibitor of a IL-15 protein (e.g., an anti-IL-15 antibody), can be used for the the treatment of hair loss disorders. Non-limiting examples of hair loss disorders include: androgenetic alopecia, Alopecia areata, telogen effluvium, alopecia areata, alopecia totalis, and alopecia universalis.

[0075] An aspect of the invention encompasses a method of treating a hair-loss disorder in a mammalian subject in need thereof, the method comprising administering to the subject an IL-15 inhibitor. In one embodiment, the inhibitor is an antibody or antibody fragment that is directed to SEQ ID NO: 1. In another embodiment, the hair-loss disorder comprises androgenetic alopecia, telogen effluvium, alopecia areata, telogen effluvium, tinea capitis, alopecia totalis, hypotrichosis, hereditary hypotrichosis simplex, or alopecia universalis.

[0076] An aspect of the invention provides for a method for inducing hair growth in a subject where the method comprises administering to the subject an effective amount of an IL-15 inhibitor, thereby controlling hair growth in the subject. In one embodiment, the inhibitor comprises an antibody that specifically binds to a protein comprising SEQ ID NO: 1. In some embodiments, the subject is afflicted with a hair-loss disorder. In other embodiments, the hair-loss disorder comprises androgenetic alopecia, telogen effluvium, alopecia areata, telogen effluvium, tinea capitis, alopecia totalis, hypotrichosis, hereditary hypotrichosis simplex, or alopecia universalis. In some embodiments, the modulating compound can also inhibit hair growth, thus it can be used for treatment of hair growth disorders, such as hypertrichosis.

[0077] An aspect of the invention encompasses a method of treating a hair-loss disorder in a mammalian subject in need thereof, the method comprising administering to the subject a NKG2D inhibitor. In one embodiment, the inhibitor is an antibody or antibody fragment that is directed to SEQ ID NO: 3. In another embodiment, the hair-loss disorder comprises androgenetic alopecia, telogen effluvium, alopecia areata, telogen effluvium, tinea capitis, alopecia totalis, hypotrichosis, hereditary hypotrichosis simplex, or alopecia universalis.

[0078] An aspect of the invention provides for a method for inducing hair growth in a subject where the method comprises administering to the subject an effective amount of a NKG2D inhibitor, thereby controlling hair growth in the subject. In one embodiment, the inhibitor comprises an antibody that specifically binds to a protein comprising SEQ ID NO: 3. In some embodiments, the subject is afflicted with a hair-loss disorder. In other embodiments, the hair-loss disorder comprises androgenetic alopecia, telogen effluvium, alopecia areata, telogen effluvium, tinea capitis, alopecia totalis, hypotrichosis, hereditary hypotrichosis simplex, or alopecia universalis. In some embodiments, the modulating compound can also inhibit hair growth, thus it can be used for treatment of hair growth disorders, such as hypertrichosis.

DNA and AminoAcid Manipulation Methods and Purification Thereof

[0079] The present invention utilizes conventional molecular biology, microbiology, and recombinant DNA techniques available to one of ordinary skill in the art. Such techniques are well known to the skilled worker and are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D. N. Glover, ed., 1985); "Oligonucleotide Synthesis" (M. J. Gait, ed., 1984); "Nucleic Acid Hybridization" (B. D. Hames & S. J. Higgins, eds., 1985); "Transcription and Translation" (B. D. Hames & S. J. Higgins, eds., 1984); "Animal Cell Culture" (R. I. Freshney, ed., 1986); "Immobilized Cells and Enzymes" (IRL Press, 1986); B. Perbal, "A Practical Guide to Molecular Cloning" (1984), and Sambrook, et al., "Molecular Cloning: a Laboratory Manual" (1989).

[0080] One skilled in the art can obtain a protein in several ways, which include, but are not limited to, isolating the protein via biochemical means or expressing a nucleotide sequence encoding the protein of interest by genetic engineering methods.

[0081] A protein is encoded by a nucleic acid (including, for example, genomic DNA, complementary DNA (cDNA), synthetic DNA, as well as any form of corresponding RNA). For example, it can be encoded by a recombinant nucleic acid of a gene. The proteins of the invention can be obtained from various sources and can be produced according to various techniques known in the art. For example, a nucleic acid that encodes a protein can be obtained by screening DNA libraries, or by amplification from a natural source. A protein can be a fragment or portion thereof. The nucleic acids encoding a protein can be produced via recombinant DNA technology and such recombinant nucleic acids can be prepared by conventional techniques, including chemical synthesis, genetic engineering, enzymatic techniques, or a combination thereof. For example, an IL-15 protein is the polypeptide encoded by the nucleic acid having the nucleotide sequence shown in SEQ ID NO: 2. An example of an IL-15 polypeptide has the amino acid sequence shown in SEQ ID NO: 1. A NKG2D protein is the polypeptide encoded by the nucleic acid having the nucleotide sequence shown in SEQ ID NO: 4. An example of a NKG2D polypeptide has the amino acid sequence shown in SEQ ID NO: 3.

[0082] The polypeptide sequence of human IL-15 is depicted in SEQ ID NO: 1. The nucleotide sequence of human IL-15 is shown in SEQ ID NO: 2. Sequence information

related to IL-15 is accessible in public databases by GenBank Accession numbers U14407 (for mRNA) and AAA21551 (for protein).

[0083] SEQ ID NO: 1 is the human wild type amino acid sequence corresponding to IL-15 (residues 1-162):

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1 MRISKPHLRS ISIQCYLCLL LNSHFLTEAG IHVFILGCF S AGLPKTEANW VNVISDLKKI
61 EDLIQSMHID ATLYTESDVH PSCKVTAMKC FLLELQVISL ESGDASIHTD VENLIILANN
121 SLSSNGNVTE SGCKECEEELE EKNIKEFLQS FVHIVQMFIN TS

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[0084] SEQ ID NO: 2 is the human wild type nucleotide sequence corresponding to IL-15 (nucleotides 1-1202), wherein the underscored bolded "ATG" denotes the beginning of the open reading frame:

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1 tgtccggcgc cccccgggag ggaactgggt ggccgcaccc tcccggctgc ggtggctgtc
61 gccccccacc ctgcagccag gactcgatgg agaatccatt ccaatatatg gccatgtggc
121 tctttggagc aatgttccat catgttccat gctgctgctg acgtcacatg gagcacagaa
181 atcaatgtta gcagatagcc agcccataca agatcgtatt gtattgtagg aggcacgtg
241 gatggatggc tgctggaaac cccttgccat agccagctct tcttcaatac ttaaggattt
301 accgtggctt tgagtaatga gaatttcgaa accacatttg agaagtattt ccatccagtg
361 ctacttggtt ttacttctaa acagtcattt tctaaactgaa gctggcattc atgtcttcat
421 tttgggctgt ttcagtgcag ggcttcctaa aacagaagcc aactgggtga atgtaataag
481 tgatttgaaa aaaattgaag atcttattca atctatgcat attgatgcta ctttatatac
541 ggaaagtgat gttcacccca gttgcaaagt aacagcaatg aagtgccttc tcttgaggtt
601 acaagtattt tcacttgagt ccggagatgc aagtattcat gatacagtag aaaatctgat
661 catcctagca aacaacagtt tgtcttctaa tgggaatgta acagaatctg gatgcaaaga
721 atgtgaggaa ctggaggaaa aaaatattaa agaatttttg cagagttttg tacatattgt
781 ccaaatgttc atcaacactt cttgattgca attgattcct tttaaagtgt ttctgttatt
841 aacaaacatc actctgctgc ttagacataa caaaacactc ggcatttaaa atgtgctgtc
901 aaaacaagtt tttctgtcaa gaagatgata agaccttgga tcagatgaac tcttagaaat
961 gaaggcagaa aaatgtcatt gagtaatata gtgactatga acttctctca gacttacttt
1021 actcattttt ttaatttatt attgaaattg tacatatttg tggaataatg taaaatggtt
1081 aataaaaata tgtacaagtg ttgtttttta agttgcaactg atattttacc tcttattgca
1141 aatagcattt tgtttaaggg tgatagtcaa attatgtatt ggtggggctg ggtaccaatg
1201 ct

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[0085] The polypeptide sequence of human IL-15 receptor-alpha is depicted in SEQ ID NO: 5. The nucleotide sequence of human IL-15 receptor-alpha is shown in SEQ ID NO: 6. Sequence information related to IL-15 receptor-alpha is accessible in public databases by GenBank Accession numbers U31628.1 (for mRNA) and Q13261 (for protein).

[0086] SEQ ID NO: 5 is the human wild type amino acid sequence corresponding to IL-15 receptor alpha (residues 1-267):

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1 MAPRRARGCR TLGLPALLLL LLLRPPATRG ITCPPMSVE HADIWVKSYS LYSRERYICN
61 SGFKRKAGTS SLTECVLNKA TNVAHWTTTS LKCI RDPALV HQRPA PPSSTV TTAGVTPQPE

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121 SLSPSGKEFA ASSPSSNNTA ATTAAIVPGS QLMPSPKSPST GTTEISSHES SHGTPSQTTA
 181 KNWELTASAS HQPPGVYPQG HSDTTVAIST STVLLCGLSA VSL LACYLKS RQTPPLASVE
 241 MEAMEALPVT WGTSSRDEDL ENCSHHL

[0087] SEQ ID NO: 6 is the human wild type nucleotide sequence corresponding to IL-15 receptor-alpha (nucleotides 1-1610), wherein the underscored bolded "ATG" denotes the beginning of the open reading frame:

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1  cccagagcag cgctcgccac ctcccccccg cctgggcagc gctcgcccg ggagtcacgc
61 ggtgtcctgt ggagctgccg ccatggcccc gcggcgggcg cgcggtgcc ggacctcgg
121 tctcccgcg ctgctactgc tgctgtgtct ccggccgccg gcgacgcgg gcacacgtg
181 ccctccccc atgtccgtgg aacacgcaga catctgggtc aagagctaca gcttgactc
241 cagggagcgg tacatttcta actctggttt caagcgtaaa gccggcacgt ccagcctgac
301 ggagtgcgtg ttgaacaagg ccacgaatgt cgcacctgg acaaccccc gtctcaaatg
361 cattagagac cctgccctgg ttcaccaaaag gccagcgcca ccctccacag taacgacggc
421 aggggtgacc ccacagccag agagcctctc cccttctgga aaagagcccg cagcttcac
481 tcccagctca aacaacacag cggccacaac agcagctatt gtcccgggct cccagctgat
541 gccttcaaaa tcaccttcca caggaaccac agagataagc agtcagtagt cctccacgg
601 caccctctct cagacaacag ccaagaactg ggaactcaca gcacccgct cccaccagcc
661 gccaggtgtg tatccacagg gccacagcga caccactgtg gctatctcca cgtccactgt
721 cctgctgtgt gggctgagcg ctgtgtctct cctggcatgc tacctcaagt caaggcaaac
781 tcccccgctg gccagcgttg aaatggaagc catggaggct ctgcccgtga cttgggggac
841 cagcagcaga gatgaagact tggaaaactg ctctcaccac ctatgaaact cggggaaacc
901 agcccagcta agtccggagt gaaggagcct ctctgcttta gctaaagacg actgagaaga
961 ggtgcaagga agcggggtcc aggagcaagc tcaccaggcc tctcagaagt cccagcagga
1021 tctcacggac tgccgggtcg gcgcctcctg cgcgaggag caggttctcc gcattcccat
1081 gggcaccacc tgctgcctg tcgtgccttg gaccacgggc ccagcttccc aggagagacc
1141 aaaggcttct gagcaggatt tttatttcat tacagtgtga gctgcctgga atacatgtgg
1201 taatgaaata aaaaccctgc ccgaatctt ccgtccctca tctaacttg cagttcacag
1261 agaaaagtga catacccaaa gctctctgtc aattacaagg cttctcctgg cgtgggagac
1321 gtctacaggg aagacaccag cgtttgggct tctaaccacc ctgtctccag ctgctctgca
1381 cacatggaca gggacctggg aaagggtggga gagatgctga gccagcgaa tcctctccat
1441 tgaaggattc aggaagaaga aaactcaact cagtgcatt ttacgaatat atgcgtttat
1501 atttatactt ccttgtctat tatatctata cattatata tatttgtatt ttgacattgt
1561 accttgtata aacaaaataa aacatctatt ttcaatatt ttaaaatgca

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[0088] The polypeptide sequence of human IL-15 receptor-beta is depicted in SEQ ID NO: 7. The nucleotide sequence of human IL-15 receptor-beta is shown in SEQ ID NO: 8. Sequence information related to IL-15 receptor-beta is accessible in public databases by GenBank Accession numbers NM_000878 (for mRNA) and NP_000869 (for protein).

[0089] SEQ ID NO: 7 is the human wild type amino acid sequence corresponding to IL-15 receptor-beta (residues 1-551):

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1  MAAPALSWRL PLLILLPLA TSWASAAVNG TSQFTCFYNS RANISCVWSQ DGALQDTSQC
61 VHAWPDRRRW NQTCELLPVS QASWACNLIL GAPDSQKLT VDIVTLRVLC REGVRWRVMA
121 IQDFKPFENL RLMAPISLQV VHVETHRCNI SWEISQASHY FERHLEFEAR TLSPGHTWEE
181 APLLTLKQKQ EWICLETLP DTQYEFQVRV KPLQGEFTTW SPWSQPLAFR TKPAALGKDT
241 IPWLGHLLVG LSGAFGFII L VYLLINCRNT GPWLKKVLKC NTPDPSKFFS QLSSEHGGDV

```

301 QKWLSSPFPS SSFSPGGLAP EISPLEVLER DKVTQILLQK DKVPEPASLS SNHSLTSCFT
 361 NQGYFFHFLP DALEIEACQV YFTYDPYSEE DPDEGVAGAP TGSSPQPLQP LSGEDDAYCT
 421 FPSRDDLLLF SPSLLGGPSP PSTAPGGSGA GEERMPPSLQ ERVPRDWDPO PLGPPTPGVP
 481 DLVDFQPPPE LVLREAGEEV PDAGPREGVS FPWSRPPGQG EFRALNARLP LNTDAYLSLQ
 541 ELQGQDPHTL V

[0090] SEQ ID NO: 8 is the human wild type nucleotide sequence corresponding to IL-15 receptor-beta (nucleotides 1-4045), wherein the underscored bolded "ATG" denotes the beginning of the open reading frame:

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1 gcagccagag ctcagcaggg ccctggagag atggccacgg tcccagcacc ggggaggact
61 ggagagcgcg cgctgccacc gcccacatgtc tcagccaggg cttccttcct cggctccacc
121 ctgtggatgt aatggcgggc cctgctctgt cctggcgtct gcccctctct atcctcctcc
181 tgcccttggc tacctcttgg gcatctgcag cggatgaatg cacttcccag ttcacatgct
241 tctacaactc gagagccaac atctcctgtg tctggagcca agatggggct ctgcaggaca
301 cttcctgcca agtccatgcc tggccggaca gacggcgggt gaaccaaacc tgtgagctgc
361 tccccgtgag tcaagcatcc tgggcctgca acctgatcct cggagcccca gattctcaga
421 aactgaccac agttgacatc gtcaccctga ggggtgctgt cctgagggg gtgcgatgga
481 ggggtgatgg catccaggac ttcaagccct ttgagaacct tcgcctgatg gccccatct
541 ccctccaagt tgtccacgtg gagaccacac gatgcaacat aagctgggaa atctcccaag
601 cctcccacta ctttgaaaga cacctggagt tcgaggcccg gacgtgtctc ccaggccaca
661 cctgggagga gggcccccctg ctgactctca agcagaagca ggaatggatc tgcctggaga
721 cgctcacccc agacaccag tatgagtttc aggtgcgggt caagcctctg caaggcgagt
781 tcacgacctg gagcccttgg agccagcccc tggccttcag gacaaagcct gcagcccttg
841 ggaaggacac cattccgtgg ctggccacc tcctcgtggg cctcagcggg gcttttggct
901 tcatcatctt agtgtacttg ctgatcaact gcaggaacac cgggccatgg ctgaagaagg
961 tcctgaagtg taacacccca gacccctcga agttcttttc ccagctgagc tcagagcatg
1021 gaggagacgt ccagaagtgg ctctcttcgc cctcccctc atcgtctctc agccctggcg
1081 cctgggcacc tgagatctcg ccactagaag tgctggagag ggacaaggat acgcagctgc
1141 tcctgcagca ggacaagggt cctgagcccg catccttaag cagcaaccac tcgctgacca
1201 gctgcttcac caaccagggt tactttcttc tcacactccc ggatgccttg gagatagagg
1261 cctgccagggt gtactttact tacgaccctt actcagagga agaccctgat gagggtgtgg
1321 ccggggcacc cacagggtct tcccccaaac ccctgcagcc tctgtcaggg gaggacgacg
1381 cctactgcac cttcccctcc agggatgacc tgctgctctt ctcctccagt ctctcgggtg
1441 gcccagccc cccaagcact gcccctgggg gcagtggggc cggatgaagg aggatgccc
1501 cttctttgca agaaagagtc cccagagagc gggaccccca gcccctgggg cctcccacc
1561 caggagtcac agacctgggt gattttcagc caccctctga gctgggtgtg cgagaggctg
1621 gggaggaggt ccctgacgtg gggccagagg agggagtcag tttccccttg tccaggcctc
1681 ctgggcaggg ggagttcagg gcccttaatg ctgcctgcc cctgaacact gatgcctact
1741 tgcctctcca agaactccag ggtcaggacc caactcactt ggtgtagaca gatggccagg
1801 gtgggaggca ggcagctgcc tgcctctgcg cgagcctcag aaggaccctg ttgagggtcc
1861 tcagtccact gctgaggaca ctcagtgtcc agttgcagct ggacttctcc acccggatgg
1921 cccccaccca gtcctgcaca cttgggccat ccatttccaa acctccactg ctgctcccgg
1981 gtcctgctgc ccgagccagg aactgtgtgt gttgcagggg ggcagtaact ccccaactcc
2041 ctctgtaatc acaggatccc acgaatttag gctcagaagc atcgtctctc tccagccctg
2101 cagctattca ccaatatcag tctcgcggc tctccagggc tcctgccct gacctcttcc
2161 ctgggttttc tgccccagcc tctccttccc ctcctccccc cgtccacagg gcagcctgag
2221 cgtgctttcc aaaacccaaa tatggccacg ctcctccctg gttcaaaacc ttgcacagggt
2281 cccactgccc tcagccccac ttctcagcct ggtacttgta cctccgggtg cgtgtgggga
2341 catcccttcc tgcaatcctc cctaccgtcc tcctgagcca ctcagagctc cctcacacc
2401 cctctgttgc acatgctatt ccctgggggt gctgtgcgct cccctctatc taggtgacaa
2461 acttccctga ctcttcaagt gccggttttg cttctcctgg agggaagcac tgctccctt
2521 aatctgccag aaacttctag cgtcagtgct ggaggagaa gctgtcaggg acccagggcg
2581 cctggagaaa gaggccctgt tactattcct ttgggatctc tgaggcctca gactgcttgg
2641 ctgctgtatc tttaatgctg gggcccaagt aagggcacag atccccccac aaagtggatg
2701 cctgctgcat cttccacag tggcttcaca gaccacaag agaagctgat ggggagtaaa
2761 ccctggagtc cgaggccag gcagcagccc cgcctagtgg tgggccctga tgcctgcagg

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2821 cctgggacct cccactgccc cctccactgg aggggtctcc tctgcagctc agggactggc
2881 acactggcct ccagaagggc agctccacag ggcagggcct cattatTTTT cactgcccc
2941 gacacagtgc ccaacacccc gtcgtatacc ctggatgaac gaattaatta cctggcacca
3001 cctcgtctgg gctccctgcg cctgacattc acacagagag gcagagtccc gtgcccatta
3061 ggtctggcat gccccctcct gcaaggggct caacccccta ccccgacccc tccacgtatc
3121 tttcctaggc agatcacgtt gcaatggctc aaacaacatt ccaccccagc aggacagtga
3181 cccagtccc agctaactct gacctgggag ccctcaggca cctgcactta caggccttgc
3241 tcacagtga ttgggcacct gaccacacgc cccacaggc tctgaccagc agcctatgag
3301 ggggtttggc accaagctct gtccaatcag gtaggctggg cctgaactag ccaatcagat
3361 caactctgtc ttgggcgttt gaactcaggg agggaggccc ttgggagcag gtgcttgtgg
3421 acaaggctcc acaagcgttg agccttggaa aggtagacaa gcgttgagcc actaagcaga
3481 ggaccttggg ttcccaatac aaaaatacct actgctgaga gggctgctga ccatttggtc
3541 aggattcctg ttgcctttat atccaaaata aactcccctt tcttgagggt gtctgagtct
3601 tgggtctatg ccttgaaaaa agctgaatta ttggacagtc tcacctcctg ccatagggtc
3661 ctgaatgttt cagaccacaa ggggtccac acctttgctg tgtgttctgg ggcaacctac
3721 taatcctctc tgcaagtcgg tctccttata cccccaatg gaaattgtat ttgccttctc
3781 cactttggga ggctccact tcttgggagg gttacatttt ttaagtctta atcatttgtg
3841 acatatgtat ctatacatcc gtatctttta atgatccgtg tgtaccatct ttgtgattat
3901 ttcttaata ttttttcttt aagtcagttc attttcgttg aaatacatTT atttaaagaa
3961 aaatctttgt tactctgtaa atgaaaaaac ccattttcgc tataaataaa aggtaactgt
4021 acaaaataag tacaatgcaa caaaa

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[0091] A NKG2D protein is encoded by a nucleic acid (including, for example, genomic DNA, complementary DNA (cDNA), synthetic DNA, as well as any form of corresponding RNA). For example, it can be encoded by a recombinant nucleic acid of a NKG2D gene. The NKG2D proteins of the invention can be obtained from various sources and can be produced according to various techniques known in the art. For example, a nucleic acid that encodes a NKG2D protein can be obtained by screening DNA libraries, or by amplification from a natural source. A NKG2D protein can be a fragment or portion thereof. The nucleic acids encoding a NKG2D protein can be produced via recombinant DNA technology and such recombinant nucleic acids can be prepared by conventional techniques, including chemical synthesis, genetic engineering, enzymatic techniques, or a combination thereof. A NKG2D protein is the polypeptide encoded by the nucleic acid having the nucleotide sequence shown in SEQ ID NO: 4. An example of a NKG2D polypeptide has the amino acid sequence shown in SEQ ID NO: 3.

[0092] The polypeptide sequence of human NKG2D is depicted in SEQ ID NO: 3. The nucleotide sequence of human NKG2D is shown in SEQ ID NO: 4. Sequence information related to NKG2D is accessible in public databases by GenBank Accession numbers NM_007360 (for mRNA) and NP_031386 (for protein).

[0093] NKG2-D type II integral membrane protein (NKG2D) is a protein encoded by the KLRK1 (killer cell lectin-like receptor subfamily K, member 1) gene. KLRK1 has also been

designated as CD314. (Nausch N, Cerwenka A. *Oncogene*. 2008 Oct 6;27(45):5944-58; and González S, et al., *Trends Immunol*. 2008 Aug;29(8):397-403).

[0094] The NKG2D axis is of widespread interest in cancer and autoimmunity (including celiac disease, RA and T1D) and many biomedical researchers have described pre-clinical methodologies that block NKG2D activation and reverse pathogenesis in animal models. Research of AA lesions in human and mouse confirms that these proteins are highly expressed by both the hair follicle target and by the attacking immune cells. In one embodiment, a therapeutic approach in AA is using commercially available NKG2D murine blocking antibodies. At least three different pharmaceutical companies have developed humanized monoclonal antibodies that can target this molecule in human autoimmunity. One of these antibodies (NN8555 (NovoNordisk; formerly called IPH 2301)) has functional blocking characteristics in vitro.

[0095] SEQ ID NO: 3 is the human wild type amino acid sequence corresponding to NKG2D (residues 1-216):

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1  MGWIRGRRSR HSWEMSEFHN YNLDLKKSD F STRWQKQRC P VVKSCKRENA SPFFFCFFIA
61  VAMGIRFIIM VTIWSAVFLN SLFNQEVQIP LTESYCGPCP KNWICYKNNC YQFFDESKNW
121 YESQASCMSQ NASLLKVYSK EDQDLLKLVK SYHWMGLVHI PTNGSWQWED GSILSPNLLT
181 IIEMQKGDCA LYASSFKGYI ENCSTPNTYI CMQRTV

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[0096] SEQ ID NO: 4 is the human wild type nucleotide sequence corresponding to NKG2D (nucleotides 1-1593), wherein the underscored bolded "ATG" denotes the beginning of the open reading frame:

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1  actttcaatt ctagatcagg aactgaggac atatctaaat tttctagttt tatagaaggc
61  ttttatccac aagaatcaag atcttccttc tctgagcagg aatcctttgt gcattgaaga
121 ctttagattc ctctctgcgg tagacgtgca cttataagta tttgatgggg tggattcggtg
181 gtcggaggtc tcgacacagc tgggagatga gtgaatttca taattataac ttggatctga
241 agaagagtga tttttcaaca cgatggcaaa agcaaagatg tccagtagtc aaaagcaaat
301 gtagagaaaa tgcactctca ttttttttct gctgcttcat cgctgtagcc atgggaatcc
361 gtttcattat tatggtaaca atatggagtg ctgtattcct aaactcatta ttcaaccaag
421 aagttcaaat tcccttgacc gaaagttact gtggcccatg tcctaaaaac tggatatgtt
481 acaaaaataa ctgctaccaa ttttttgatg agagtaaaaa ctggtatgag agccaggcctt
541 cttgtatgtc tcaaaatgcc agccttctga aagtatacag caaagaggac caggatttac
601 ttaaaactgg gaagtcatat cattggatgg gactagtaca cattccaaca aatggatcctt
661 ggcagtggga agatggctcc attctctcac ccaacctact aacaataatt gaaatgcaga
721 agggagactg tgcactctat gcctcgagct ttaaaggcta tatagaaaac tgttcaactc
781 caaatcagta catctgcatg caaaggactg tgtaaagatg atcaaccatc tcaataaaaag
841 ccaggaacag agaagagatt acaccagcgg taacactgcc aactgagact aaaggaaaca
901 acaaaaaaca ggacaaaatg accaaagact gtcagatttc ttagactcca caggaccaa
961 ccatagaaaca atttcactgc aaacatgcat gattctccaa gacaaaagaa gaggattcctt
1021 aaaggcaatt cagatatccc caaggctgcc tctcccacca caagcccaga gtggatgggc
1081 tgggggaggg gtgctgtttt aatttctaaa ggtaggacca acaccagggt gatcagtgaa
1141 ggaagagaag gccagcagat cactgagagt gcaacccac cctccacagg aaattgcctc

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1201 atgggcaggg ccacagcaga gagacacagc atgggcagtg ccttccttgc ctgtgggggt
1261 catgctgcca cttttaatgg gtcctccacc caacggggtc agggaggtgg tgctgccccca
1321 gtggggccatg attatcttaa aggcattatt ctccagcctt aagtaagatc ttaggacggt
1381 tcctttgcta tgatttgtac ttgcttgagt cccatgactg tttctcttcc tctctttctt
1441 ccttttggaa tagtaatatc catcctatgt ttgtcccact attgtatttt ggaagcacat
1501 aacttgtttg gtttcacagg ttcacagtta agaaggaatt ttgcctctga ataaatagaa
1561 tcttgagtct catgcaaaaa aaaaaaaaaa aaa

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[0097] Protein Variants: Protein variants can include amino acid sequence modifications. For example, amino acid sequence modifications fall into one or more of three classes: substitutional, insertional or deletional variants. Insertions can include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. These variants ordinarily are prepared by site-specific mutagenesis of nucleotides in the DNA encoding the protein, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture.

[0098] Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis. Amino acid substitutions can be single residues, but can occur at a number of different locations at once. In one non-limiting embodiment, insertions can be on the order of about from 1 to about 10 amino acid residues, while deletions can range from about 1 to about 30 residues. Deletions or insertions can be made in adjacent pairs (for example, a deletion of about 2 residues or insertion of about 2 residues). Substitutions, deletions, insertions, or any combination thereof can be combined to arrive at a final construct. The mutations cannot place the sequence out of reading frame and should not create complementary regions that can produce secondary mRNA structure. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place.

[0099] Substantial changes in function or immunological identity are made by selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions that can produce the greatest changes in the protein properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is

substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine, in this case, (e) by increasing the number of sites for sulfation and/or glycosylation.

[00100] Minor variations in the amino acid sequences of proteins are provided by the present invention. The variations in the amino acid sequence can be when the sequence maintains at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, at least about 95%, or at least about 99% identity to SEQ ID NO: 1, 3, 5 or 7. For example, conservative amino acid replacements can be utilized. Conservative replacements are those that take place within a family of amino acids that are related in their side chains, wherein the interchangeability of residues have similar side chains.

[00101] Genetically encoded amino acids are generally divided into families: (1) acidic amino acids are aspartate, glutamate; (2) basic amino acids are lysine, arginine, histidine; (3) non-polar amino acids are alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, and (4) uncharged polar amino acids are glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. The hydrophilic amino acids include arginine, asparagine, aspartate, glutamine, glutamate, histidine, lysine, serine, and threonine. The hydrophobic amino acids include alanine, cysteine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine and valine. Other families of amino acids include (i) a group of amino acids having aliphatic-hydroxyl side chains, such as serine and threonine; (ii) a group of amino acids having amide-containing side chains, such as asparagine and glutamine; (iii) a group of amino acids having aliphatic side chains such as glycine, alanine, valine, leucine, and isoleucine; (iv) a group of amino acids having aromatic side chains, such as phenylalanine, tyrosine, and tryptophan; and (v) a group of amino acids having sulfur-containing side chains, such as cysteine and methionine. Useful conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine valine, glutamic-aspartic, and asparagine-glutamine.

[00102] For example, the replacement of one amino acid residue with another that is biologically and/or chemically similar is known to those skilled in the art as a conservative

substitution. For example, a conservative substitution would be replacing one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Substitutional or deletional mutagenesis can be employed to insert sites for N-glycosylation (Asn-X-Thr/Ser) or O-glycosylation (Ser or Thr). Deletions of cysteine or other labile residues also can be desirable. Deletions or substitutions of potential proteolysis sites, e.g. Arg, is accomplished for example by deleting one of the basic residues or substituting one by glutamyl or histidyl residues.

[00103] Bacterial and Yeast Expression Systems. In bacterial systems, a number of expression vectors can be selected. For example, when a large quantity of a protein encoded by a gene, such as NKG2D or IL-15, is needed for the induction of antibodies, vectors which direct high level expression of proteins that are readily purified can be used. Non-limiting examples of such vectors include multifunctional *E. coli* cloning and expression vectors such as BLUESCRIPT (Stratagene). pIN vectors or pGEX vectors (Promega, Madison, Wis.) also can be used to express foreign polypeptide molecules as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems can be designed to include heparin, thrombin, or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

[00104] Plant and Insect Expression Systems. If plant expression vectors are used, the expression of sequences encoding a NKG2D or IL-15 protein can be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV can be used alone or in combination with the omega leader sequence from TMV. Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters, can be used. These constructs can be introduced into plant cells by direct DNA transformation or by pathogen-mediated transfection.

[00105] An insect system also can be used to express NKG2D or IL-15 proteins. For example, in one such system *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. Sequences encoding a polypeptide of NKG2D or IL-15 can be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the

polyhedrin promoter. Successful insertion of nucleic acid sequences, such as a sequence corresponding to a gene, such as a NKG2D or IL-15 gene, will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses can then be used to infect *S. frugiperda* cells or *Trichoplusia* larvae in which the protein or a variant thereof can be expressed.

[00106] Mammalian Expression Systems. An expression vector can include a nucleotide sequence that encodes a NKG2D or IL-15 polypeptide linked to at least one regulatory sequence in a manner allowing expression of the nucleotide sequence in a host cell. A number of viral-based expression systems can be used to express a NKG2D or IL-15 protein or a variant thereof in mammalian host cells. For example, if an adenovirus is used as an expression vector, sequences encoding a protein can be ligated into an adenovirus transcription/translation complex comprising the late promoter and tripartite leader sequence. Insertion into a non-essential E1 or E3 region of the viral genome can be used to obtain a viable virus which expresses a NKG2D or IL-15 protein in infected host cells. Transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, can also be used to increase expression in mammalian host cells.

[00107] Regulatory sequences are well known in the art, and can be selected to direct the expression of a protein or polypeptide of interest in an appropriate host cell as described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990). Non-limiting examples of regulatory sequences include: polyadenylation signals, promoters (such as CMV, ASV, SV40, or other viral promoters such as those derived from bovine papilloma, polyoma, and Adenovirus 2 viruses (Fiers, et al., 1973, *Nature* 273:113; Hager GL, et al., *Curr Opin Genet Dev*, 2002, 12(2):137-41) enhancers, and other expression control elements.

[00108] Enhancer regions, which are those sequences found upstream or downstream of the promoter region in non-coding DNA regions, are also known in the art to be important in optimizing expression. If needed, origins of replication from viral sources can be employed, such as if a prokaryotic host is utilized for introduction of plasmid DNA. However, in eukaryotic organisms, chromosome integration is a common mechanism for DNA replication.

[00109] For stable transfection of mammalian cells, a small fraction of cells can integrate introduced DNA into their genomes. The expression vector and transfection method utilized

can be factors that contribute to a successful integration event. For stable amplification and expression of a desired protein, a vector containing DNA encoding a protein of interest is stably integrated into the genome of eukaryotic cells (for example mammalian cells, such as cells from the end bulb of the hair follicle), resulting in the stable expression of transfected genes. An exogenous nucleic acid sequence can be introduced into a cell (such as a mammalian cell, either a primary or secondary cell) by homologous recombination as disclosed in U.S. Patent 5,641,670, the contents of which are herein incorporated by reference.

[00110] A gene that encodes a selectable marker (for example, resistance to antibiotics or drugs, such as ampicillin, neomycin, G418, and hygromycin) can be introduced into host cells along with the gene of interest in order to identify and select clones that stably express a gene encoding a protein of interest. The gene encoding a selectable marker can be introduced into a host cell on the same plasmid as the gene of interest or can be introduced on a separate plasmid. Cells containing the gene of interest can be identified by drug selection wherein cells that have incorporated the selectable marker gene will survive in the presence of the drug. Cells that have not incorporated the gene for the selectable marker die. Surviving cells can then be screened for the production of the desired protein molecule (for example, a protein encoded by a gene, such as NKG2D or IL-15).

Cell Transfection

[00111] A eukaryotic expression vector can be used to transfect cells in order to produce proteins encoded by nucleotide sequences of the vector. Mammalian cells (such as isolated cells from the hair bulb; for example dermal sheath cells and dermal papilla cells) can contain an expression vector (for example, one that contains a gene encoding a NKG2D or IL-15 protein or polypeptide) via introducing the expression vector into an appropriate host cell via methods known in the art.

[00112] A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed polypeptide encoded by a gene, such as a NKG2D or IL-15 gene, in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a “prepro” form of the polypeptide also can be used to facilitate correct insertion, folding and/or function. Different

host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and WI38), are available from the American Type Culture Collection (ATCC; 10801 University Boulevard, Manassas, Va. 20110-2209) and can be chosen to ensure the correct modification and processing of the foreign protein.

[00113] An exogenous nucleic acid can be introduced into a cell via a variety of techniques known in the art, such as lipofection, microinjection, calcium phosphate or calcium chloride precipitation, DEAE-dextran-mediated transfection, or electroporation. Electroporation is carried out at approximate voltage and capacitance to result in entry of the DNA construct(s) into cells of interest (such as cells of the end bulb of a hair follicle, for example dermal papilla cells or dermal sheath cells). Other transfection methods also include modified calcium phosphate precipitation, polybrene precipitation, liposome fusion, and receptor-mediated gene delivery.

[00114] Cells that will be genetically engineered can be primary and secondary cells obtained from various tissues, and include cell types which can be maintained and propagated in culture. Non-limiting examples of primary and secondary cells include epithelial cells (for example, dermal papilla cells, hair follicle cells, inner root sheath cells, outer root sheath cells, sebaceous gland cells, epidermal matrix cells), neural cells, endothelial cells, glial cells, fibroblasts, muscle cells (such as myoblasts) keratinocytes, formed elements of the blood (e.g., lymphocytes, bone marrow cells), and precursors of these somatic cell types.

[00115] Vertebrate tissue can be obtained by methods known to one skilled in the art, such as a punch biopsy or other surgical methods of obtaining a tissue source of the primary cell type of interest. In one embodiment, a punch biopsy or removal can be used to obtain a source of keratinocytes, fibroblasts, endothelial cells, or mesenchymal cells (for example, hair follicle cells or dermal papilla cells). In another embodiment, removal of a hair follicle can be used to obtain a source of fibroblasts, keratinocytes, endothelial cells, or mesenchymal cells (for example, hair follicle cells or dermal papilla cells). A mixture of primary cells can be obtained from the tissue, using methods readily practiced in the art, such as explanting or enzymatic digestion (for examples using enzymes such as pronase, trypsin, collagenase, elastase dispase, and chymotrypsin). Biopsy methods have also been described in United States Patent Application Publication 2004/0057937 and PCT application publication WO 2001/32840, and are hereby incorporated by reference.

[00116] Primary cells can be acquired from the individual to whom the genetically engineered primary or secondary cells are administered. However, primary cells can also be obtained from a donor, other than the recipient, of the same species. The cells can also be obtained from another species (for example, rabbit, cat, mouse, rat, sheep, goat, dog, horse, cow, bird, or pig). Primary cells can also include cells from an isolated vertebrate tissue source grown attached to a tissue culture substrate (for example, flask or dish) or grown in a suspension; cells present in an explant derived from tissue; both of the aforementioned cell types plated for the first time; and cell culture suspensions derived from these plated cells. Secondary cells can be plated primary cells that are removed from the culture substrate and replated, or passaged, in addition to cells from the subsequent passages. Secondary cells can be passaged one or more times. These primary or secondary cells can contain expression vectors having a gene that encodes a protein of interest (for example, a NKG2D or IL-15 protein or polypeptide).

Cell Culturing

[00117] Various culturing parameters can be used with respect to the host cell being cultured. Appropriate culture conditions for mammalian cells are well known in the art (Cleveland WL, et al., *J Immunol Methods*, 1983, 56(2): 221-234) or can be determined by the skilled artisan (see, for example, Animal Cell Culture: A Practical Approach 2nd Ed., Rickwood, D. and Hames, B. D., eds. (Oxford University Press: New York, 1992)). Cell culturing conditions can vary according to the type of host cell selected. Commercially available medium can be utilized. Non-limiting examples of medium include, for example, Minimal Essential Medium (MEM, Sigma, St. Louis, Mo.); Dulbecco's Modified Eagles Medium (DMEM, Sigma); Ham's F10 Medium (Sigma); HyClone cell culture medium (HyClone, Logan, Utah); RPMI-1640 Medium (Sigma); and chemically-defined (CD) media, which are formulated for various cell types, e.g., CD-CHO Medium (Invitrogen, Carlsbad, Calif.).

[00118] The cell culture media can be supplemented as necessary with supplementary components or ingredients, including optional components, in appropriate concentrations or amounts, as necessary or desired. Cell culture medium solutions provide at least one component from one or more of the following categories: (1) an energy source, usually in the form of a carbohydrate such as glucose; (2) all essential amino acids, and usually the basic set of twenty amino acids plus cysteine; (3) vitamins and/or other organic compounds required at

low concentrations; (4) free fatty acids or lipids, for example linoleic acid; and (5) trace elements, where trace elements are defined as inorganic compounds or naturally occurring elements that can be required at very low concentrations, usually in the micromolar range.

[00119] The medium also can be supplemented electively with one or more components from any of the following categories: (1) salts, for example, magnesium, calcium, and phosphate; (2) hormones and other growth factors such as, serum, insulin, transferrin, and epidermal growth factor; (3) protein and tissue hydrolysates, for example peptone or peptone mixtures which can be obtained from purified gelatin, plant material, or animal byproducts; (4) nucleosides and bases such as, adenosine, thymidine, and hypoxanthine; (5) buffers, such as HEPES; (6) antibiotics, such as gentamycin or ampicillin; (7) cell protective agents, for example pluronic polyol; and (8) galactose. In one embodiment, soluble factors can be added to the culturing medium.

[00120] The mammalian cell culture that can be used with the present invention is prepared in a medium suitable for the type of cell being cultured. In one embodiment, the cell culture medium can be any one of those previously discussed (for example, MEM) that is supplemented with serum from a mammalian source (for example, fetal bovine serum (FBS)). In another embodiment, the medium can be a conditioned medium to sustain the growth of epithelial cells or cells obtained from the hair bulb of a hair follicle (such as dermal papilla cells or dermal sheath cells). For example, epithelial cells can be cultured according to Barnes and Mather in Animal Cell Culture Methods (Academic Press, 1998), which is hereby incorporated by reference in its entirety. In a further embodiment, epithelial cells or hair follicle cells can be transfected with DNA vectors containing genes that encode a polypeptide or protein of interest (for example, a NKG2D or IL-15 protein or polypeptide). In other embodiments of the invention, cells are grown in a suspension culture (for example, a three-dimensional culture such as a hanging drop culture) in the presence of an effective amount of enzyme, wherein the enzyme substrate is an extracellular matrix molecule in the suspension culture. For example, the enzyme can be a hyaluronidase. Epithelial cells or hair follicle cells can be cultivated according to methods practiced in the art, for example, as those described in PCT application publication WO 2004/044188 and in U.S. Patent Application Publication No. 2005/0272150, or as described by Harris in Handbook in Practical Animal Cell Biology: Epithelial Cell Culture (Cambridge Univ. Press, Great Britain; 1996; see Chapter 8), which are hereby incorporated by reference.

[00121] A suspension culture is a type of culture wherein cells, or aggregates of cells (such as aggregates of DP cells), multiply while suspended in liquid medium. A suspension culture comprising mammalian cells can be used for the maintenance of cell types that do not adhere or to enable cells to manifest specific cellular characteristics that are not seen in the adherent form. Some types of suspension cultures can include three-dimensional cultures or a hanging drop culture. A hanging-drop culture is a culture in which the material to be cultivated is inoculated into a drop of fluid attached to a flat surface (such as a coverglass, glass slide, Petri dish, flask, and the like), and can be inverted over a hollow surface. Cells in a hanging drop can aggregate toward the hanging center of a drop as a result of gravity. However, according to the methods of the invention, cells cultured in the presence of a protein that degrades the extracellular matrix (such as collagenase, chondroitinase, hyaluronidase, and the like) will become more compact and aggregated within the hanging drop culture, for degradation of the ECM will allow cells to become closer in proximity to one another since less of the ECM will be present. See also International PCT Publication No. WO2007/100870, which is incorporated by reference.

[00122] Cells obtained from the hair bulb of a hair follicle (such as dermal papilla cells or dermal sheath cells) can be cultured as a single, homogenous population (for example, comprising DP cells) in a hanging drop culture so as to generate an aggregate of DP cells. Cells can also be cultured as a heterogeneous population (for example, comprising DP and DS cells) in a hanging drop culture so as to generate a chimeric aggregate of DP and DS cells. Epithelial cells can be cultured as a monolayer to confluency as practiced in the art. Such culturing methods can be carried out essentially according to methods described in Chapter 8 of the Handbook in Practical Animal Cell Biology: Epithelial Cell Culture (Cambridge Univ. Press, Great Britain; 1996); Underhill CB, *J Invest Dermatol*, 1993, 101(6):820-6; in Armstrong and Armstrong, (1990) *J Cell Biol* 110:1439-55; or in Animal Cell Culture Methods (Academic Press, 1998), which are all hereby incorporated by reference in their entireties.

[00123] Three-dimensional cultures can be formed from agar (such as Gey's Agar), hydrogels (such as matrigel, agarose, and the like; Lee et al., (2004) *Biomaterials* 25: 2461-2466) or polymers that are cross-linked. These polymers can comprise natural polymers and their derivatives, synthetic polymers and their derivatives, or a combination thereof. Natural polymers can be anionic polymers, cationic polymers, amphipathic polymers, or neutral

polymers. Non-limiting examples of anionic polymers can include hyaluronic acid, alginic acid (alginate), carageenan, chondroitin sulfate, dextran sulfate, and pectin. Some examples of cationic polymers, include but are not limited to, chitosan or polylysine. (Peppas et al., (2006) *Adv Mater.* 18: 1345-60; Hoffman, A. S., (2002) *Adv Drug Deliv Rev.* 43: 3-12; Hoffman, A. S., (2001) *Ann NY Acad Sci* 944: 62-73). Examples of amphipathic polymers can include, but are not limited to collagen, gelatin, fibrin, and carboxymethyl chitin. Non-limiting examples of neutral polymers can include dextran, agarose, or pullulan. (Peppas et al., (2006) *Adv Mater.* 18: 1345-60; Hoffman, A. S., (2002) *Adv Drug Deliv Rev.* 43: 3-12; Hoffman, A. S., (2001) *Ann NY Acad Sci* 944: 62-73).

[00124] Cells suitable for culturing according to methods of the invention can harbor introduced expression vectors, such as plasmids. The expression vector constructs can be introduced via transformation, microinjection, transfection, lipofection, electroporation, or infection. The expression vectors can contain coding sequences, or portions thereof, encoding the proteins for expression and production. Expression vectors containing sequences encoding the produced proteins and polypeptides, as well as the appropriate transcriptional and translational control elements, can be generated using methods well known to and practiced by those skilled in the art. These methods include synthetic techniques, *in vitro* recombinant DNA techniques, and *in vivo* genetic recombination which are described in J. Sambrook et al., 2001, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y. and in F. M. Ausubel et al., 1989, Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y.

Obtaining and Purifying Polypeptides

[00125] A polypeptide molecule encoded by a gene, such as a NKG2D or IL-15 gene, or a variant thereof, can be obtained by purification from human cells expressing a protein or polypeptide encoded by a NKG2D or IL-15 gene via *in vitro* or *in vivo* expression of a nucleic acid sequence encoding a NKG2D or IL-15 protein or polypeptide; or by direct chemical synthesis.

[00126] Detecting Polypeptide Expression. Host cells which contain a nucleic acid encoding a NKG2D or IL-15 protein or polypeptide, and which subsequently express a protein encoded by a NKG2D or IL-15 gene, can be identified by various procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or

DNA-RNA hybridizations and protein bioassay or immunoassay techniques which include membrane, solution, or chip-based technologies for the detection and/or quantification of nucleic acid or protein. For example, the presence of a nucleic acid encoding a NKG2D or IL-15 protein or polypeptide can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes or fragments of nucleic acids encoding a NKG2D or IL-15 protein or polypeptide. In one embodiment, a fragment of a nucleic acid of a NKG2D or IL-15 gene can encompass any portion of at least about 8 consecutive nucleotides of SEQ ID NO: 2 or 4. In another embodiment, the fragment can comprise at least about 10 consecutive nucleotides, at least about 15 consecutive nucleotides, at least about 20 consecutive nucleotides, or at least about 30 consecutive nucleotides of SEQ ID NO: 2 or 4. Fragments can include all possible nucleotide lengths between about 8 and about 100 nucleotides, for example, lengths between about 15 and about 100 nucleotides, or between about 20 and about 100 nucleotides. Nucleic acid amplification-based assays involve the use of oligonucleotides selected from sequences encoding a polypeptide encoded by a NKG2D or IL-15 gene to detect transformants which contain a nucleic acid encoding a NKG2D or IL-15 protein or polypeptide.

[00127] Protocols for detecting and measuring the expression of a polypeptide encoded by a gene, such as a NKG2D or IL-15 gene, using either polyclonal or monoclonal antibodies specific for the polypeptide are well established. Non-limiting examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay using monoclonal antibodies reactive to two non-interfering epitopes on a polypeptide encoded by a gene, such as a NKG2D or IL-15 gene, can be used, or a competitive binding assay can be employed.

[00128] Labeling and conjugation techniques are known by those skilled in the art and can be used in various nucleic acid and amino acid assays. Methods for producing labeled hybridization or PCR probes for detecting sequences related to nucleic acid sequences encoding a protein, such as NKG2D or IL-15, include, but are not limited to, oligolabeling, nick translation, end-labeling, or PCR amplification using a labeled nucleotide.

Alternatively, nucleic acid sequences encoding a polypeptide encoded by a gene, such as a NKG2D or IL-15 gene, can be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and can be used to synthesize RNA probes *in vitro* by addition of labeled nucleotides and an appropriate RNA polymerase such as T7, T3, or SP6. These procedures can be conducted using a variety of commercially

available kits (Amersham Pharmacia Biotech, Promega, and US Biochemical). Suitable reporter molecules or labels which can be used for ease of detection include radionuclides, enzymes, and fluorescent, chemiluminescent, or chromogenic agents, as well as substrates, cofactors, inhibitors, and/or magnetic particles.

[00129] Expression and Purification of Polypeptides. Host cells transformed with a nucleic acid sequence encoding a polypeptide, such as NKG2D or IL-15, can be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The polypeptide produced by a transformed cell can be secreted or contained intracellularly depending on the sequence and/or the vector used. Expression vectors containing a nucleic acid sequence encoding a polypeptide, such as NKG2D or IL-15, can be designed to contain signal sequences which direct secretion of soluble polypeptide molecules encoded by a gene, such as a NKG2D or IL-15 gene, or a variant thereof, through a prokaryotic or eukaryotic cell membrane or which direct the membrane insertion of membrane-bound a polypeptide molecule encoded by a NKG2D or IL-15 gene or a variant thereof.

[00130] Other constructions can also be used to join a gene sequence encoding a NKG2D or IL-15 polypeptide to a nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, Wash.). Including cleavable linker sequences (i.e., those specific for Factor Xa or enterokinase (Invitrogen, San Diego, Calif.)) between the purification domain and a polypeptide encoded by a NKG2D or IL-15 gene also can be used to facilitate purification. One such expression vector provides for expression of a fusion protein containing a polypeptide encoded by a NKG2D or IL-15 gene and 6 histidine residues preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification by immobilized metal ion affinity chromatography, while the enterokinase cleavage site provides a means for purifying the polypeptide encoded by a NKG2D or IL-15 gene.

[00131] A NKG2D or IL-15 polypeptide can be purified from any human or non-human cell which expresses the polypeptide, including those which have been transfected with expression constructs that express a NKG2D or IL-15 protein. A purified NKG2D or IL-15

protein can be separated from other compounds which normally associate with a protein encoded by a NKG2D or IL-15 gene in the cell, such as certain proteins, carbohydrates, or lipids, using methods practiced in the art. Non-limiting methods include size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, and preparative gel electrophoresis.

[00132] Chemical Synthesis. Nucleic acid sequences comprising a gene, such as a NKG2D or IL-15 gene, that encodes a polypeptide can be synthesized, in whole or in part, using chemical methods known in the art. Alternatively, a polypeptide, such as NKG2D or IL-15, can be produced using chemical methods to synthesize its amino acid sequence, such as by direct peptide synthesis using solid-phase techniques. Protein synthesis can either be performed using manual techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Optionally, fragments of NKG2D or IL-15 polypeptides can be separately synthesized and combined using chemical methods to produce a full-length molecule. In one embodiment, a fragment of a nucleic acid sequence that comprises a NKG2D or IL-15 gene can encompass any portion of at least about 8 consecutive nucleotides of SEQ ID NO: 2 or 4. In one embodiment, the fragment can comprise at least about 10 nucleotides, at least about 15 nucleotides, at least about 20 nucleotides, or at least about 30 nucleotides of SEQ ID NO: 2 or 4. Fragments include all possible nucleotide lengths between about 8 and about 100 nucleotides, for example, lengths between about 15 and about 100 nucleotides, or between about 20 and about 100 nucleotides.

[00133] A NKG2D or IL-15 fragment can be a fragment of a protein, such as NKG2D or IL-15. For example, the NKG2D or IL-15 fragment can encompass any portion of at least about 8 consecutive amino acids of SEQ ID NO: 1 or 3. The fragment can comprise at least about 10 consecutive amino acids, at least about 20 consecutive amino acids, at least about 30 consecutive amino acids, at least about 40 consecutive amino acids, at least about 50 consecutive amino acids, at least about 60 consecutive amino acids, at least about 70 consecutive amino acids, or at least about 75 consecutive amino acids of SEQ ID NO: 1 or 3. Fragments include all possible amino acid lengths between about 8 and about 100 amino acids, for example, lengths between about 10 and about 100 amino acids, between about 15 and about 100 amino acids, between about 20 and about 100 amino acids, between about 35 and about 100 amino acids, between about 40 and about 100 amino acids, between about 50

and about 100 amino acids, between about 70 and about 100 amino acids, between about 75 and about 100 amino acids, or between about 80 and about 100 amino acids.

[00134] A synthetic peptide can be substantially purified via high performance liquid chromatography (HPLC). The composition of a synthetic polypeptide of NKG2D or IL-15 can be confirmed by amino acid analysis or sequencing. Additionally, any portion of an amino acid sequence comprising a protein encoded by a NKG2D or IL-15 gene can be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins to produce a variant polypeptide or a fusion protein.

Identifying NKG2D or IL-15 Modulating Compounds

[00135] The invention provides methods for identifying compounds which can be used for controlling and/or regulating hair growth (for example, hair density) or hair pigmentation in a subject. Since the invention has provided the identification of the genes listed herein as genes associated with a hair loss disorder, the invention also provides methods for identifying compounds that modulate the expression or activity of a gene and/or protein of NKG2D or IL-15. In addition, the invention provides methods for identifying compounds which can be used for the treatment of a hair loss disorder. The invention also provides methods for identifying compounds which can be used for the treatment of hypotrichosis (for example, hereditary hypotrichosis simplex (HHS)). Non-limiting examples of hair loss disorders include: androgenetic alopecia, Alopecia areata, telogen effluvium, alopecia areata, alopecia totalis, and alopecia universalis. The methods can comprise the identification of test compounds or agents (e.g., peptides (such as antibodies or fragments thereof), small molecules, nucleic acids (such as siRNA or antisense RNA), or other agents) that can bind to a polypeptide molecule encoded by a NKG2D or IL-15 gene and/or have a stimulatory or inhibitory effect on the biological activity of a protein encoded by a NKG2D or IL-15 gene or its expression, and subsequently determining whether these compounds can regulate hair growth in a subject or can have an effect on symptoms associated with the hair loss disorders in an *in vivo* assay (i.e., examining an increase or reduction in hair growth).

[00136] As used herein, a “NKG2D or IL-15 modulating compound” refers to a compound that interacts with a NKG2D or IL-15 gene or a NKG2D or IL-15 protein or polypeptide and modulates its activity and/or its expression. The compound can either increase the activity or expression of a protein encoded by a NKG2D or IL-15 gene. Conversely, the compound can

decrease the activity or expression of a protein encoded by a NKG2D or IL-15 gene. The compound can be a NKG2D or IL-15 agonist or a NKG2D or IL-15 antagonist (e.g., an IL-15 inhibitor or an NKG2D inhibitor). Some non-limiting examples of NKG2D or IL-15 modulating compounds include peptides (such as peptide fragments comprising a polypeptide encoded by a NKG2D or IL-15 gene, or antibodies or fragments thereof), small molecules, and nucleic acids (such as siRNA or antisense RNA specific for a nucleic acid comprising a NKG2D or IL-15 gene). Agonists of a NKG2D or IL-15 protein can be molecules which, when bound to a NKG2D or IL-15 protein, increase or prolong the activity of the NKG2D or IL-15 protein. NKG2D or IL-15 agonists include, but are not limited to, proteins, nucleic acids, small molecules, or any other molecule which activates a NKG2D or IL-15 protein. Antagonists of a NKG2D or IL-15 protein can be molecules which, when bound to a NKG2D or IL-15 protein decrease the amount or the duration of the activity of the NKG2D or IL-15 protein. Antagonists include proteins, nucleic acids, antibodies, small molecules, or any other molecule which decrease the activity of a NKG2D or IL-15 protein.

[00137] The term “modulate,” as it appears herein, refers to a change in the activity or expression of a gene or protein of NKG2D or IL-15. For example, modulation can cause an increase or a decrease in protein activity, binding characteristics, or any other biological, functional, or immunological properties of a NKG2D or IL-15 protein.

[00138] In one embodiment, a NKG2D or IL-15 modulating compound can be a peptide fragment of a NKG2D or IL-15 protein that binds to the protein. For example, the NKG2D or IL-15 polypeptide can encompass any portion of at least about 8 consecutive amino acids of SEQ ID NO: 1, 3, 5 or 7. The fragment can comprise at least about 10 consecutive amino acids, at least about 20 consecutive amino acids, at least about 30 consecutive amino acids, at least about 40 consecutive amino acids, at least about 50 consecutive amino acids, at least about 60 consecutive amino acids, or at least about 75 consecutive amino acids of SEQ ID NO: 1, 3 5 or 7. Fragments include all possible amino acid lengths between and including about 8 and about 100 amino acids, for example, lengths between about 10 and about 100 amino acids, between about 15 and about 100 amino acids, between about 20 and about 100 amino acids, between about 35 and about 100 amino acids, between about 40 and about 100 amino acids, between about 50 and about 100 amino acids, between about 70 and about 100 amino acids, between about 75 and about 100 amino acids, or between about 80 and about 100 amino acids. These peptide fragments can be obtained commercially or synthesized via

liquid phase or solid phase synthesis methods (Atherton et al., (1989) Solid Phase Peptide Synthesis: a Practical Approach. IRL Press, Oxford, England). The NKG2D or IL-15 peptide fragments can be isolated from a natural source, genetically engineered, or chemically prepared. These methods are well known in the art.

[00139] A NKG2D or IL-15 modulating compound can be a protein, such as an antibody (monoclonal, polyclonal, humanized, chimeric, or fully human), or a binding fragment thereof, directed against a polypeptide encoded by a NKG2D or IL-15 gene. An antibody fragment can be a form of an antibody other than the full-length form and includes portions or components that exist within full-length antibodies, in addition to antibody fragments that have been engineered. Antibody fragments can include, but are not limited to, single chain Fv (scFv), diabodies, Fv, and (Fab')₂, triabodies, Fc, Fab, CDR1, CDR2, CDR3, combinations of CDR's, variable regions, tetrabodies, bifunctional hybrid antibodies, framework regions, constant regions, and the like (*see*, Maynard et al., (2000) *Ann. Rev. Biomed. Eng.* 2:339-76; Hudson (1998) *Curr. Opin. Biotechnol.* 9:395-402). In one embodiment, an antibody or binding fragment thereof is directed against SEQ ID NO: 1, 3, 5 or 7. Antibodies can be obtained commercially, custom generated, or synthesized against an antigen of interest according to methods established in the art (Janeway et al., (2001) Immunobiology, 5th ed., Garland Publishing). For example, Antibodies directed to IL-15 as well as NKG2D can be obtained commercially from Abcam, Santa Cruz Biotechnology, Abnova Corp., BD Biosciences, Antigenix America Inc., etc. Human antibodies directed to either IL-15 or NKG2D (such as monoclonal, humanized, or chimeric antibodies) can be useful antibody therapeutics for use in humans. In one embodiment, blocking the IL-15 receptor and/or its downstream signaling would also benefit AA patients. To block IL-15 receptors, humanized, chimeric, or monoclonal blocking antibodies are available. For example, IL-15 can be blocked using an antibody against CD122 (IL-15Rbeta).

[00140] Inhibition of RNA encoding a polypeptide encoded by a NKG2D or IL-15 gene can effectively modulate the expression of a NKG2D or IL-15 gene from which the RNA is transcribed. Inhibitors are selected from the group comprising: siRNA; interfering RNA or RNAi; dsRNA; RNA Polymerase III transcribed DNAs; ribozymes; and antisense nucleic acids, which can be RNA, DNA, or an artificial nucleic acid.

[00141] Antisense oligonucleotides, including antisense DNA, RNA, and DNA/RNA molecules, act to directly block the translation of mRNA by binding to targeted mRNA and

preventing protein translation. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the DNA sequence encoding a polypeptide encoded by a NKG2D or IL-15 gene can be synthesized, e.g., by conventional phosphodiester techniques (Dallas et al., (2006) *Med. Sci. Monit.* 12(4):RA67-74; Kalota et al., (2006) *Handb. Exp. Pharmacol.* 173:173-96; Lutzelburger et al., (2006) *Handb. Exp. Pharmacol.* 173:243-59). Antisense nucleotide sequences include, but are not limited to: morpholinos, 2'-O-methyl polynucleotides, DNA, RNA and the like.

[00142] siRNA comprises a double stranded structure containing from about 15 to about 50 base pairs, for example from about 21 to about 25 base pairs, and having a nucleotide sequence identical or nearly identical to an expressed target gene or RNA within the cell. The siRNA comprise a sense RNA strand and a complementary antisense RNA strand annealed together by standard Watson-Crick base-pairing interactions. The sense strand comprises a nucleic acid sequence which is substantially identical to a nucleic acid sequence contained within the target miRNA molecule. "Substantially identical" to a target sequence contained within the target mRNA refers to a nucleic acid sequence that differs from the target sequence by about 3% or less. The sense and antisense strands of the siRNA can comprise two complementary, single-stranded RNA molecules, or can comprise a single molecule in which two complementary portions are base-paired and are covalently linked by a single-stranded "hairpin" area. See also, McMnaus and Sharp (2002) *Nat Rev Genetics*, 3:737-47, and Sen and Blau (2006) *FASEB J.*, 20:1293-99, the entire disclosures of which are herein incorporated by reference.

[00143] The siRNA can be altered RNA that differs from naturally-occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siRNA or to one or more internal nucleotides of the siRNA, or modifications that make the siRNA resistant to nuclease digestion, or the substitution of one or more nucleotides in the siRNA with deoxyribo-nucleotides. One or both strands of the siRNA can also comprise a 3' overhang. As used herein, a 3' overhang refers to at least one unpaired nucleotide extending from the 3'-end of a duplexed RNA strand. For example, the siRNA can comprise at least one 3' overhang of from 1 to about 6 nucleotides (which includes ribonucleotides or deoxyribonucleotides) in length, or from 1 to about 5 nucleotides in length, or from 1 to about 4 nucleotides in length, or from about 2 to about 4 nucleotides in length. For example, each

strand of the siRNA can comprise 3' overhangs of dithymidylic acid ("TT") or diuridylic acid ("uu").

[00144] siRNA can be produced chemically or biologically, or can be expressed from a recombinant plasmid or viral vector (for example, see U.S. Patent No. 7,294,504 and U.S. Patent No. 7,422,896, the entire disclosures of which are herein incorporated by reference). Exemplary methods for producing and testing dsRNA or siRNA molecules are described in U.S. Patent Application Publication No. 2002/0173478 to Gewirtz, U.S. Patent Application Publication No. 2007/0072204 to Hannon et al., and in U.S. Patent Application Publication No. 2004/0018176 to Reich et al., the entire disclosures of which are herein incorporated by reference.

[00145] In one embodiment, an siRNA directed to a human nucleic acid sequence comprising a NKG2D or IL-15 gene can be generated against any one of SEQ ID NOS: 2, 4, 6 or 8. In another embodiment, an siRNA directed to a human nucleic acid sequence comprising a NKG2D gene can comprise any one of the sequences listed in Table 13. In another embodiment, an siRNA directed to a human nucleic acid sequence comprising a IL-15 gene can comprise any one of the sequences listed in Table 11. In another embodiment, an siRNA directed to a human nucleic acid sequence comprising a IL-15RA gene can comprise any one of the sequences listed in Table 12.

[00146] In another embodiment, the siRNA directed to IL-15 is listed in Table 11.

Table 11.		
	siRNA SEQUENCES for IL-15	

Table 11.		
	siRNA SEQUENCES for IL-15	
	CCAATGTGGCCCAGGGAAA CAGATGAACTCTTAGAAAT GCAAACAGAGGAAGAATTT TCTTAGAAATGAAGGCAGA GTTTAAGGGTGATAGTCAA CTAATGGAAGTGAAGAAA GGGTGAATGTAATAAGTGA AAACCACATTTGAGAAGTA GAATCTGGATGCAAAGAA TGCAATAACTGGCATATAA GGCTTTGAGTAATGAGAAT CCACATTTGAGAAGTATTT CATAGCAAACAGAGGAAGA CCAACTGGGTGAATGTAAT TCAGATGAACTCTTAGAAA CATATAATGTCCATCAGTA AGCCAGGACTCGATGGAGA GCACAGAAATCAATGTTAG GATGGATGGCTGCTGGAAA AGAAATCAATGTTAGCAGA CCACTGGGTGAATGTAATA GCAATAACTGGCATATAAT TAGGTAGAATGGTGTGCAA GAACTGTAAGAAAGATGAA	AGACCTTGGATCAGATGAA TGTTTAAGGGTGATAGTCA TCTTGGTGGTGGTGGCAAT GAGCAATGTCCATCATGT ACAGAAGCCAACTGGGTGA GATGCAAGTATTCATGATA CTGCTTAGACATAACAAAA AAGAAGATGATCAGACCTT TGAACCTCTCTCAGACTTA CAGAGGAAGAATTTGTTAT GTACATGGAGCACAGAAA TCTCTAATGGGAATGTAA AGATGAACTCTTAGAAATG TAGAAATGAAGGCAGAAAA TTTAAGGGTGATAGTCAA GTACCAATGCTGCAGGTCA TCATTGACTTCCTTACTAA AGCAAACAGAGGAAGAATT GTGCAAGCTTGTCCAATCA AAGTGTGGCCCAAGACAAT GTATTTCCATCCASTGCTA GCCAACTGGGTGAATGTAA GGTGAATGTAATAAGTGAT GCTGCTTAGACATAACAAA GCCAGGACTCGATGGAGAA TTAGAAATGAAGGCAGAAA

[00147] In another embodiment, the siRNA directed to IL-15RA is listed in Table 12.

Table 12.		
	siRNA SEQUENCES for IL-15RA	

Table 12.		
	siRNA SEQUENCES for IL-15RA	
	GAGATGAAGACTTGGAAAA	GCTCTCACCACCTATGAAA
	CACAGGAACCCACAGAGATA	CTAAAGACGACTGAGAAGA
	ACATCTGGGTCAAGAGCTA	AGTGTGAGCTGCCTGGAA
	GAATACATGTGTAATGAA	AAGGATTCAGGAAGAAGAA
	CTGGAATACATGTGGTAAT	GAGATAAGCAGTCATGAGT
	GGATTTCAGGAAGAAGAAAA	GGCCAGCGTTGAAATGGAA
	CAGCAGAGATGAAGACTTG	GGGACCAGCAGCAGAGATG
	AGGGAAGACACCAGCGTTT	AGCTAAAGACGACTGAGAA
	GCTAAAGACGACTGAGAAG	CAAAGCTCTCTGTCAATTA
	TGACATTGTACCTTGATA	GCTTCATCTCCAGCTCAA
	ACTCTGGTTTCAAGCGTAA	CCACAGGAACCCACAGAGAT
	CCACAGAGATAAGCAGTCA	CAGCTAAGTCCGGAGTGAA
	AGAGATGAAGACTTGGAAA	CAGCAGGATCTCAGGACT
	CAGGAGAGACCAAAGGCTT	GACCAAAGGCTTCTGAGCA
	GACCAGCAGCAGAGATGAA	GAAGAAAACCTCAACTCAGT
	TAGCTAAAGACGACTGAGA	GCATGCTACCTCAAGTCAA
	GTGTGAGCTGCCTGGAATA	CACAGAGAAAAGTGACATA
	ACCCAAAGCTCTCTGTCAA	CATTGTACCTTGTATAAAC
	TACCTCAAGTCAAGGCAAA	CAGCTGATGCCTTCAAAAT
	GAAAAGTGACATACCCAAA	ACAGGAACCACAGAGATAA
	GAAGGATTCAGGAAGAAGA	GCAGAGATGAAGACTTGGAA
	GGCCACAACAGCAGCTATT	TGAAGGAGCCTCTCTGCTT
	GCAGTAGAGATGAAGACTT	ACCAAAGGCTTCTGAGCAG
	CCAGCTAAGTCCGGAGTGA	AGGCTTCTGAGCAGGATTT
		CCCAAAGCTCTCTGTCAAT
		TCTCCAGCTCAAACAACA

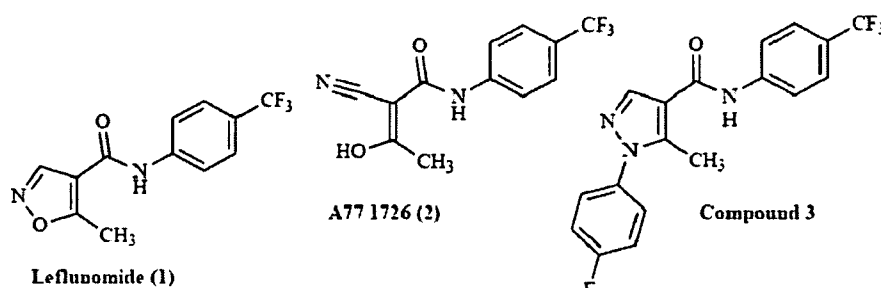
[00148] In another embodiment, the siRNA directed to NKG2D is listed in Table 13.

Table 13.		
	siRNA SEQUENCES for NKG2D	

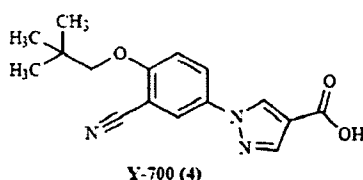
Table 13.		
	siRNA SEQUENCES for NKG2D	
	GGACCAAACCATAGAACAA	GCAAAAGGACTGTGTAAAGA
	GCAAAGAGGACCAGGATTT	GAACTGAGGACATATCTAA
	GCACATAACTTGTGTTGGTT	GAAGAGAAGGCCAGCAGAT
	GATCTGAAGAAGAGTGATT	GGGCCATGATTATCTTAAA
	CGGTAGACGTGCACTTATA	CGACACAGCTGGGAGATGA
	GACCAAAGACTGTCAGATT	CCTCGAGCTTTAAAGGCTA
	GATCAGTGAAGGAAGAGAA	GCTTTAAAGGCTATATAGA
	GCATTATTCTCCAGCCTTA	CATGCAAAGGACTGTGTAA
	GCATTGAAGACTTTAGATT	TGATCAACCATCTCAATAA
	TTGAAATGCAGAAAGGAGA	GGAACAGAGAAGAGATTAC
	GTGTAAAGATGATCAACCA	AGACTAAAGGAAACAAACA
	GCATGATTCTCCAAGACAA	GACTAAAGGAAACAAACA
	AGACAAAAGAAGAGAGATC	TCACAGTTAAGAAGGAATT
	GGGCCACAGCAGAGAGACA	GCAAAGATGTCCAGTAGTC
	TTTCACAGGTTACAGTTA	GGATGGGACTAGTACACAT
	CAACCAAGAAGTTCAAATT	AGAACAATTTCACTGCAAA
	CAAATTCCCTTGACCGAAA	GGGGATCAGTGAAGGAAGA
	CAGGAACAGAGAAGAGATT	ACTCATTTATTCAACCAAGA
	CATGATTCTCCAAGACAAA	GGATCTTGGCAGTGGGAAG
	ACAAATGGATCTTGGCAGT	TAGAACAATTTCACTGCAA
	GATCAACCATCTCAATAAA	CAGCAGAGAGACACAGCAT
	AGAGATTACACCAGCGGTA	GGTTCACAGTTAAGAAGGA
	GAATTTTGCTCTGAATAA	ATRACTTGGATCTGAAGAA
	TTAAACTGGTGAAGTCATA	TCGCTGTAGCCATGGGAAT
		CGAAAGTTACTGTGGCCCA
		TTCTGAAAGTATACAGCAA

[00149] RNA polymerase III transcribed DNAs contain promoters, such as the U6 promoter. These DNAs can be transcribed to produce small hairpin RNAs in the cell that can function as siRNA or linear RNAs that can function as antisense RNA. The NKG2D or IL-15 modulating compound can contain ribonucleotides, deoxyribonucleotides, synthetic nucleotides, or any suitable combination such that the target RNA and/or gene is inhibited. In addition, these forms of nucleic acid can be single, double, triple, or quadruple stranded. (see for example Bass (2001) *Nature*, 411, 428-429; Elbashir et al., (2001) *Nature*, 411, 494-498; and PCT Publication Nos. WO 00/44895, WO 01/36646, WO 99/32619, WO 00/01846, WO 01/29058, WO 99/07409, WO 00/44914).

[00150] A NKG2D or IL-15 modulating compound can be a small molecule that binds to a NKG2D or IL-15 protein and disrupts its function, or conversely, enhances its function. Small molecules are a diverse group of synthetic and natural substances generally having low molecular weights. They can be isolated from natural sources (for example, plants, fungi, microbes and the like), are obtained commercially and/or available as libraries or collections, or synthesized. Candidate small molecules that modulate a NKG2D or IL-15 protein can be identified via *in silico* screening or high-through-put (HTP) screening of combinatorial libraries. Most conventional pharmaceuticals, such as aspirin, penicillin, and many chemotherapeutics, are small molecules, can be obtained commercially, can be chemically synthesized, or can be obtained from random or combinatorial libraries as described below (Werner et al., (2006) *Brief Funct. Genomic Proteomic* 5(1):32-6). In one embodiment, the antagonist can be an inhibitor of NKG2D or an inhibitor of IL-15. Such an inhibitor can be a small molecule. Non-limiting examples of IL-15 inhibitors include phenylpyazoleanilides as described by Ushio et al., (Letters in Drug Design & Discovery, 2008, 5, 111-115), such as leflunomide (Arava®), the active metabolite of leflunomide, A77 1726, Y-700, and Compound 3 cited therein. Structures for these compounds are depicted below:



[00151]



[00152] Knowledge of the primary sequence of a molecule of interest, such as a polypeptide encoded by a NKG2D or IL-15 gene, and the similarity of that sequence with proteins of known function, can provide information as to the inhibitors or antagonists of the protein of interest in addition to agonists. Identification and screening of agonists and antagonists is further facilitated by determining structural features of the protein, e.g., using

X-ray crystallography, neutron diffraction, nuclear magnetic resonance spectrometry, and other techniques for structure determination. These techniques provide for the rational design or identification of agonists and antagonists.

[00153] Test compounds, such as NKG2D or IL-15 modulating compounds, can be screened from large libraries of synthetic or natural compounds (see Wang et al., (2007) *Curr Med Chem*, 14(2):133-55; Mannhold (2006) *Curr Top Med Chem*, 6 (10):1031-47; and Hensen (2006) *Curr Med Chem* 13(4):361-76). Numerous means are currently used for random and directed synthesis of saccharide, peptide, and nucleic acid based compounds. Synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), AMRI (Albany, NY), ChemBridge (San Diego, CA), and MicroSource (Gaylordsville, CT). A rare chemical library is available from Aldrich (Milwaukee, Wis.). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from e.g. Pan Laboratories (Bothell, Wash.) or MycoSearch (N.C.), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle et al., (1996) *Tib Tech* 14:60).

[00154] Methods for preparing libraries of molecules are well known in the art and many libraries are commercially available. Libraries of interest in the invention include peptide libraries, randomized oligonucleotide libraries, synthetic organic combinatorial libraries, and the like. Degenerate peptide libraries can be readily prepared in solution, in immobilized form as bacterial flagella peptide display libraries or as phage display libraries. Peptide ligands can be selected from combinatorial libraries of peptides containing at least one amino acid. Libraries can be synthesized of peptoids and non-peptide synthetic moieties. Such libraries can further be synthesized which contain non-peptide synthetic moieties, which are less subject to enzymatic degradation compared to their naturally-occurring counterparts. For example, libraries can also include, but are not limited to, peptide-on-plasmid libraries, synthetic small molecule libraries, aptamer libraries, in vitro translation-based libraries, polysome libraries, synthetic peptide libraries, neurotransmitter libraries, and chemical libraries.

[00155] Examples of chemically synthesized libraries are described in Fodor et al., (1991) *Science* 251:767-773; Houghten et al., (1991) *Nature* 354:84-86; Lam et al., (1991) *Nature* 354:82-84; Medynski, (1994) *BioTechnology* 12:709-710; Gallop et al., (1994) *J. Medicinal*

Chemistry 37(9):1233-1251; Ohlmeyer et al., (1993) *Proc. Natl. Acad. Sci. USA* 90:10922-10926; Erb et al., (1994) *Proc. Natl. Acad. Sci. USA* 91:11422-11426; Houghten et al., (1992) *Biotechniques* 13:412; Jayawickreme et al., (1994) *Proc. Natl. Acad. Sci. USA* 91:1614-1618; Salmon et al., (1993) *Proc. Natl. Acad. Sci. USA* 90:11708-11712; PCT Publication No. WO 93/20242, dated Oct. 14, 1993; and Brenner et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:5381-5383.

[00156] Examples of phage display libraries are described in Scott et al., (1990) *Science* 249:386-390; Devlin et al., (1990) *Science*, 249:404-406; Christian, et al., (1992) *J. Mol. Biol.* 227:711-718; Lenstra, (1992) *J. Immunol. Meth.* 152:149-157; Kay et al., (1993) *Gene* 128:59-65; and PCT Publication No. WO 94/18318.

[00157] *In vitro* translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058; and Mattheakis et al., (1994) *Proc. Natl. Acad. Sci. USA* 91:9022-9026.

[00158] As used herein, the term “ligand source” can be any compound library described herein, or tissue extract prepared from various organs in an organism’s system, that can be used to screen for compounds that would act as an agonist or antagonist of a NKG2D or IL-15 protein. Screening compound libraries listed herein [also see U.S. Patent Application Publication No. 2005/0009163, which is hereby incorporated by reference in its entirety], in combination with *in vivo* animal studies, functional and signaling assays described below can be used to identify NKG2D or IL-15 modulating compounds that regulate hair growth or treat hair loss disorders.

[00159] Screening the libraries can be accomplished by any variety of commonly known methods. See, for example, the following references, which disclose screening of peptide libraries: Parmley and Smith, (1989) *Adv. Exp. Med. Biol.* 251:215-218; Scott and Smith, (1990) *Science* 249:386-390; Fowlkes et al., (1992) *BioTechniques* 13:422-427; Oldenburg et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:5393-5397; Yu et al., (1994) *Cell* 76:933-945; Staudt et al., (1988) *Science* 241:577-580; Bock et al., (1992) *Nature* 355:564-566; Tuerk et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:6988-6992; Ellington et al., (1992) *Nature* 355:850-852; U.S. Patent Nos. 5,096,815; 5,223,409; and 5,198,346, all to Ladner et al.; Rebar et al., (1993) *Science* 263:671-673; and PCT Pub. WO 94/18318.

[00160] Small molecule combinatorial libraries can also be generated and screened. A combinatorial library of small organic compounds is a collection of closely related analogs that differ from each other in one or more points of diversity and are synthesized by organic techniques using multi-step processes. Combinatorial libraries include a vast number of small organic compounds. One type of combinatorial library is prepared by means of parallel synthesis methods to produce a compound array. A compound array can be a collection of compounds identifiable by their spatial addresses in Cartesian coordinates and arranged such that each compound has a common molecular core and one or more variable structural diversity elements. The compounds in such a compound array are produced in parallel in separate reaction vessels, with each compound identified and tracked by its spatial address. Examples of parallel synthesis mixtures and parallel synthesis methods are provided in U.S. Ser. No. 08/177,497, filed Jan. 5, 1994 and its corresponding PCT published patent application W095/18972, published Jul. 13, 1995 and U.S. Pat. No. 5,712,171 granted Jan. 27, 1998 and its corresponding PCT published patent application W096/22529, which are hereby incorporated by reference.

[00161] In one non-limiting example, non-peptide libraries, such as a benzodiazepine library (see e.g., Bunin et al., (1994) *Proc. Natl. Acad. Sci. USA* 91:4708-4712), can be screened. Peptoid libraries, such as that described by Simon et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:9367-9371, can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (1994), *Proc. Natl. Acad. Sci. USA* 91:11138-11142.

[00162] Computer modeling and searching technologies permit the identification of compounds, or the improvement of already identified compounds, that can modulate the expression or activity of a NKG2D or IL-15 protein. Having identified such a compound or composition, the active sites or regions of a NKG2D or IL-15 protein can be subsequently identified via examining the sites to which the compounds bind. These sites can be ligand binding sites and can be identified using methods known in the art including, for example, from the amino acid sequences of peptides, from the nucleotide sequences of nucleic acids, or from study of complexes of the relevant compound or composition with its natural ligand. In the latter case, chemical or X-ray crystallographic methods can be used to find the active site by finding where on the factor the complexed ligand is found.

[00163] The three dimensional geometric structure of a site, for example that of a polypeptide encoded by a NKG2D or IL-15 gene, can be determined by known methods in the art, such as X-ray crystallography, which can determine a complete molecular structure. Solid or liquid phase NMR can be used to determine certain intramolecular distances. Any other experimental method of structure determination can be used to obtain partial or complete geometric structures. The geometric structures can be measured with a complexed ligand, natural or artificial, which can increase the accuracy of the active site structure determined.

[00164] Other methods for preparing or identifying peptides that bind to a target are known in the art. Molecular imprinting, for instance, can be used for the de novo construction of macromolecular structures such as peptides that bind to a molecule. See, for example, Kenneth J. Shea, Molecular Imprinting of Synthetic Network Polymers: The De Novo synthesis of Macromolecular Binding and Catalytic Sites, TRIP Vol. 2, No. 5, May 1994; Mosbach, (1994) *Trends in Biochem. Sci.*, 19(9); and Wulff, G., in Polymeric Reagents and Catalysts (Ford, W. T., Ed.) ACS Symposium Series No. 308, pp 186-230, American Chemical Society (1986). One method for preparing mimics of a NKG2D or IL-15 modulating compound involves the steps of: (i) polymerization of functional monomers around a known substrate (the template) that exhibits a desired activity; (ii) removal of the template molecule; and then (iii) polymerization of a second class of monomers in, the void left by the template, to provide a new molecule which exhibits one or more desired properties which are similar to that of the template. In addition to preparing peptides in this manner other binding molecules such as polysaccharides, nucleosides, drugs, nucleoproteins, lipoproteins, carbohydrates, glycoproteins, steroids, lipids, and other biologically active materials can also be prepared. This method is useful for designing a wide variety of biological mimics that are more stable than their natural counterparts, because they are prepared by the free radical polymerization of functional monomers, resulting in a compound with a nonbiodegradable backbone. Other methods for designing such molecules include for example drug design based on structure activity relationships, which require the synthesis and evaluation of a number of compounds and molecular modeling.

Screening Assays

[00165] NKG2D or IL-15 Modulating Compounds. A NKG2D or IL-15 modulating compound can be a compound that affects the activity and/or expression of a NKG2D or IL-

15 protein *in vivo* and/or *in vitro*. NKG2D or IL-15 modulating compounds can be agonists and antagonists of a NKG2D or IL-15 protein, and can be compounds that exert their effect on the activity of a NKG2D or IL-15 protein via the expression, via post-translational modifications, or by other means.

[00166] Test compounds or agents which bind to a NKG2D or IL-15 protein, and/or have a stimulatory or inhibitory effect on the activity or the expression of a NKG2D or IL-15 protein, can be identified by two types of assays: (a) cell-based assays which utilize cells expressing a NKG2D or IL-15 protein or a variant thereof on the cell surface; or (b) cell-free assays, which can make use of isolated NKG2D or IL-15 proteins. These assays can employ a biologically active fragment of a NKG2D or IL-15 protein, full-length proteins, or a fusion protein which includes all or a portion of a polypeptide encoded by a NKG2D or IL-15 gene. A NKG2D or IL-15 protein can be obtained from any suitable mammalian species (e.g., human, rat, chick, xenopus, equine, bovine or murine). The assay can be a binding assay comprising direct or indirect measurement of the binding of a test compound. The assay can also be an activity assay comprising direct or indirect measurement of the activity of a NKG2D or IL-15 protein. The assay can also be an expression assay comprising direct or indirect measurement of the expression of NKG2D or IL-15 mRNA nucleic acid sequences or a protein encoded by a NKG2D or IL-15 gene. The various screening assays can be combined with an *in vivo* assay comprising measuring the effect of the test compound on the symptoms of a hair loss disorder or disease in a subject (for example, androgenetic alopecia, alopecia areata, alopecia totalis, or alopecia universalis), loss of hair pigmentation in a subject, or even hypotrichosis.

[00167] An *in vivo* assay can also comprise assessing the effect of a test compound on regulating hair growth in known mammalian models that display defective or aberrant hair growth phenotypes or mammals that contain mutations in the open reading frame (ORF) of nucleic acid sequences comprising a NKG2D or IL-15 gene that affects hair growth regulation or hair density, or hair pigmentation. In one embodiment, controlling hair growth can comprise an induction of hair growth or density in the subject. Here, the compound's effect in regulating hair growth can be observed either visually via examining the organism's physical hair growth or loss, or by assessing protein or mRNA expression using methods known in the art.

[00168] Assays for screening test compounds that bind to or modulate the activity of a NKG2D or IL-15 protein can also be carried out. The test compound can be obtained by any suitable means, such as from conventional compound libraries. Determining the ability of the test compound to bind to a membrane-bound form of the NKG2D or IL-15 protein can be accomplished via coupling the test compound with a radioisotope or enzymatic label such that binding of the test compound to the cell expressing a NKG2D or IL-15 protein can be measured by detecting the labeled compound in a complex. For example, the test compound can be labeled with ^3H , ^{14}C , ^{35}S , or ^{125}I , either directly or indirectly, and the radioisotope can be subsequently detected by direct counting of radioemission or by scintillation counting. Alternatively, the test compound can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

[00169] Cell-based assays can comprise contacting a cell expressing NKG2D or IL-15 with a test agent and determining the ability of the test agent to modulate (such as increase or decrease) the activity or the expression of the membrane-bound molecule. Determining the ability of the test agent to modulate the activity of the membrane-bound NKG2D or IL-15 molecule can be accomplished by any method suitable for measuring the activity of such a molecule, such as monitoring downstream signaling events (e.g., You et al., *Ann N Y Acad Sci.* 2008 Dec;1150:300-10; Posadas et al., *Expert Rev Clin Immunol.* 2009 Jan;5(1):9-17; Korhonen et al., *Basic Clin Pharmacol Toxicol.* 2009 Apr;104(4):276-84; Vital et al., *Ther Clin Risk Manag.* 2006 Dec;2(4):365-75; Malek and Castro, *Immunity.* 2010 Aug 27;33(2):153-65; Cheng et al., *Immunol Rev.* 2011 May;241(1):63-76; Lanier, *Nat Immunol.* 2008 May;9(5):495-502; Lowell, *Cold Spring Harb Perspect Biol.* 2011 Mar 1;3(3). pii: a002352; Mócsai et al., *Nat Rev Immunol.* 2010 Jun;10(6):387-402; Bradshaw, *Cell Signal.* 2010 Aug;22(8):1175-84; Ivanenkov et al., *Mini Rev Med Chem.* 2011 Jan;11(1):55-78; Himpe et al., *Biofactors.* 2009 Jan-Feb;35(1):76-81, each of which are incorporated by reference in their entirety).

[00170] In one embodiment, cell-based assays can comprise contacting a cell expressing NKG2D with a test agent and determining the ability of the test agent to modulate (such as increase or decrease) the activity or the expression of the membrane-bound NKG2D molecule. Determining the ability of the test agent to modulate the activity of the membrane-bound NKG2D molecule can be accomplished by any method suitable for measuring the

activity of such a molecule, such as monitoring downstream signaling events described in Lanier (*Nat Immunol.* 2008 May;9(5):495-502). Non-limiting examples include DAP10 phosphorylation, p85 PI3 kinase activity, Akt kinase activity, alteration in IFN γ concentration, of a NKG2D-ligand+ target cell, or a combination thereof (see also Roda-Navarro P, Reyburn HT., *J Biol Chem.* 2009 Jun 12;284(24):16463-72; Tassi et al., *Eur J Immunol.* 2009 Apr;39(4):1129-35; Coudert JD, et al., *Blood.* 2008 Apr 1;111(7):3571-8; Coudert JD, et al., *Blood.* 2005 106: 1711-1717; and Horng T, et al., *Nat Immunol.* 2007 Dec;8(12):1345-52, which describe methods and protocols that are all hereby incorporated by reference in their entireties).

[00171] A NKG2D or IL-15 protein or the target of a NKG2D or IL-15 protein can be immobilized to facilitate the separation of complexed from uncomplexed forms of one or both of the proteins. Binding of a test compound to a NKG2D or IL-15 protein or a variant thereof, or interaction of a NKG2D or IL-15 protein with a target molecule in the presence and absence of a test compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix (for example, glutathione-S-transferase (GST) fusion proteins or glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical; St. Louis, Mo.) or glutathione derivatized microtiter plates).

[00172] A NKG2D or IL-15 protein, or a variant thereof, can also be immobilized via being bound to a solid support. Non-limiting examples of suitable solid supports include glass or plastic slides, tissue culture plates, microtiter wells, tubes, silicon chips, or particles such as beads (including, but not limited to, latex, polystyrene, or glass beads). Any method known in the art can be used to attach a polypeptide (or polynucleotide) corresponding to NKG2D or IL-15 or a variant thereof, or test compound to a solid support, including use of covalent and non-covalent linkages, or passive absorption.

[00173] The expression of a NKG2D or IL-15 protein can also be monitored. For example, regulators of the expression of a NKG2D or IL-15 protein can be identified via contacting a cell with a test compound and determining the expression of a protein encoded by a NKG2D or IL-15 gene or NKG2D or IL-15 mRNA nucleic acid sequences in the cell. The expression level of a protein encoded by a NKG2D or IL-15 gene or NKG2D or IL-15

mRNA nucleic acid sequences in the cell in the presence of the test compound is compared to the protein or mRNA expression level in the absence of the test compound. The test compound can then be identified as a regulator of the expression of a NKG2D or IL-15 protein based on this comparison. For example, when expression of a protein encoded by a NKG2D or IL-15 gene or NKG2D or IL-15 mRNA nucleic acid sequences in the cell is statistically or significantly greater in the presence of the test compound than in its absence, the test compound is identified as a stimulator/enhancer of expression of a protein encoded by a NKG2D or IL-15 gene or NKG2D or IL-15 mRNA nucleic acid sequences in the cell. The test compound can be said to be a NKG2D or IL-15 modulating compound (such as an agonist).

[00174] Alternatively, when expression of a protein encoded by a NKG2D or IL-15 gene or NKG2D or IL-15 mRNA nucleic acid sequences in the cell is statistically or significantly less in the presence of the test compound than in its absence, the compound is identified as an inhibitor of the expression of a protein encoded by a NKG2D or IL-15 gene or NKG2D or IL-15 mRNA nucleic acid sequences in the cell. The test compound can also be said to be a NKG2D or IL-15 modulating compound (such as an antagonist). The expression level of a protein encoded by a NKG2D or IL-15 gene or NKG2D or IL-15 mRNA nucleic acid sequences in the cell in cells can be determined by methods previously described.

[00175] For binding assays, the test compound can be a small molecule which binds to and occupies the binding site of a polypeptide encoded by a NKG2D or IL-15 gene, or a variant thereof. This can make the ligand binding site inaccessible to substrate such that normal biological activity is prevented. Examples of such small molecules include, but are not limited to, small peptides or peptide-like molecules. In binding assays, either the test compound or a polypeptide encoded by a NKG2D or IL-15 gene can comprise a detectable label, such as a fluorescent, radioisotopic, chemiluminescent, or enzymatic label (for example, alkaline phosphatase, horseradish peroxidase, or luciferase). Detection of a test compound which is bound to a polypeptide encoded by a NKG2D or IL-15 gene can then be determined via direct counting of radioemission, by scintillation counting, or by determining conversion of an appropriate substrate to a detectable product.

[00176] Determining the ability of a test compound to bind to a NKG2D or IL-15 protein also can be accomplished using real-time Biomolecular Interaction Analysis (BIA)

[McConnell et al., 1992, *Science* 257, 1906-1912; Sjolander, Urbaniczky, 1991, *Anal.*

Chem. 63, 2338-2345]. BIA is a technology for studying biospecific interactions in real time, without labeling any of the interactants (for example, BIA-core™). Changes in the optical phenomenon surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

[00177] To identify other proteins which bind to or interact with a NKG2D or IL-15 protein and modulate its activity, a polypeptide encoded by a NKG2D or IL-15 gene can be used as a bait protein in a two-hybrid assay or three-hybrid assay (Szabo et al., 1995, *Curr. Opin. Struct. Biol.* 5, 699-705; U.S. Pat. No. 5,283,317), according to methods practiced in the art. The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains.

[00178] Functional Assays. Test compounds can be tested for the ability to increase or decrease the activity of a NKG2D or IL-15 protein, or a variant thereof. Activity can be measured after contacting a purified NKG2D or IL-15 protein, a cell membrane preparation, or an intact cell with a test compound. A test compound that decreases the activity of a NKG2D or IL-15 protein by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, at least about 95% or 100% is identified as a potential agent for decreasing the activity of a NKG2D or IL-15 protein, for example an antagonist. A test compound that increases the activity of a NKG2D or IL-15 protein by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, at least about 95% or 100% is identified as a potential agent for increasing the activity of a NKG2D or IL-15 protein, for example an agonist.

Treatment and Prevention

[00179] The invention also provides a method for treating or preventing a hair-loss disorder in a subject. In one embodiment, the method comprises detecting the presence of an alteration in a NKG2D or IL-15 gene in a sample from the subject, the presence of the alteration being indicative of a hair-loss disorder, or the predisposition to a hair-loss disorder, and, administering to the subject in need a therapeutic treatment against a hair-loss disorder. The therapeutic treatment can be a drug administration (for example, a pharmaceutical composition comprising a siRNA directed to a NKG2D or IL-15 nucleic acid). In one

embodiment, the therapeutic molecule to be administered comprises a polypeptide encoded by a NKG2D or IL-15 gene, comprising at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 97%, at least about 98%, at least about 99%, or 100% of the amino acid sequence of SEQ ID NO: 1 or 3, and exhibits the function of decreasing expression of a protein encoded by a NKG2D or IL-15 gene. This can restore the capacity to initiate hair growth in cells derived from hair follicles or skin. In another embodiment, the therapeutic molecule to be administered comprises a nucleic acid sequence comprising a NKG2D or IL-15 gene that encodes a polypeptide, comprising at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 97%, at least about 98%, at least about 99%, or 100% of the nucleic acid sequence of SEQ ID NO: 2 or 4, and encodes a polypeptide with the function of decreasing expression of a protein encoded by a NKG2D or IL-15 gene, thus restoring the capacity to initiate hair growth in cells derived from hair follicle cells or skin.

[00180] The alteration can be determined at the level of the DNA, RNA, or polypeptide. Optionally, detection can be determined by performing an oligonucleotide ligation assay, a confirmation based assay, a hybridization assay, a sequencing assay, an allele-specific amplification assay, a microsequencing assay, a melting curve analysis, a denaturing high performance liquid chromatography (DHPLC) assay (for example, see Jones et al, (2000) Hum Genet., 106(6):663-8), or a combination thereof. In another embodiment, the detection is performed by sequencing all or part of a NKG2D or IL-15 gene or by selective hybridization or amplification of all or part of a NKG2D or IL-15 gene. A NKG2D or IL-15 gene specific amplification can be carried out before the alteration identification step.

[00181] An alteration in a chromosome region occupied by a NKG2D or IL-15 gene can be any form of mutation(s), deletion(s), rearrangement(s) and/or insertions in the coding and/or non-coding region of the locus, alone or in various combination(s). Mutations can include point mutations. Insertions can encompass the addition of one or several residues in a coding or non-coding portion of the gene locus. Insertions can comprise an addition of between 1 and 50 base pairs in the gene locus. Deletions can encompass any region of one, two or more residues in a coding or non-coding portion of the gene locus, such as from two residues up to the entire gene or locus. Deletions can affect smaller regions, such as domains (introns) or repeated sequences or fragments of less than about 50 consecutive base pairs,

although larger deletions can occur as well. Rearrangement includes inversion of sequences. The alteration in a chromosome region occupied by a NKG2D or IL-15 gene can result in amino acid substitutions, RNA splicing or processing, product instability, the creation of stop codons, frame-shift mutations, and/or truncated polypeptide production. The alteration can result in the production of a polypeptide encoded by a NKG2D or IL-15 gene with altered function, stability, targeting or structure. The alteration can also cause a reduction, or even an increase in protein expression. In one embodiment, the alteration in the chromosome region occupied by a NKG2D or IL-15 gene can comprise a point mutation, a deletion, or an insertion in a NKG2D or IL-15 gene or corresponding expression product. In another embodiment, the alteration can be a deletion or partial deletion of a NKG2D or IL-15 gene. The alteration can be determined at the level of the DNA, RNA, or polypeptide.

[00182] In another embodiment, the method can comprise detecting the presence of altered RNA expression. Altered RNA expression includes the presence of an altered RNA sequence, the presence of an altered RNA splicing or processing, or the presence of an altered quantity of RNA. These can be detected by various techniques known in the art, including sequencing all or part of the RNA or by selective hybridization or selective amplification of all or part of the RNA. In a further embodiment, the method can comprise detecting the presence of altered expression of a polypeptide encoded by a NKG2D or IL-15 gene. Altered polypeptide expression includes the presence of an altered polypeptide sequence, the presence of an altered quantity of polypeptide, or the presence of an altered tissue distribution. These can be detected by various techniques known in the art, including by sequencing and/or binding to specific ligands (such as antibodies).

[00183] Various techniques known in the art can be used to detect or quantify altered gene or RNA expression or nucleic acid sequences, which include, but are not limited to, hybridization, sequencing, amplification, and/or binding to specific ligands (such as antibodies). Other suitable methods include allele-specific oligonucleotide (ASO), oligonucleotide ligation, allele-specific amplification, Southern blot (for DNAs), Northern blot (for RNAs), single-stranded conformation analysis (SSCA), PFGE, fluorescent in situ hybridization (FISH), gel migration, clamped denaturing gel electrophoresis, denaturing HPLC, melting curve analysis, heteroduplex analysis, RNase protection, chemical or enzymatic mismatch cleavage, ELISA, radio-immunoassays (RIA) and immuno-enzymatic assays (IEMA). Some of these approaches (such as SSCA and CGGE) are based on a change

in electrophoretic mobility of the nucleic acids, as a result of the presence of an altered sequence. According to these techniques, the altered sequence is visualized by a shift in mobility on gels. The fragments can then be sequenced to confirm the alteration. Some other approaches are based on specific hybridization between nucleic acids from the subject and a probe specific for wild type or altered gene or RNA. The probe can be in suspension or immobilized on a substrate. The probe can be labeled to facilitate detection of hybrids. Some of these approaches are suited for assessing a polypeptide sequence or expression level, such as Northern blot, ELISA and RIA. These latter require the use of a ligand specific for the polypeptide, for example, the use of a specific antibody.

[00184] Sequencing. Sequencing can be carried out using techniques well known in the art, using automatic sequencers. The sequencing can be performed on the complete NKG2D or IL-15 gene or on specific domains thereof, such as those known or suspected to carry deleterious mutations or other alterations.

[00185] Amplification. Amplification is based on the formation of specific hybrids between complementary nucleic acid sequences that serve to initiate nucleic acid reproduction. Amplification can be performed according to various techniques known in the art, such as by polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA). These techniques can be performed using commercially available reagents and protocols. Useful techniques in the art encompass real-time PCR, allele-specific PCR, or PCR-SSCP. Amplification usually requires the use of specific nucleic acid primers, to initiate the reaction. Nucleic acid primers useful for amplifying sequences from a NKG2D or IL-15 gene or locus are able to specifically hybridize with a portion of a NKG2D or IL-15 gene locus that flank a target region of the locus, wherein the target region is altered in certain subjects having a hair-loss disorder. In one embodiment, amplification can comprise using forward and reverse PCR primers comprising nucleotide sequences of SEQ ID NOS: 2, 4, 6 or 8.

[00186] The invention provides for a nucleic acid primer, wherein the primer can be complementary to and hybridize specifically to a portion of a NKG2D or IL-15 coding sequence (e.g., gene or RNA) altered in certain subjects having a hair-loss disorder. Primers of the invention can be specific for altered sequences in a NKG2D or IL-15 gene or RNA. By using such primers, the detection of an amplification product indicates the presence of an alteration in a NKG2D or IL-15 gene or the absence of such gene. Primers can also be used

to identify single nucleotide polymorphisms (SNPs) located in or around a NKG2D or IL-15 gene locus; SNPs can comprise a single nucleotide change, or a cluster of SNPs in and around a NKG2D or IL-15 gene. Examples of primers of this invention can be single-stranded nucleic acid molecules of about 5 to 60 nucleotides in length, or about 8 to about 25 nucleotides in length. The sequence can be derived directly from the sequence of a NKG2D or IL-15 gene. Perfect complementarity is useful to ensure high specificity; however, certain mismatch can be tolerated. For example, a nucleic acid primer or a pair of nucleic acid primers as described above can be used in a method for detecting the presence of or a predisposition to a hair-loss disorder in a subject.

[00187] Amplification methods include, e.g., polymerase chain reaction, PCR (PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS, ed. Innis, Academic Press, N.Y., 1990 and PCR STRATEGIES, 1995, ed. Innis, Academic Press, Inc., N.Y., ligase chain reaction (LCR) (see, e.g., Wu, Genomics 4:560, 1989; Landegren, Science 241:1077, 1988; Barringer, Gene 89:117, 1990); transcription amplification (see, e.g., Kwoh, Proc. Natl. Acad. Sci. USA 86:1173, 1989); and, self-sustained sequence replication (see, e.g., Guatelli, Proc. Natl. Acad. Sci. USA 87:1874, 1990); Q Beta replicase amplification (see, e.g., Smith, J. Clin. Microbiol. 35:1477-1491, 1997), automated Q-beta replicase amplification assay (see, e.g., Burg, Mol. Cell. Probes 10:257-271, 1996) and other RNA polymerase mediated techniques (e.g., NASBA, Cangene, Mississauga, Ontario); see also Berger, Methods Enzymol. 152:307-316, 1987; Sambrook; Ausubel; U.S. Pat. Nos. 4,683,195 and 4,683,202; Sooknanan, Biotechnology 13:563-564, 1995. All the references stated above, an throughout the description, are incorporated by reference in their entireties.

[00188] Selective Hybridization. Hybridization detection methods are based on the formation of specific hybrids between complementary nucleic acid sequences that serve to detect nucleic acid sequence alteration(s). A detection technique involves the use of a nucleic acid probe specific for wild type or altered gene or RNA, followed by the detection of the presence of a hybrid. The probe can be in suspension or immobilized on a substrate or support (for example, as in nucleic acid array or chips technologies). The probe can be labeled to facilitate detection of hybrids. For example, a sample from the subject can be contacted with a nucleic acid probe specific for a wild type NKG2D or IL-15 gene or an altered NKG2D or IL-15 gene, and the formation of a hybrid can be subsequently assessed. In one embodiment, the method comprises contacting simultaneously the sample with a set of

probes that are specific, respectively, for a wild type NKG2D or IL-15 gene and for various altered forms thereof. Thus, it is possible to detect directly the presence of various forms of alterations in a NKG2D or IL-15 gene in the sample. Also, various samples from various subjects can be treated in parallel.

[00189] According to the invention, a probe can be a polynucleotide sequence which is complementary to and can specifically hybridize with a (target portion of a) NKG2D or IL-15 gene or RNA, and that is suitable for detecting polynucleotide polymorphisms associated with alleles of a NKG2D or IL-15 gene (or genes) which predispose to or are associated with a hair-loss disorder. Useful probes are those that are complementary to a NKG2D or IL-15 gene, RNA, or target portion thereof. Probes can comprise single-stranded nucleic acids of between 8 to 1000 nucleotides in length, for instance between 10 and 800, between 15 and 700, or between 20 and 500. Longer probes can be used as well. A useful probe of the invention is a single stranded nucleic acid molecule of between 8 to 500 nucleotides in length, which can specifically hybridize to a region of a NKG2D or IL-15 gene or RNA that carries an alteration. For example, the probe can be directed to a chromosome region occupied by a NKG2D or IL-15 gene.

[00190] The sequence of the probes can be derived from the sequences of a NKG2D or IL-15 gene and RNA as provided herein. Nucleotide substitutions can be performed, as well as chemical modifications of the probe. Such chemical modifications can be accomplished to increase the stability of hybrids (e.g., intercalating groups) or to label the probe. Some examples of labels include, without limitation, radioactivity, fluorescence, luminescence, and enzymatic labeling.

[00191] A guide to the hybridization of nucleic acids is found in e.g., Sambrook, ed., Molecular Cloning: A Laboratory Manual (3rd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory, 2001; Current Protocols in Molecular Biology, Ausubel, ed. John Wiley & Sons, Inc., New York, 1997; Laboratory Techniques in Biochemistry and Molecular Biology: Hybridization with Nucleic Acid Probes, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y., 1993.

Specific Ligand Binding

[00192] As discussed herein, alteration in a chromosome region occupied by a NKG2D or IL-15 gene or alteration in expression of a NKG2D or IL-15 gene, can also be detected by

screening for alteration(s) in a sequence or expression level of a polypeptide encoded by a NKG2D or IL-15 gene. Different types of ligands can be used, such as specific antibodies. In one embodiment, the sample is contacted with an antibody specific for a polypeptide encoded by a NKG2D or IL-15 gene and the formation of an immune complex is subsequently determined. Various methods for detecting an immune complex can be used, such as ELISA, radioimmunoassays (RIA) and immuno-enzymatic assays (IEMA).

[00193] For example, an antibody can be a polyclonal antibody, a monoclonal antibody, as well as fragments or derivatives thereof having substantially the same antigen specificity. Fragments include Fab, Fab'2, or CDR regions. Derivatives include single-chain antibodies, humanized antibodies, or poly-functional antibodies. An antibody specific for a polypeptide encoded by a NKG2D or IL-15 gene can be an antibody that selectively binds such a polypeptide, namely, an antibody raised against a polypeptide encoded by a NKG2D or IL-15 gene or an epitope-containing fragment thereof. Although non-specific binding towards other antigens can occur, binding to the target polypeptide occurs with a higher affinity and can be reliably discriminated from non-specific binding. In one embodiment, the method can comprise contacting a sample from the subject with an antibody specific for a wild type or an altered form of a polypeptide encoded by a NKG2D or IL-15 gene, and determining the presence of an immune complex. Optionally, the sample can be contacted to a support coated with antibody specific for the wild type or altered form of a polypeptide encoded by a NKG2D or IL-15 gene. In one embodiment, the sample can be contacted simultaneously, or in parallel, or sequentially, with various antibodies specific for different forms of a polypeptide encoded by a NKG2D or IL-15 gene, such as a wild type and various altered forms thereof.

Gene Therapy and Protein Replacement Methods

[00194] Delivery of nucleic acids into viable cells can be effected *ex vivo*, *in situ*, or *in vivo* by use of vectors, such as viral vectors (e.g., lentivirus, adenovirus, adeno-associated virus, or a retrovirus), or *ex vivo* by use of physical DNA transfer methods (e.g., liposomes or chemical treatments). Non-limiting techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, and the calcium phosphate precipitation method (See, for example, Anderson, Nature, supplement to vol. 392, no. 6679, pp. 25-20 (1998)). Introduction of a nucleic acid or a gene encoding a polypeptide of the invention can also be accomplished with

extrachromosomal substrates (transient expression) or artificial chromosomes (stable expression). Cells can also be cultured *ex vivo* in the presence of therapeutic compositions of the present invention in order to proliferate or to produce a desired effect on or activity in such cells. Treated cells can then be introduced *in vivo* for therapeutic purposes.

[00195] Nucleic acids can be inserted into vectors and used as gene therapy vectors. A number of viruses have been used as gene transfer vectors, including papovaviruses, e.g., SV40 (Madzak et al., 1992), adenovirus (Berkner, 1992; Berkner et al., 1988; Gorziglia and Kapikian, 1992; Quantin et al., 1992; Rosenfeld et al., 1992; Wilkinson et al., 1992; Stratford-Perricaudet et al., 1990), vaccinia virus (Moss, 1992), adeno-associated virus (Muzyczka, 1992; Ohi et al., 1990), herpesviruses including HSV and EBV (Margolskee, 1992; Johnson et al., 1992; Fink et al., 1992; Breakfield and Geller, 1987; Freese et al., 1990), and retroviruses of avian (Biandyopadhyay and Temin, 1984; Petropoulos et al., 1992), murine (Miller, 1992; Miller et al., 1985; Sorge et al., 1984; Mann and Baltimore, 1985; Miller et al., 1988), and human origin (Shimada et al., 1991; Helseth et al., 1990; Page et al., 1990; Buchschacher and Panganiban, 1992). Non-limiting examples of *in vivo* gene transfer techniques include transfection with viral (e.g., retroviral) vectors (see U.S. Pat. No. 5,252,479, which is incorporated by reference in its entirety) and viral coat protein-liposome mediated transfection (Dzau et al., Trends in Biotechnology 11:205-210 (1993), incorporated entirely by reference). For example, naked DNA vaccines are generally known in the art; see Brower, Nature Biotechnology, 16:1304-1305 (1998), which is incorporated by reference in its entirety. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see, e.g., U.S. Pat. No. 5,328,470) or by stereotactic injection (see, e.g., Chen, et al., 1994. Proc. Natl. Acad. Sci. USA 91: 3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can include one or more cells that produce the gene delivery system.

[00196] For reviews of gene therapy protocols and methods see Anderson et al., Science 256:808-813 (1992); U.S. Pat. Nos. 5,252,479, 5,747,469, 6,017,524, 6,143,290, 6,410,010 6,511,847; and U.S. Application Publication Nos. 2002/0077313 and 2002/00069, which are all hereby incorporated by reference in their entireties. For additional reviews of gene

therapy technology, see Friedmann, *Science*, 244:1275-1281 (1989); Verma, *Scientific American*: 68-84 (1990); Miller, *Nature*, 357: 455-460 (1992); Kikuchi et al., *J Dermatol Sci.* 2008 May;50(2):87-98; Isaka et al., *Expert Opin Drug Deliv.* 2007 Sep;4(5):561-71; Jager et al., *Curr Gene Ther.* 2007 Aug;7(4):272-83; Waehler et al., *Nat Rev Genet.* 2007 Aug;8(8):573-87; Jensen et al., *Ann Med.* 2007;39(2):108-15; Herweijer et al., *Gene Ther.* 2007 Jan;14(2):99-107; Eliyahu et al., *Molecules*, 2005 Jan 31;10(1):34-64; and Altaras et al., *Adv Biochem Eng Biotechnol.* 2005;99:193-260, all of which are hereby incorporated by reference in their entireties.

[00197] Protein replacement therapy can increase the amount of protein by exogenously introducing wild-type or biologically functional protein by way of infusion. A replacement polypeptide can be synthesized according to known chemical techniques or can be produced and purified via known molecular biological techniques. Protein replacement therapy has been developed for various disorders. For example, a wild-type protein can be purified from a recombinant cellular expression system (e.g., mammalian cells or insect cells-see U.S. Pat. No. 5,580,757 to Desnick et al.; U.S. Pat. Nos. 6,395,884 and 6,458,574 to Selden et al.; U.S. Pat. No. 6,461,609 to Calhoun et al.; U.S. Pat. No. 6,210,666 to Miyamura et al.; U.S. Pat. No. 6,083,725 to Selden et al.; U.S. Pat. No. 6,451,600 to Rasmussen et al.; U.S. Pat. No. 5,236,838 to Rasmussen et al. and U.S. Pat. No. 5,879,680 to Ginns et al.), human placenta, or animal milk (see U.S. Pat. No. 6,188,045 to Reuser et al.), or other sources known in the art. After the infusion, the exogenous protein can be taken up by tissues through non-specific or receptor-mediated mechanism.

[00198] A polypeptide encoded by a NKG2D or IL-15 gene can also be delivered in a controlled release system. For example, the polypeptide can be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump can be used (see Langer, supra; Sefton, *CRC Crit. Ref. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery* 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Fla. (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61 (1983); see also Levy et al., *Science* 228:190 (1985); During et al., *Ann. Neurol.* 25:351 (1989); Howard et al., *J. Neurosurg.* 71:105

(1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984)). Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

Pharmaceutical Compositions and Administration for Therapy

[00199] NKG2D or IL-15 proteins and NKG2D or IL-15 modulating compounds of the invention can be administered to the subject once (e.g., as a single injection or deposition). Alternatively, NKG2D or IL-15 proteins and NKG2D or IL-15 modulating compounds can be administered once or twice daily to a subject in need thereof for a period of from about two to about twenty-eight days, or from about seven to about ten days. NKG2D or IL-15 proteins and NKG2D or IL-15 modulating compounds can also be administered once or twice daily to a subject for a period of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 times per year, or a combination thereof. Furthermore, NKG2D or IL-15 proteins and NKG2D or IL-15 modulating compounds of the invention can be co-administered with another therapeutic. Where a dosage regimen comprises multiple administrations, the effective amount of the NKG2D or IL-15 proteins and NKG2D or IL-15 modulating compounds administered to the subject can comprise the total amount of gene product administered over the entire dosage regimen.

[00200] NKG2D or IL-15 proteins NKG2D or IL-15 modulating compounds can be administered to a subject by any means suitable for delivering the NKG2D or IL-15 proteins and NKG2D or IL-15 modulating compounds to cells of the subject, such as the dermis, epidermis, dermal papilla cells, or hair follicle cells. For example, NKG2D or IL-15 proteins and NKG2D or IL-15 modulating compounds can be administered by methods suitable to transfect cells. Transfection methods for eukaryotic cells are well known in the art, and include direct injection of the nucleic acid into the nucleus or pronucleus of a cell; electroporation; liposome transfer or transfer mediated by lipophilic materials; receptor mediated nucleic acid delivery, bioballistic or particle acceleration; calcium phosphate precipitation, and transfection mediated by viral vectors.

[00201] The compositions of this invention can be formulated and administered to reduce the symptoms associated with a hair-loss disorder by any means that produces contact

of the active ingredient with the agent's site of action in the body of a subject, such as a human or animal (e.g., a dog, cat, or horse). They can be administered by any conventional means available for use in conjunction with pharmaceuticals, either as individual therapeutic active ingredients or in a combination of therapeutic active ingredients. They can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

[00202] A therapeutically effective dose of NKG2D or IL-15 modulating compounds can depend upon a number of factors known to those of ordinary skill in the art. The dose(s) of the NKG2D or IL-15 modulating compounds can vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the NKG2D or IL-15 modulating compounds to have upon the nucleic acid or polypeptide of the invention. These amounts can be readily determined by a skilled artisan. Any of the therapeutic applications described herein can be applied to any subject in need of such therapy, including, for example, a mammal such as a dog, a cat, a cow, a horse, a rabbit, a monkey, a pig, a sheep, a goat, or a human.

[00203] Pharmaceutical compositions for use in accordance with the invention can be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. The therapeutic compositions of the invention can be formulated for a variety of routes of administration, including systemic and topical or localized administration. Techniques and formulations generally can be found in Remington's Pharmaceutical Sciences, Meade Publishing Co., Easton, Pa (20th Ed., 2000), the entire disclosure of which is herein incorporated by reference. For systemic administration, an injection is useful, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the therapeutic compositions of the invention can be formulated in liquid solutions, for example in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the therapeutic compositions can be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included. Pharmaceutical compositions of the present invention are characterized as being at least sterile and pyrogen-free. These pharmaceutical formulations include formulations for human and veterinary use.

[00204] According to the invention, a pharmaceutically acceptable carrier can comprise any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic

and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Any conventional media or agent that is compatible with the active compound can be used. Supplementary active compounds can also be incorporated into the compositions.

[00205] The invention also provides for a kit that comprises a pharmaceutically acceptable carrier and a NKG2D or IL-15 modulating compound identified using the screening assays of the invention packaged with instructions for use. For modulators that are antagonists of the activity of a NKG2D or IL-15 protein, or which reduce the expression of a NKG2D or IL-15 protein, the instructions would specify use of the pharmaceutical composition for promoting the loss of hair on the body surface of a mammal (for example, arms, legs, bikini area, face).

[00206] For NKG2D or IL-15 modulating compounds that are agonists of the activity of a NKG2D or IL-15 protein or increase the expression of one or more proteins encoded by NKG2D or IL-15 genes, the instructions would specify use of the pharmaceutical composition for regulating hair growth. In one embodiment, the instructions would specify use of the pharmaceutical composition for the treatment of hair loss disorders. In a further embodiment, the instructions would specify use of the pharmaceutical composition for restoring hair pigmentation. For example, administering an agonist can reduce hair graying in a subject.

[00207] A pharmaceutical composition containing a NKG2D or IL-15 modulating compound can be administered in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic effects discussed herein. Such pharmaceutical compositions can comprise, for example antibodies directed to polypeptides encoded by genes comprising a NKG2D or IL-15 gene, or variants thereof, or agonists and antagonists of a polypeptide encoded by a NKG2D or IL-15 gene. The compositions can be administered alone or in combination with at least one other agent, such as a stabilizing compound, which can be administered in any sterile, biocompatible pharmaceutical carrier including, but not limited to, saline, buffered saline, dextrose, and water. The compositions can be administered to a patient alone, or in combination with other agents, drugs or hormones.

[00208] Sterile injectable solutions can be prepared by incorporating the NKG2D or IL-15 modulating compound (e.g., a polypeptide or antibody) in the required amount in an

appropriate solvent with one or a combination of ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated herein. In the case of sterile powders for the preparation of sterile injectable solutions, examples of useful preparation methods are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[00209] In some embodiments, the NKG2D or IL-15 modulating compound can be applied via transdermal delivery systems, which slowly releases the active compound for percutaneous absorption. Permeation enhancers can be used to facilitate transdermal penetration of the active factors in the conditioned media. Transdermal patches are described in for example, U.S. Pat. No. 5,407,713; U.S. Pat. No. 5,352,456; U.S. Pat. No. 5,332,213; U.S. Pat. No. 5,336,168; U.S. Pat. No. 5,290,561; U.S. Pat. No. 5,254,346; U.S. Pat. No. 5,164,189; U.S. Pat. No. 5,163,899; U.S. Pat. No. 5,088,977; U.S. Pat. No. 5,087,240; U.S. Pat. No. 5,008,110; and U.S. Pat. No. 4,921,475.

[00210] Various routes of administration and various sites of cell implantation can be utilized, such as, subcutaneous or intramuscular, in order to introduce the aggregated population of cells into a site of preference. Once implanted in a subject (such as a mouse, rat, or human), the aggregated cells can then stimulate the formation of a hair follicle and the subsequent growth of a hair structure at the site of introduction. In another embodiment, transfected cells (for example, cells expressing a protein encoded by a NKG2D or IL-15 gene are implanted in a subject to promote the formation of hair follicles within the subject. In further embodiments, the transfected cells are cells derived from the end bulb of a hair follicle (such as dermal papilla cells or dermal sheath cells). Aggregated cells (for example, cells grown in a hanging drop culture) or transfected cells (for example, cells produced as described herein) maintained for 1 or more passages can be introduced (or implanted) into a subject (such as a rat, mouse, dog, cat, human, and the like).

[00211] “Subcutaneous” administration can refer to administration just beneath the skin (i.e., beneath the dermis). Generally, the subcutaneous tissue is a layer of fat and connective tissue that houses larger blood vessels and nerves. The size of this layer varies throughout the body and from person to person. The interface between the subcutaneous and muscle layers can be encompassed by subcutaneous administration.

[00212] This mode of administration can be feasible where the subcutaneous layer is sufficiently thin so that the factors present in the compositions can migrate or diffuse from the locus of administration and contact the hair follicle cells responsible for hair formation. Thus, where intradermal administration is utilized, the bolus of composition administered is localized proximate to the subcutaneous layer.

[00213] Administration of the cell aggregates (such as DP or DS aggregates) is not restricted to a single route, but can encompass administration by multiple routes. For instance, exemplary administrations by multiple routes include, among others, a combination of intradermal and intramuscular administration, or intradermal and subcutaneous administration. Multiple administrations can be sequential or concurrent. Other modes of application by multiple routes will be apparent to the skilled artisan.

[00214] In other embodiments, this implantation method will be a one-time treatment for some subjects. In further embodiments of the invention, multiple cell therapy implantations will be required. In some embodiments, the cells used for implantation will generally be subject-specific genetically engineered cells. In another embodiment, cells obtained from a different species or another individual of the same species can be used. Thus, using such cells can require administering an immunosuppressant to prevent rejection of the implanted cells. Such methods have also been described in United States Patent Application Publication 2004/0057937 and PCT application publication WO 2001/32840, and are hereby incorporated by reference.

Inhibitors

[00215] Cytokines are produced to activate neighboring cells to communicate danger signals to one another and spread and amplify the inflammatory response. Over the years, it was learned how to both neutralize these cytokines with blocking antibodies and inhibit their signaling in responding cells by small molecule protein tyrosine kinase inhibitors. FDA approved drugs exist for both approaches, e.g., IL-2 and TNF blocking antibodies and the small orally available molecule GleevecTM that blocks cytokine signaling. Whenever possible, the center will pursue topical small molecule formulations that should improve efficacy while limiting systemic toxicity (improved therapeutic indexes) encouraging clinical evaluation in AA of other small molecule inhibitors in the biopharmaceutical pipeline.

[00216] The inhibitors can comprise peptides (such as antibodies or fragments thereof), small molecules, nucleic acids (such as siRNA or antisense RNA), or other agents) that can bind to a polypeptide molecule encoded by a gene of interest and/or molecules that have an inhibitory effect on the biological activity of a protein of interest or its expression.

[00217] As used herein, a “IL-15 inhibitor” refers to a compound that interacts with a IL-15 gene or a IL-15 protein or polypeptide and inhibits its activity and/or its expression. The compound can decrease the activity or expression of a protein encoded by IL-15.

[00218] In one embodiment, an IL-15 inhibitor can be a peptide fragment that binds a protein comprising SEQ ID NO: 1. For example, the fragment can encompass any portion of at least about 8 consecutive amino acids of SEQ ID NO: 1. The fragment can comprise at least about 10 consecutive amino acids, at least about 20 consecutive amino acids, at least about 30 consecutive amino acids, at least about 40 consecutive amino acids, at least about 50 consecutive amino acids, at least about 60 consecutive amino acids, or at least about 75 consecutive amino acids of SEQ ID NO: 1. Fragments include all possible amino acid lengths between and including about 8 and about 100 amino acids, for example, lengths between about 10 and about 100 amino acids, between about 15 and about 100 amino acids, between about 20 and about 100 amino acids, between about 35 and about 100 amino acids, between about 40 and about 100 amino acids, between about 50 and about 100 amino acids, between about 70 and about 100 amino acids, between about 75 and about 100 amino acids, or between about 80 and about 100 amino acids. These peptide fragments can be obtained commercially or synthesized via liquid phase or solid phase synthesis methods (Atherton et al., (1989) Solid Phase Peptide Synthesis: a Practical Approach. IRL Press, Oxford, England).

[00219] In another embodiment, an IL-15 inhibitor can be a peptide fragment that binds a protein comprising SEQ ID NO: 5. For example, the fragment can encompass any portion of at least about 8 consecutive amino acids of SEQ ID NO: 5. The fragment can comprise at least about 10 consecutive amino acids, at least about 20 consecutive amino acids, at least about 30 consecutive amino acids, at least about 40 consecutive amino acids, at least about 50 consecutive amino acids, at least about 60 consecutive amino acids, or at least about 75 consecutive amino acids of SEQ ID NO: 5. Fragments include all possible amino acid lengths between and including about 8 and about 100 amino acids, for example, lengths between about 10 and about 100 amino acids, between about 15 and about 100 amino acids, between about 20 and about 100 amino acids, between about 35 and about 100 amino acids,

between about 40 and about 100 amino acids, between about 50 and about 100 amino acids, between about 70 and about 100 amino acids, between about 75 and about 100 amino acids, or between about 80 and about 100 amino acids. These peptide fragments can be obtained commercially or synthesized via liquid phase or solid phase synthesis methods (Atherton et al., (1989) Solid Phase Peptide Synthesis: a Practical Approach. IRL Press, Oxford, England).

[00220] As used herein, a “NKG2D inhibitor” refers to a compound that interacts with a NKG2D gene or a NKG2D protein or polypeptide and inhibits its activity and/or its expression. The compound can decrease the activity or expression of a protein encoded by NKG2D.

[00221] In one embodiment, a NKG2D inhibitor can be a peptide fragment that binds a protein comprising SEQ ID NO: 3. For example, the fragment can encompass any portion of at least about 8 consecutive amino acids of SEQ ID NO: 3. The fragment can comprise at least about 10 consecutive amino acids, at least about 20 consecutive amino acids, at least about 30 consecutive amino acids, at least about 40 consecutive amino acids, at least about 50 consecutive amino acids, at least about 60 consecutive amino acids, or at least about 75 consecutive amino acids of SEQ ID NO: 3. Fragments include all possible amino acid lengths between and including about 8 and about 100 amino acids, for example, lengths between about 10 and about 100 amino acids, between about 15 and about 100 amino acids, between about 20 and about 100 amino acids, between about 35 and about 100 amino acids, between about 40 and about 100 amino acids, between about 50 and about 100 amino acids, between about 70 and about 100 amino acids, between about 75 and about 100 amino acids, or between about 80 and about 100 amino acids. These peptide fragments can be obtained commercially or synthesized via liquid phase or solid phase synthesis methods (Atherton et al., (1989) Solid Phase Peptide Synthesis: a Practical Approach. IRL Press, Oxford, England).

[00222] An inhibitor of the invention can be a protein, such as an antibody (monoclonal, polyclonal, humanized, chimeric, or fully human), or a binding fragment thereof, directed against a polypeptide encoded by SEQ ID NO: 1 or 3. An antibody fragment can be a form of an antibody other than the full-length form and includes portions or components that exist within full-length antibodies, in addition to antibody fragments that have been engineered. Antibody fragments can include, but are not limited to, single chain Fv (scFv), diabodies, Fv, and (Fab')₂, triabodies, Fc, Fab, CDR1, CDR2, CDR3, combinations of CDR's, variable regions, tetrabodies, bifunctional hybrid antibodies, framework regions, constant regions, and

the like (*see*, Maynard et al., (2000) *Ann. Rev. Biomed. Eng.* 2:339-76; Hudson (1998) *Curr. Opin. Biotechnol.* 9:395-402). Antibodies can be obtained commercially, custom generated, or synthesized against an antigen of interest according to methods established in the art (Janeway et al., (2001) *Immunobiology*, 5th ed., Garland Publishing).

[00223] An inhibitor of the invention can also be a small molecule that binds to a protein and disrupts its function. Small molecules are a diverse group of synthetic and natural substances generally having low molecular weights. They can be isolated from natural sources (for example, plants, fungi, microbes and the like), are obtained commercially and/or available as libraries or collections, or synthesized. Candidate small molecules that modulate a protein can be identified via *in silico* screening or high-through-put (HTP) screening of combinatorial libraries. Most conventional pharmaceuticals, such as aspirin, penicillin, and many chemotherapeutics, are small molecules, can be obtained commercially, can be chemically synthesized, or can be obtained from random or combinatorial libraries (Werner et al., (2006) *Brief Funct. Genomic Proteomic* 5(1):32-6). In some embodiments, the agent is a small molecule that binds, interacts, or associates with a target protein or RNA. Such a small molecule can be an organic molecule that, when the target is an intracellular target, is capable of penetrating the lipid bilayer of a cell to interact with the target. Small molecules include, but are not limited to, toxins, chelating agents, metals, and metalloid compounds. Small molecules can be attached or conjugated to a targeting agent so as to specifically guide the small molecule to a particular cell.

Pharmaceutical Compositions and Administration for Therapy

[00224] An inhibitor or agonist of the invention can be incorporated into pharmaceutical compositions suitable for administration, for example the inhibitor and a pharmaceutically acceptable carrier.

[00225] According to the invention, a pharmaceutically acceptable carrier can comprise any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Any conventional media or agent that is compatible with the active compound can be used. Supplementary active compounds can also be incorporated into the compositions.

[00226] Any of the therapeutic applications described herein can be applied to any subject in need of such therapy, including, for example, a mammal such as a dog, a cat, a cow, a horse, a rabbit, a monkey, a pig, a sheep, a goat, or a human.

[00227] A pharmaceutical composition of the invention can be administered in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic effects discussed herein. Such pharmaceutical compositions can comprise, for example antibodies directed to polypeptides. The compositions can be administered alone or in combination with at least one other agent, such as a stabilizing compound, which can be administered in any sterile, biocompatible pharmaceutical carrier including, but not limited to, saline, buffered saline, dextrose, and water. The compositions can be administered to a patient alone, or in combination with other agents, drugs or hormones.

[00228] A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[00229] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EM™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or

dispersion medium containing, for example, water, ethanol, a pharmaceutically acceptable polyol like glycerol, propylene glycol, liquid polyethylene glycol, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it can be useful to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[00230] Sterile injectable solutions can be prepared by incorporating the inhibitor (e.g., a polypeptide or antibody or small molecule) or agonist of the invention in the required amount in an appropriate solvent with one or a combination of ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated herein. In the case of sterile powders for the preparation of sterile injectable solutions, examples of useful preparation methods are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[00231] Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed.

[00232] Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as

magnesium stearate or sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

[00233] Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

[00234] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention.

[00235] Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

[00236] All publications and other references mentioned herein are incorporated by reference in their entirety, as if each individual publication or reference were specifically and individually indicated to be incorporated by reference. Publications and references cited herein are not admitted to be prior art.

EXAMPLES

[00237] Examples are provided below to facilitate a more complete understanding of the invention. The following examples illustrate the exemplary modes of making and practicing the invention. However, the scope of the invention is not limited to specific embodiments disclosed in these Examples, which are for purposes of illustration only, since alternative methods can be utilized to obtain similar results.

EXAMPLE 1

[00238] Interferon γ has been shown to be a target in murine AA (*see Nakamura, et al.* 2008, *Am J Pathol*, 172(3): 650-658; *see also Freyschmidt-Paul et al., Br J Dermatol.*, 155(3):515-521; *see also Gilhar et al., Journal Invest. Dermatol.*, 124(1):288-289). In human AA, AA PBMCs show Th1 skewing, AA skin shows IFN signature and GWAS genes (SOCS/IFN- γ).but also IL-2/6/13/21/26.

[00239] The interferon γ pathway (**FIG. 11**) induces NKG2DL in target HF cells, enhances antigen presentation by DCs, promotes Th1 cellular autoimmunity and augments NK and CTL mediated- cytotoxicity.

[00240] Clinical programs in AA include the following:

[00241] Mild-Moderate AA: Phase 2/3 Psoriasis and oral Phase 3 Myelofibrosis, using a topical JAK inhibitor

[00242] Moderate-Severe AA: Systemic Therapy, using Anti-NKG2D and Anti-IL15.

- o Anti-NKG2D Phase I IBD/MS
- o Anti-IL-15 Phase I/2 RA

[00243] A population-based cohort evaluated cases ascertained through National Alopecia Areata Registry vs controls ascertained through New York City Cancer project. They were matched for Northern European ancestry using AIMS.

[00244] Cases: n=1080 (Alopecia Universalis=482; Alopecia Totalis=120; Alopecia Areata Transient=176; Alopecia Areata Patchy=302)

[00245] Controls: n=1053

[00246] FIG. 14 presents a Genomewide Association Study (GWAS) in alopecia areata.

Region	Gene	Function	Strongest association (pvalue)	Maximum odds ratio
2q33.2	CTLA4	T-cell proliferation	3.55×10^{-13}	1.44
	ICOS	T-cell proliferation	4.33×10^{-08}	1.32
4q27	IL21/IL2	T-, B- and NK-cell proliferation	4.27×10^{-08}	1.34
6q25.1	ULBP6	NKG2D activating ligand	4.49×10^{-19}	1.65
	ULBP3	NKG2D activating ligand	4.43×10^{-17}	1.52
9q31.1	STX17	premature hair graying	3.60×10^{-07}	1.33
10p15.1	IL2RA (CD25)	T-cell proliferation	1.74×10^{-12}	1.41
11q13	PRDX5	antioxidant enzyme	4.14×10^{-07}	1.33
12q13	Eos (IKZF4)	T-cell proliferation	3.21×10^{-08}	1.34
	ERBB3	epidermal growth factor receptor	1.27×10^{-07}	1.34
6p21.32 (HLA)	MICA	NKG2D activating ligand	1.19×10^{-07}	1.44
	NOTCH4	T-cell differentiation	1.03×10^{-08}	1.61
	BTNL2	T-cell proliferation	2.11×10^{-26}	2.70
	HLA-DRA	Antigen presentation	2.93×10^{-31}	2.62
	HLA-DQA1	Antigen presentation	3.60×10^{-17}	2.15
	HLA-DQA2	Antigen presentation	1.38×10^{-35}	5.43
	HLA-DQB2	Antigen presentation	1.73×10^{-13}	1.60
	HLA-DOB	Antigen presentation	2.07×10^{-08}	1.72

Table 2: Genes identified in the Genomewide Association Study (GWAS) in alopecia areata.

Region	Gene	Strongest association (pvalue)	Maximum odds ratio	Involved in other autoimmune disease
2q33.2	CTLA4	3.55×10^{-13}	1.44	T1D, RA, CeD, MS, SLE, GD
	ICOS	4.33×10^{-08}	1.32	T1D, RA, CeD, MS, SLE, GD
4q27	IL21/IL2	4.27×10^{-08}	1.34	T1D, RA, CeD, PS
6q25.1	ULBP6	4.49×10^{-19}	1.65	none
	ULBP3	4.43×10^{-17}	1.52	none
9q31.1	STX17	3.60×10^{-07}	1.33	none
10p15.1	IL2RA (CD25)	1.74×10^{-12}	1.41	T1D, MS, GD, GV
11q13	PRDX5	4.14×10^{-07}	1.33	MS
12q13	Eos (IKZF4)	3.21×10^{-08}	1.34	T1D, SLE
	ERBB3	1.27×10^{-07}	1.34	T1D, SLE
6p21.32	MICA	1.19×10^{-07}	1.44	T1D, RA, CeD, UC, PS, SLE
(HLA)	NOTCH4	1.03×10^{-08}	1.61	T1D, RA, MS
	C6orf10	1.45×10^{-16}	2.36	T1D, RA, PS, GV
	BTNL2	2.11×10^{-26}	2.70	T1D, RA, UC, CD, SLE, MS, GV
	HLA-DRA	2.93×10^{-31}	2.62	T1D, RA, CeD, MS, GV
	HLA-DQA1	3.60×10^{-17}	2.15	T1D, RA, CeD, MS, SLE, PS, CD, UC, GD, GV
	HLA-DQA2	1.38×10^{-35}	5.43	T1D, RA
	HLA-DQB2	1.73×10^{-13}	1.60	RA
	HLA-DOB	2.07×10^{-08}	1.72	

Type I diabetes (T1D), rheumatoid arthritis (RA), celiac disease (CeD), multiple sclerosis (MS), system lupus erythematosus (SLE), Graves disease (GD), psoriasis (PS), Generalized Vitiligo (GV).

Table 3: Common Cause Model of Skin Autoimmune Diseases. See also

<http://www.genome.gov/gwastudies> (for psoriasis) and Jin *et al.*, 2010, *NEJM*, 362:1686-1697.

Region	Gene	Strongest association (pvalue)	Maximum odds ratio		Other autoimmune disease
2q33.2	CTLA4	3.55×10^{-13}	1.44		T1D, RA, CeD, MS, SLE, GD
	ICOS	4.33×10^{-08}	1.32		T1D, RA, CeD, MS, SLE, GD
4q27	IL21/IL2	4.27×10^{-08}	1.34		T1D, RA, CeD, MS
6q25.1	ULBP6	4.49×10^{-19}	1.65		none
	ULBP3	4.43×10^{-17}	1.52		none
9q31.1	STX17	3.60×10^{-07}	1.33		none
10p15.1	IL2RA (CD25)	1.74×10^{-12}	1.41		T1D, MS, GD, GV
11q13	PRDX5	4.14×10^{-07}	1.33		MS
12q13	Eos (IKZF4)	3.21×10^{-08}	1.34		T1D, SLE
	ERBB3	1.27×10^{-07}	1.34		T1D, SLE
6p21.32	MICA	1.19×10^{-07}	1.44		T1D, RA, CeD, MS, PS, SLE
(HLA)	NOTCH4	1.03×10^{-08}	1.61		T1D, RA, MS
	C6orf10	1.45×10^{-18}	2.36		T1D, RA, PS, GV
	BTNL2	2.11×10^{-28}	2.70		T1D, RA, UC, MS, SLE, MS, GV
	HLA-DRA	2.93×10^{-31}	2.62		T1D, RA, CeD, MS, GV
	HLA-DQA1	3.60×10^{-17}	2.15		T1D, RA, CeD, MS, SLE, PS, CD, UC, GD, GV
	HLA-DQA2	1.38×10^{-35}	5.43		T1D, RA
	HLA-DQB2	1.73×10^{-13}	1.60		RA
	HLA-DOB	2.07×10^{-08}	1.72		

Type I diabetes (T1D), rheumatoid arthritis (RA), celiac disease (CeD), multiple sclerosis (MS), system lupus erythematosus (SLE), Graves disease (GD), psoriasis (PS), Crohn's disease (CD), ulcerative colitis (UC), vitiligo (GV).

Table 4: Common Cause Model of Other Autoimmune Diseases. See also

<http://www.genome.gov/gwastudies> (for psoriasis) and Jin *et al.*, 2010, *NEJM*, 362:1686-1697.

Region	Gene	Function	Strongest association (pvalue)	Maximum odds ratio
2q33.2	⊕ CTLA4	T-cell proliferation	3.55×10^{-13}	1.44
	ICOS	T-cell proliferation	4.33×10^{-08}	1.32
4q27	⊖ IL21/IL2	T-, B- and NK-cell proliferation	4.27×10^{-08}	1.34
6q25.1	● ULBP6	NKG2D activating ligand	4.49×10^{-19}	1.65
	● ULBP3	NKG2D activating ligand	4.43×10^{-17}	1.52
9q31.1	★● STX17	premature hair graying	3.60×10^{-07}	1.33
10p15.1	⊕ IL2RA (CD25)	T-cell proliferation	1.74×10^{-12}	1.41
11q13	★● PRDX5	antioxidant enzyme	4.14×10^{-07}	1.33
12q13	⊕ Eos (IKZF4)	T-cell proliferation	3.21×10^{-08}	1.34
	ERBB3	epidermal growth factor receptor	1.27×10^{-07}	1.34
6p21.32 (HLA)	● MICA	NKG2D activating ligand	1.19×10^{-07}	1.44
	NOTCH4	T-cell differentiation	1.03×10^{-08}	1.61
	C6orf10		1.45×10^{-16}	2.36
	BTNL2	T-cell proliferation	2.11×10^{-26}	2.70
	HLA-DRA	Antigen presentation	2.93×10^{-31}	2.62
	HLA-DQA1	Antigen presentation	3.60×10^{-17}	2.15
	HLA-DQA2	Antigen presentation	1.38×10^{-35}	5.43
	HLA-DQB2	Antigen presentation	1.73×10^{-13}	1.60
	HLA-DOB	Antigen presentation	2.07×10^{-08}	1.72

Table 5: Genes identified in the Genomewide Association Study (GWAS) in alopecia areata. Black circles indicate genes that affect NKG2D; gray circles indicate genes that affect T-cell regulation; black circles with star indicate genes that affect end organs.

[00247] Common pathways with other autoimmune diseases, including NK ligands in the target organs include the following:

[00248] (1) In RA, synoviocytes aberrantly express MIC ligands, leading to autoreactive T cell stimulation.

[00249] (2) In Celiac disease, MIC ligands are overexpressed in the gut epithelium during active disease.

[00250] (3) In Type 1 diabetes, Islet cells in prediabetic NOD mice express RAE-1 ligand.

[00251] The aberrant expression of NK activating ligands in genetically predisposed individuals can induce or exacerbate disease.

[00252] Candidate pathways in AA that present new opportunities for therapy include the following:

[00253] (1) Members of the costimulatory receptor family, which have complementary effects on T cell activation, and their balance controls the overall outcome of immune and autoimmune responses.

[00254] (2) Genes which enhance the NK cell cytotoxic response; triggers T cells to produce proinflammatory cytokines; and induces CD4(+) T cells to differentiate into Th17 cells.

[00255] (3) Genes which pivotal role in regulating the adaptive immune system by controlling the survival and proliferation of regulatory T (Treg) cells, which are required for the maintenance of immune tolerance.

[00256] (4) Activating receptors expressed on natural killer (NK) cells, NK1.1 (+) T cells, and T cells. NKG2D ligands include MHC class I chain-related (MIC)A, MICB.

[00257] Candidate compounds for treatment of AA have been discussed herein. Anti-NKG2D antibody blockade prevents autoimmune diabetes in NOD mice.

EXAMPLE 2

[00258] The following genetic studies will be performed: (1) A replication study with another 1000 AA patients; (2) Deep sequencing of candidate regions, (3) Collection of 10,000 patients, and (4) Common Genetic Studies T1D, CeO and AA. An immunology study using NK activating ligands will be performed using ULBP3, ULBP6 - inducible transgenics, new mouse models.

Gene	Mb	Count of SNPs <1x10 ⁻⁴	Min p-value observed	Min p-value imputed	Autoimmune Reports	GO classification
Chromosome 2						
HDAC4	240.03	1	8.10E-05	5.59E-05		inflammatory response
Chromosome 3						
CACNA2D3	55.02	1	7.28E-05	1.47E-05	CeD	
Chromosome 5						
IL13	132.02	2	1.87E-06		Asthma	immune response
Chromosome 6						
HLA-G	29.94	1	1.07E-04	4.54E-06	RA, MS, SLE, PS, T1D, Asthma,	immune response
HLA-A	30.01	1	1.00E-04	2.72E-05	MS, T1D, PS, GD, Asthma, Vitiligo	immune response
MICB	31.59	2	1.89E-05	1.97E-05	MS, T1D, UC, RA, CeD, Asthma	immune response
TAP2	32.91	1	6.42E-06	1.28E-05	T1D, RA, SLE, PS, GD	immune response
Chromosome 7						
IL6	22.72	2	7.72E-05	4.84E-05	RA, T1D, CeD	inflammatory response
CHCHD3	132.44	1	3.07E-05	2.02E-05	CeD	
Chromosome 8						
CSMD1	3.02	1	8.65E-05	8.38E-05	CeD, MS, PS	
Chromosome 12						
IFNG	66.84	1	1.55E-05	1.29E-05	CeD, T1D, RA, MS, SLE, PS, GD, Asthma	
IL26	66.87	2	7.18E-05	6.45E-05	MS, Asthma	immune response
Chromosome 16						
KIAA0350 (CLEC16A)	11.11	6	1.77E-05	1.15E-05	T1D, MS, Thyroid Disease	
SOCS1	11.24	2	1.16E-05	8.66E-06	CeD, T1D, Asthma	
Chromosome 18						
ANKRD12	9.25	2	3.59E-05	1.55E-05		
PTPN2	12.80	1	4.09E-06	3.38E-07	CD, T1D	

Table 6: Genes with Nominal Significance on the GWAS studies

Category	Term	Genes
hsa05332	Graft-versus-host disease	IL6, IFNG, HLA-A, HLA-DMA, HLA-G
hsa04612	Antigen processing and presentation	HSPA1L, TAP2, HLA-A, HLA-DMA, HLA-G
hsa04630	Jak-STAT signaling pathway	SPRY2, IL6, SOCS1, IFNG, IL26, IL13
hsa05330	Allograft rejection	IFNG, HLA-A, HLA-DMA, HLA-G
hsa04940	Type I diabetes mellitus	IFNG, HLA-A, HLA-DMA, HLA-G
hsa04650	Natural killer cell mediated cytotoxicity	MICB, IFNG, HLA-A, HLA-G
hsa05320	Autoimmune thyroid disease	HLA-A, HLA-DMA, HLA-G
hsa05416	Viral myocarditis	HLA-A, HLA-DMA, HLA-G
hsa04060	Cytokine-cytokine receptor interaction	IL6, IFNG, IL26, IL13

Table 7: Pathway analysis of nominal genes (see <http://david.abcc.ncifcrf.gov/>)

EXAMPLE 3- Study samples, genotyping, quality control and population stratification

[00259] Alopecia areata (AA) is a major medical problem and is the most prevalent autoimmune disease in the U.S. (**Table 8**), affecting approximately 4.6 million people, including males and females across all ethnic groups, with a lifetime risk of 1.7% (Kyriakis KP, Paltatzidou K, Kosma E, Sofouri E, Tadros A, Rachioti E. Alopecia areata prevalence by gender and age. *J Eur Acad Dermatol Venereol.* 23:572-3, 2009). Additionally, AA represents the second most common form of human hair loss, second only to androgenetic alopecia, and causes significant disfigurement and psychological distress to affected individuals (**FIG. 1**). AA affects more individuals than most other autoimmune diseases combined, including lupus erythematosus (LE), type 1 diabetes (T1D), psoriasis, multiple sclerosis (MS) and rheumatoid arthritis (RA) (Table 1).

[00260] **Table 8. Automimmune Disease Prevalence**

Autoimmune Disease	Rate per 100,000
Alopecia Areata	1700
Psoriasis	696-1527
Rheumatoid arthritis	310-800
Type 1 diabetes	227-355
Multiple sclerosis	177-358
Systemic lupus erythematosus	34-150

[00261] In stark contrast to these other conditions, research into the pathogenesis and the development of innovative therapies in AA has lagged far behind. This can be due in part to the perception that AA is merely a cosmetic disorder. In reality, AA carries one of the highest burdens among any skin diseases, particularly among children and adolescents whose self-image is so closely linked to their appearance (Bickers DR, Lim HW, Margolis D, Weinstock MA, Goodman C, Faulkner E, Gould C, Gemmen E, Dall T; American Academy of Dermatology Association; Society for Investigative Dermatology. The burden of skin diseases: 2004 a joint project of the American Academy of Dermatology Association and the Society for Investigative Dermatology. *J Am Acad Dermatol.* 55:490-5, 2006).

[00262] Despite its high prevalence, there are no evidence-based treatments for AA. A comprehensive Cochrane analysis assessment of seventeen randomized clinical trials (RCTs) involving a total of 540 participants found no proven treatment of AA (Delamere FM,

Sladden MM, Dobbins HM, Leonardi-Bee J. Interventions for alopecia areata. *Cochrane Database Syst Rev*. 2:CD004413, 2008). Each trial included from 6 to 85 participants and assessed a range of interventions that included topical and oral corticosteroids, topical cyclosporin, photodynamic therapy and topical minoxidil. Overall, none of the interventions showed significant treatment benefit in terms of hair growth when compared with placebo. It was concluded that, few, (if any), treatments for AA are proven to be effective. No RCTs on the use of diphencyprone, dinitrochlorobenzene, intralesional corticosteroids or dithranol were found, although these drugs are commonly used for the treatment of AA. Similarly, although topical corticosteroids and minoxidil are widely prescribed and appear to be safe, there is no convincing evidence that they are beneficial in the long-term. Most trials have been poorly reported and/or are so small that any important clinical benefits are inconclusive. These observations underscore the importance of defining the genetic basis of AA and determining its pathogenesis, so that rational therapeutic approaches can be developed and translational research can begin.

[00263] Pre-clinical Evaluation of NKG2D Targeting in Alopecia Areata

[00264] The NKG2D receptor (NKG2D), functions to eliminate cells emitting danger signals and dysregulation of this recognition process often leads to development of autoimmunity. Preclinical evaluation of the NKG2D axis as a therapeutic target will be pursued on two fronts:

[00265] i) Inhibition of NKG2DL Upregulation: NKG2DL is regulated at the transcriptional and post-translational levels and is shed from the cell surface upon proteolytic cleavage.

[00266] ii) Inhibition of NKG2D Signaling: NKG2D is a member of the NK immunoreceptor family, which signals through DAP10 and DAP12 adapters. IL-15 is intimately involved with NKG2D signaling, both upregulating its expression and providing co-stimulation.

[00267] **NKG2D-Fc/NKG2D Mab:** Preclinical. Humanized antibodies have been developed for clinical studies.

[00268] **IL-15 Antibodies:** Phase II evaluation in Rheumatoid Arthritis and other autoimmune disorders.

[00269] Table 9: Biologics useful for treatment of AA

Biologics				
Treatment	Action	Target	Administration	Stage of Clinical Development
NKG2D-Fc	Soluble receptor	NKG2DL	i.v.	
Anti-NKG2D mAb	Blocking Antibody	NKG2D	i.v.	Humanized mAbs developed
Anti-IL-15	Neutralizing Antibody	IL-15	i.v.	Phase II/RA

[00270] GWAS studies revealed a number of risk loci shared by with other forms of autoimmunity, such as rheumatoid arthritis (RA), type I diabetes (T1D), celiac disease (CeD), systemic lupus erythematosus (SLE), multiple sclerosis (MS) and psoriasis (PS). The commonality with RA, T1D, and CeD is noteworthy in light of the significance of the expression of an NK ligand in the end organ, and the involvement of the NKG2D receptor in the pathogenesis of each of these three diseases.

[00271] A genetic basis of AA provide avenues of exploration for therapies based on the underlying mechanisms of AA. Such therapies will focus not only on T cell subsets and mechanisms common to other forms of autoimmunity, but also on unique mechanisms that involve signaling pathways downstream of the NKG2D receptor.

[00272] The origin of autoimmunity in AA can reside in the hair follicle itself. The studies herein are focused on defining putative danger signals in the hair follicle that contribute to the pathogenesis of AA. For example, ULBPs are a new class of activating ligands for the NKG2D receptor, which is present on innate immune cells. NKG2D functions to eliminate target cells emitting danger signals (such as ULBPs), and dysregulation of this recognition process often leads to the development of autoimmunity. Pathogenic alleles that reside within the MHC, which can contribute to immune dysregulation driving the pathogenesis of AA, will also be identified. This can be due in part to the importance of antigen-presenting cells (APCs) in the presentation of danger signals as neoantigens to the immune system.

[00273] It will also be determined whether dysregulation of NKG2D ligand expression, and NKG2D signaling in skin resident cells, has a causative role in AA. The NKG2D ligands are upregulated under conditions of cellular stress that are well-known triggers of the NF- κ B transcription factor family, and NKG2D ligands can be upregulated by NF- κ B activation. Whether NF- κ B plays an important role in NKG2D ligand expression and NKG2D signaling will also be examined, and the possibility that targeting NF- κ B therapeutically in lesional skin can be beneficial for treatment of AA will be explored.

[00274] AA is characterized by both cellular and humoral immune responses directed against the anagen hair follicle (HF) resulting in non-scarring hair loss. NKG2D is an activating NK cell receptor expressed in several skin resident cells including NK cells, NKT cells, γ/δ^+ T cells and $CD8^+\alpha/\beta^+$ T cells. NKG2D recognizes MHC family proteins including the ULBP/RAET1 (UL-16 binding protein; Rae1 and H60 in mice) and MICA/MICB families of proteins. The NKG2D ligands are upregulated under conditions of cellular stress that are well-known triggers of the NF- κ B transcription factor family, and NKG2D ligands can be upregulated by NF- κ B activation. Acute upregulation of NKG2D ligands in the skin is sufficient to trigger an inflammatory response characterized initially by activation of NKG2D bearing resident T cells and NK cells followed by activation of adaptive immune responses. It was discovered that NF- κ B activation can directly drive transcription from a ULBP promoter. Upregulation of NKG2D ligands, MICA, ULBP3 and ULBP6 has been demonstrated in human AA lesions and NK cells triggered through these same ligands have been implicated in the breakdown of HF immune privilege. Whether dysregulation of the NKG2D ligand expression, and NKG2D signaling in skin resident cells, has a causative role in AA, will be explored.

Transcriptional regulation of NKG2D ligands by NF- κ B

[00275] Previous studies have linked activation of NF- κ B to the activation of transcription from a ULBP3/6 promoter and demonstrate upregulation of NKG2DL in AA skin. A systematic and thorough analysis of the transcriptional regulation of NKG2D ligands by the NF- κ B family of transcription factors will therefore be conducted. The promoter and enhancer structures of ULBP and MIC family genes will be analyzed and the function of target regions containing κ B binding sites will be interrogated. The role of NF- κ B in the upregulation of NKG2DL expression in AA will be examined by examining the activation

status of the NF- κ B signaling pathway in lesional and non-lesional AA skin and correlating these findings with the expression levels of NKG2DL. The contribution of specific transcription factors to the promoter and enhancer regions defined will be analyzed by chromatin Immunoprecipitation techniques.

Determining whether NKG2D signaling to NF- κ B contributes to the pathogenesis of AA

[00276] Signaling from NKG2D activates PI3K and Src family kinase pathways leading to the activation of AKT, ERK, JAK/STAT, Rac/Rho, and PLC γ . Despite the parallels to TCR signaling pathways, the details of NKG2D signaling remain poorly characterized. NKG2D signaling to NF- κ B can be mediated by PDK1 downstream of PI3K and activation of NF- κ B can be crucial to NKG2D-mediated inflammation. Therefore, the contribution of NKG2D signaling to NF- κ B in the development of inflammation in AA will be investigated. The activation of proinflammatory gene programs in NK, NKT, g/d, and conventional a/b T cells will be investigated using conditional gene deletion in mice. The signaling pathways activated by NKG2D ligation in NK cells and g/d and conventional a/b T cells will be examined using phosphoproteomics. The contribution of NF- κ B to the transcriptional response to NKG2D ligation will be further analyzed by examining the gene expression profiles generated in the presence or absence of NF- κ B signaling components.

Treatment of AA using the cell permeable NEMO binding domain peptide

[00277] It was previously shown that canonical NF- κ B signaling can be selectively blocked, both in vitro and in vivo, through the use of a cell permeable peptide that disrupts the interaction between IKK β and the crucial regulatory protein NEMO. It was shown that the NEMO binding domain (NBD) peptide is efficacious in several models of autoimmune inflammatory conditions. In preliminary studies, it was found that topical application of NBD is an effective means of delivery in mice. Therefore, the efficacy of NF- κ B inhibition using the NBD peptide in the treatment of AA will be investigated. The ability of NBD to affect NKG2DL expression *ex vivo* in AA HF organ cultures will be examined. The efficacy of the NBD peptide in inhibiting the production of cytokines by NK cells and T cells in response to NKG2D ligation will be further tested. Finally, the therapeutic potential of the NBD peptide in the C3H/HeJ allograft mouse model of AA will be examined.

Blocking the induction and pathogenicity of CD8 NKT cell effectors in Alopecia Areata

[00278] Specific autoimmune mechanisms underlying Alopecia Areata (AA) have remained obscure. In the normal state, hair follicles (HF) represent an immuneprivileged (IP) sanctuary, expressing little or no classical HLA class I molecules and instead expressing the inhibitory HLA ligands HLA-E and HLA-G, thus simultaneously avoiding immune recognition by class I restricted CD8 cells while negatively regulating NK cells, that might otherwise react to “missing self”. In active AA, the IP status is lost, since HLA class I expression and NKG2D ligand expression are markedly upregulated. Given the strong association of NKG2D ligand loci with human AA (1), without being bound by theory, aberrant NKG2DL upregulation and persistent NKG2D activation can drive inflammatory AA initiation and progression. Microbial triggers as drivers of the innate inflammatory response have been well studied. However the activators of “sterile” inflammation, pivotal to autoimmune disease development are incompletely understood.

[00279] Previous data shed light on pathways shared by AA in humans and C3H mice, namely:

[00280] IL-15 and NKG2DL are highly upregulated in HFs from diseased but not adjacent normal skin revealing the probable tissue-specific disease-initiating events.

[00281] CD8⁺ DX5⁺ NKT cells are the dominant infiltrating NKG2D-bearing immune effectors likely responsible for HF cytotoxicity.

[00282] The mechanisms of NKG2DL upregulation will be investigated, the importance of NKG2DL-NKG2D interactions as drivers of disease induction will be evaluated, and the NKG2D-bearing cells responsible for triggering inflammation will be determined. Pharmacologic approaches interrupting the NKG2D axis will also be pursued. CD8 NKT cells are the dominant NKG2D-bearing population in active AA lesions. The effector function and antigen specificities of this cellular subset in the skin will be characterized and therapeutic avenues to reverse their pathogenic consequences will be evaluated.

[00283] Without being bound by theory, activation of the NKG2DL/NKG2D axis, accompanied by local interferon production, can be responsible for initiating and sustaining the autoimmune adaptive response. To investigate this further; the following will be done:

[00284] (1) The primacy of the NKG2D pathway will be examined by treating mice with anti-NKG2D blocking antibodies which should prevent innate activation, and can prevent and reverse AA pathogenesis.

[00285] (2) CD8 effector cells are armed by NKG2D in response to IL-15 which are found to be highly upregulated in alopecic HF's in the C3H mouse. Thus the expression of IL-15 will now be examined in human AA skin and IL-15 blockade will be examined therapeutically in C3H mice.

[00286] (3) C3H IFN- γ deficient mice are protected from AA development. It is shown that IFNs are capable of upregulating NKG2DL in cultured human dermal keratinocytes and fibroblasts. Further NKG2D-cross-linking induces IFN- γ production by innate NK, NKT and $\gamma\delta$ T cells, thus creating a potential positive feedback loop, driving adaptive autoreactive immunity. Thus adoptive transfer and antibody depletion/blockade experiments will define the NKG2D-bearing cells involved.

[00287] Pharmacologic interventions aimed at interruption of the CD8 NKG2D axis will be examined. Interfering with the upregulation of NKG2DL or the activation of NKG2D-bearing cells are both rational approaches that will be pursued.

[00288] It is shown that NKG2D bearing CD8 T cells are the dominant inflammatory cellular subset found in active AA lesional skin in both the human and mouse. The effector function and antigen specificities of this cellular subset will be characterized in the skin and *in vitro* approaches to inactivate their pathogenicity will be evaluated. The pathogenic potential of NKG2D bearing CD8 T cells will be determined *in vivo* and in human explant models. Persistent dependence on IL-15 and NKG2D will be examined. The TCR repertoire of the NKG2D bearing CD8 T cells in the lesional skin and peripheral blood will be assessed.

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EXAMPLE 4

[00290] Specific autoimmune mechanisms underlying Alopecia Areata (AA) have remained obscure and therefore clinical investigation of AA has historically lagged behind other autoimmune diseases. Despite the large number of new biotherapeutics available for clinical evaluation in autoimmunity, intralesional or systemic steroids remain the standard of care and only two small randomized clinical trials have been published in the last decade. The insights provided by the discovery of GWAS genes provide a roadmap for closing this gap, by investigating GWAS-identified immunological pathways both for mechanistic and therapeutic relevance.

[00291] Identify Effective, Clinically-Relevant, Therapies in Mouse Models of Alopecia Areata. In the normal state, hair follicles (HF) represent an immuneprivileged (IP) sanctuary, expressing little or no classical HLA class I molecules. In active AA, the IP status is lost, since HLA class I expression and NKG2D ligand expression are markedly upregulated. Given the strong genetic association of NKG2D ligand loci with human AA, without being bound by theory, aberrant NKG2DL upregulation and persistent NKG2D activation on immune effector cells drives inflammatory AA initiation and progression. Previous data have shown that NKG2DL and IL-15 are highly upregulated in HFs in AA skin revealing key tissue-specific disease-initiating events, shared by both the human and mouse disease. Without being bound by theory, in AA, as for Celiac Disease, IL-15 can drive CD8 T cell effector differentiation enabling promiscuous “NK-type” CD8 cytotoxicity. Indeed, the local HF IL-15/NKG2DL inflammatory signals are accompanied by a dense infiltrate of CD8⁺ T cells expressing NK markers, likely the critical AA immune effectors responsible for IFN γ

production and HF cytotoxicity. These collective observations, made in both the human and mouse, invite therapeutic evaluation.

[00292] The therapeutic potential of blocking NK-like reprogramming and cytotoxicity by interfering with relevant cytokines (IL-15/IFN- γ and relevant immunoreceptor signaling pathways (NKG2D). A graft model of alopecia in which all grafted C3H mice develop AA in a timely manner, within 12 weeks, will be used. This greatly accelerates drug screening since evaluation of prevention/reversal of spontaneous disease in C3H/HeJ is otherwise impractical given the late onset (>6 months of age) and low cumulative incidence (15%). Both small molecule approaches, that have the advantage of potential topical delivery, and biotherapeutic proteins (antibodies) that have superior biologic specificity, will be evaluated. The overarching goals are to use biotherapeutics to establish critical immune pathways that might be targeted by more clinically tenable, small molecule topical approaches.

[00293] Identify disease-associated AA biomarkers and their reversal with effective therapy in C3H mice. Approaches using GWAS and transcriptional profiling, for example, as well as approaches using immunostains and FACs of T cell subsets, for example, have been taken to identify several pathogenic AA biomarkers in the skin and blood of AA mice. These mouse studies will continue to inform developing a translational biomarker platform in human AA. In the skin, the studies in alopecic C3H/HeJ mice demonstrated that IFN-induced genes are dramatically upregulated, including the chemokines CXCL9-11, which are likely responsible for recruitment of a dense infiltrate of HF-associated IFN- γ -producing CXCR3⁺ NKG2D-bearing CD8 T effectors. Spectratype data provide striking evidence that these circulating NKG2D⁺ CD8 T effectors are *bona fide* autoantigen-driven effector T cells that increase with disease progression. Temporal assessment of these serologic, cutaneous and cellular biomarkers in mice during treatment will enable one to identify dynamic, mechanistically important, prescient inflammatory biomarkers that predict therapeutic outcome and inform clinical biomarker discovery/utilization.

[00294] Preclinical validation of targeted approaches using AA human tissues ex vivo. To expedite clinical development of treatments that demonstrate “proof-of-concept” in the AA mouse model, human AA tissues will be used for target validation and correlative studies. For instance, correlative evidence between IL-15 expression/activity in human AA skin and clinicopathological parameters of AA disease severity will be sought, e.g., SALT score and “NK-type” CD8 T cell involvement in the skin and blood. IL-15 is a known potent activator

of CTL survival/activity and is routinely used to promote expansion of dermal T cell “crawl-outs”. This system will provide a convenient *ex vivo* human bioassay to validate that agents that reverse mouse AA translate to the human, effectively inhibiting human AA T cell survival/expansion/function.

[00295] Just as the human DNA from thousands of alopecic subjects provided GWAS genes and unique insight into the human disease, human tissues, blood and skin from AA subjects will be used to continue to interrogate these genetically implicated immune pathways ultimately for therapeutic utility. Conceptually, the mouse model of alopecia areata, providing the pre-clinical testing grounds for therapeutic approaches in alopecia areata.

[00296] Next-generation topicals targeting NF- κ b, aimed at unraveling and undermining the mechanistic underpinnings of NKG2DL/NKG2D signaling, will be developed. Extensive evaluation in mouse models and validation using human tissues *ex vivo* will be done. Conversely, protein biotherapeutics, that demonstrate proof of concept in reversing AA in the mouse, will be tested and will lead to testing small molecules that interrupt the signaling of that same pathway.

[00297] Autoimmune diseases affect an estimated 23 million individuals in the US (1) and although the prevalence of AA is not established, it is one of the most prevalent autoimmune diseases (2, 3). In 1999-2000, there were an estimated 2.4 million office visits for AA, half of which by patients in their 20s and 30s. AA causes significant disfigurement and psychological distress to affected individuals and carries one of the highest burdens among any skin diseases, particularly among children and adolescents whose self-image is so closely linked to their appearance (4). At present, the prognosis of AA is unpredictable and there is no definitive treatment. Current therapies include steroids and topical immune therapy, and induce durable remissions in only one third of patients (5, 6). Despite its high prevalence, a comprehensive Cochrane analysis assessment concluded that there are *no* evidence-based treatments for AA (7).

[00298] In contrast to other autoimmune conditions, research into the pathogenesis and the development of innovative therapies in AA has lagged far behind. In the era of targeted immunological therapies, only two clinical studies evaluating protein biotherapeutics in AA have been reported (8, 9) (both targeting T cell trafficking). In part this has been due to the limited understanding of specific AA immune pathways that would prompt evaluation of

rational therapeutic approaches. Based on new understanding of the genetic and pathogenic basis of AA pre-clinical proof-of-concept data that will prompt clinical evaluation of rational therapeutic approaches in this disease will be obtained.

[00299] The GWAS (10) study revealed a number of risk loci shared by with other forms of autoimmunity, such as RA, T1D, celiac disease (CeD), SLE, MS and PS. The genetic commonality with RA, T1D, and CeD is especially noteworthy in light of the pathogenic significance of the expression of an NK ligand in the end organ (synovium, islet, gut and skin), and the involvement of the NKG2DL/NKG2D pathway in the pathogenesis of each of these three diseases (11, 14). One clear advantage of immunotherapeutic studies in the skin is the relative ease of access of the target organ. Thus, the studies herein examining the skin can provide important insight into NKG2D and IL-15 triggered CD8 cytotoxicity, IFN-triggered injury, etc., that impact the understanding of these other related human diseases in which the target organ is not accessible. Indeed positive studies in any one of these autoimmune diseases that share a common cause can serve as the basis for common treatments.

[00300] Specific autoimmune mechanisms underlying AA have remained obscure beyond the prevailing view that it is the result of T cell mediated attack of the hair follicle. In the normal state, hair follicles represent an immuneprivileged (IP) sanctuary (15-17), expressing little or no classical HLA class I molecules and instead express the inhibitory HLA ligands HLA-E and HLA-G, thus simultaneously avoiding immune recognition by class I restricted CD8 cells while negatively regulating NK cells, that might otherwise react to “missing self” (16, 18-21). In active AA, the IP status of the normal hair follicle is not operative, since HLA class I expression is markedly upregulated. Previous studies have noted both CD4 and CD8 T cell infiltrates surround the hair follicle in alopecic areas of skin in the mouse and human (22, 23). Indeed transfer of total T cells, but not B cells or sera, can transfer disease from an alopecic mouse or human to a normal WT (24) or SCID mouse (25), implicating effector T cells as the pathogenic cells. However molecular mediators of site-specific inflammation have not been previously identified. Guided by the GWAS findings and previous immunobiological studies, new insight into this common autoimmune disorder is provided, that will lead to the development of innovative, rationally targeted treatments to fill the unmet needs of patients with AA.

[00301] AA Immunological Pathways Identified by GWAS Studies The most highly-significant genetic loci found associated with alopecia areata are shown in **Table 10**: (the gray area denotes “nominally significant” genes). These seminal studies have been validated in a confirmatory study in a distinct population (REF). Underlined genes are HF expressed genes while **bold** genes are genes expressed in immune cells. Overall, many susceptible loci are shared by other tissue-specific autoimmune states, with Type I diabetes exhibiting the most striking overlap (right column). The commonality with RA, T1D and CeD is particularly noteworthy, as NKG2D has been shown to have a significant role in the pathogenesis of each of these three diseases (11-14). Thus insight in any one of these disease/model systems is likely to shed light on common pathogenic pathways shared by multiple autoimmune states. The most highly significant alleles identified (other than HLA) were four NKG2D ligands (UBLP-3/UBLP-6/MICA/MICB in **Bold italics**) and one inhibitory ligand (HLA-G). The NKG2D receptor (NKG2D), functions to eliminate cells emitting danger signals and dysregulation of this recognition process often leads to development of autoimmunity (26). Other HF expressed genes that contribute to risk include HLA, antigen processing genes (TAP) and PRDX/STX17.

2q33.2	CTLA4	Inhibits costim	3.6×10^{-13}	T1D, RA, CeD, MS, SLE, GD
	ICOS	T-cell costim	4.3×10^{-08}	T1D, RA, CeD, MS, SLE
4q27	IL21	NK function/Th17	4.3×10^{-08}	T1D, RA, CeD, PS
	IL2	T-cell./Treg prolif.	3.0×10^{-04}	T1D, RA, CD, MS, CD, SLE, PS, GD
6q25.1	ULBP6	NKG2D ligand	4.5×10^{-19}	None
	ULBP3	NKG2D ligand	4.4×10^{-17}	None
9q31.1	STX17	hair graying	3.6×10^{-07}	None
10p15	IL2RA	T-cell prolif./Treg	1.7×10^{-12}	T1D, MS, GD
11q13	PRDX5	antiox enz	4.1×10^{-07}	MS
12q13	Eos	T-reg programming	3.2×10^{-08}	T1D, SLE
6p21.3	MICA	NKG2D ligand	1.2×10^{-07}	T1D, RA, CeD, UC, PS, SLE
(HLA)	NOTCH4	Hematopoiesis	1.0×10^{-08}	T1D, RA, MS
	BTNL2	T cell costim.	2.1×10^{-26}	T1D, RA, UC, CD, SLE, M
	HLA-DRA	Ag presentation	2.9×10^{-31}	T1D, RA, CeD, MS
	HLA-DQA1	Ag presentation	3.6×10^{-17}	T1D, RA, CeD, MS, SLE, S, CD UC, GD
	HLA-DQA2	Ag presentation	1.4×10^{-35}	T1D, RA
	HLA-DQB2	Ag presentation	1.7×10^{-13}	RA
	HLA-DOB	Ag presentation	2.1×10^{-08}	SLE
5	IL-13	Th2 polarization	1.9×10^{-08}	Asthma
6	HLA-G	NKG2A inhibition	1.0×10^{-04}	RA, MS, SLE, PS, T1D, Asthma,
	HLA-A	Ag presentation	1.0×10^{-04}	MS, T1D, PS, GD, Asthma, Vitiligo
	MICB	NKG2D ligand	1.9×10^{-05}	MS, T1D, UC, RA, CeD, Asthma
	TAP2	Ag presentation	6.4×10^{-08}	T1D, RA, SLE, PS, GD
7	IL6	Inflammation	7.7×10^{-05}	RA, T1D, CeD
12	IFNG	Th1/inflamm.	1.5×10^{-05}	CD, T1D, RA, MS, SLE, PS, GD, Asthm
	IL26	T polarization	7.2×10^{-05}	MS, Asthma
16	CLEC16A	C-type lectin receptor	1.8×10^{-05}	T1D, MS, GD
	SOCS1	JAK/STAT inh	1.2×10^{-05}	CeD, T1D, Asthma
18	PTPN2	phosphatase	4.2×10^{-08}	CeD, T1D

Table 10: Genetic loci associated with AA.

[00302] Given the GWAS findings strongly associating NKG2D ligand genetic loci with human AA, without being bound by theory, aberrant NKG2DL upregulation and persistent NKG2D immunoreceptor innate activation can drive AA initiation and progression. The AACORT data point to two tissue-specific immunostimulatory events; upregulation of NKG2DL and IL-15 on HF targets. These factors can provide the major molecular drivers of cytolytic NKG2D-bearing CD8 T cells which are found to densely infiltrate the diseased HF. Closing the vicious circle, IFN- γ production by activated NKG2D-bearing CD8 effectors may reverse immunologic privilege and perpetuate the inflammatory loop.

[00303] These pathways can be undermined with existing therapeutics under clinical investigation, expediting the path to translation. Both anti-NKG2D and anti-IL-15 mAbs are currently under clinical investigation in other autoimmune conditions. These biologics require systemic delivery, carrying with it the associated benefit of systemic potency but as well the added risk of systemic adverse effects. Topical agents with small molecules would carry significantly less risk.

Identify clinically-relevant therapies targeting effector T cell responses in alopecic mice.

[00304] Rationale for Targeting NKG2D expressing CD8 T cells: Based on the GWAS study identifying NKG2D ligands (ULBP3, ULBP6, MICA) as AA genes and the evidence for CD8⁺NKG2D⁺ T cell infiltration in human AA (10) (FIG. 19) their relevance in the alopecic C3H/HeJ mouse was pursued. Using immunostaining and flow cytometric analysis of total cellular populations from AA skin, CD8⁺NKG2D⁺ T cells were found infiltrating the hair follicle (FIG. 19) and expanded in AA but not normal skin (FIG. 20 and FIG. 7A). Furthermore AA mice exhibit cutaneous lymphadenopathy, at least in part due to dramatic systemic expansion of the CD8⁺NKG2D⁺ population, in both the AA cutaneous lymph node and in the blood, representing fully 6+/-1% of total LN cells are 2.4+/-1.3% of total PBMCs, each more than ten-fold higher than that seen in normal C3H mice. These CD8⁺NKG2D⁺ T cells are potently cytotoxic against HF dermal sheath cells (FIG. 26). Further immunophenotypic analysis of CD8⁺NKG2D⁺ T cells revealed that this population expressed the T memory marker CD44⁺ and multiple "NK markers", including DX5, NKG2A/C/E, and NKp46 (FIG. 21) reminiscent of IL-15-driven "CD8 effector NK re-programming", described previously for celiac disease (11, 27, 28).

[00305] To provide proof that the circulating CD8⁺NKG2D⁺ population represented the bona fide "AA-specific" effector population, without being bound by theory, the CD8⁺NKG2D⁺ T cells found in the lymph node can express a similar TCR repertoire to the total T cells found infiltrating AA skin. FIG. 22 shows that CD8⁺NKG2D⁺ lymph node populations exhibit a normal distribution of CDR lengths, a pattern reflective of an unrestricted polyclonal T cell population. In contrast, the TCR repertoire of skin T cells are highly restricted with nearly all Vβ family members dominated by a small number of CDR lengths, indicative of an oligoclonal population of antigen-driven T cells. CD8⁺NKG2D⁺ T cells from the cutaneous lymph node cells are also highly restricted and show a strikingly similar pattern with total cutaneous T cells.

[00306] These data confirm that CD8⁺NKG2D⁺ T cells contain most of the oligoclonal T cell populations found in the skin. Moreover the evidence for the expansion of common oligoclonal T cells in the skin and lymph node argues against the possibility that these LN populations (or even markers) are expanded/activated as a reactive process to inflammatory events. For instance, Treg cells (CD4⁺CD25⁺FoxP3⁺) are also increased in numbers in AA lymph nodes, however, this is likely a reactive process, since their spectratype is diverse and unlike those present in the skin. Note that some TCR/clones are found in skin but not in draining cutaneous lymph nodes, e.g., Vβ10. These likely include CD4⁺ T cell populations found in AA skin but not present in the *sorted* CD8⁺ LN fraction. These data establish that the CD8⁺NKG2D⁺ T cells are the pathogenic NKG2D⁺ effectors in mouse AA, establishing a parallel of the mouse model to human AA,

Pre-clinical AA model to establish proof of concept for therapeutic approaches *in vivo*

[00307] C3H/HeJ Skin Graft Model: The C3H/HeJ mouse model for alopecia areata has been studied since it was first reported this model in 1994 (30, 31). While other mouse models for alopecia areata were found (32), this model remains the most studied and used in the field today. Having done a variety of drug trials with this model over the years (33), a variety of protocols have been optimized (34).

[00308] Full thickness skin grafting from alopecic C3H/HeJ mice to 2-3 month-old unaffected C3H/HeJ female mice results in patchy alopecia within 10 weeks of grafting in all recipients, progressing to generalized alopecia at 20 weeks. A variety of comprehensive and efficient histological scoring systems are employed building on the traditional training of histopathologists where the adjective used in descriptions (normal, 0; mild, 1; moderate, 2; severe, 3; and extreme, 4) are converted to a numerical value for importation into a relational database (Mouse Disease Information System [or MoDIS]: <http://research.jax.org/faculty/sundberg/registration.php>) (35, 36). This database is exportable via Excel for importation into a statistical analysis or other software program. Alopecia areata is graded based on severity, location of inflammatory cells, types and relative numbers of inflammatory cells (granulocytes, mast cells, lymphocytes), follicular dystrophy, etc. Molecular RNA and protein biomarkers will be incorporated in this panel, and generation and analysis of this multiple variable database will be guided by the biostatistical group in the TTR Core.

[00309] Protocol#1: In the prevention setting, recipient mice will be treated beginning the first week after grafting.

[00310] Protocol#2: For treatment, grafted mice with early-established AA will be treated to reverse AA progression.

[00311] Agents will first be examined in the prevention model. Agents that successfully prevent AA will then move forward for secondary evaluation in the setting of established disease, while those that fail the first test will be discarded, permitting entry of other innovative approaches. The following are the initial therapeutic approaches, both specific biologics and small molecules, chosen for preclinical evaluation.

[00312] Therapeutic interventions with biologics: Targeting the immune effectors in AA: CD8⁺NKG2D⁺ “NK-like” T cells have been identified as the causal alopecic effectors. Two approaches have been taken to eliminating the activity of these cells: 1) blocking their *induction* (anti-IL15 therapy), or; 2) blocking their *activation* (anti-NKG2D therapy).

1) Targeting IL15:

[00313] IL-15 immunobiology: IL-15 is a potent pro-inflammatory cytokine produced locally in tissues by epithelial cells and monocyte/dendritic cells. IL-15 is exocytosed together with IL-15R α and trans-presented to IL-15R β bearing cells driving innate and adaptive immunity. Notably IL-15 induces “NK-type” CD8 T cell activation at several points: 1) IL-15 induces NKG2DL expression on epithelial cells (37); 2) CD8 effector cells are armed by IL-15 to express NKG2D (27; 38-41); 3) IL-15 provides co-stimulation for TCR and/or NKG2D mediated activation of NK cells and intraepithelial T cells lowering the TCR threshold of self-reactive T cells (27; 38-42); 4) IL-15 promotes T and NK survival/expansion (37, 39, 43-47).

[00314] IL-15 in autoimmunity: In the inflamed rheumatoid arthritic joint, IL-15 is upregulated on synovial membrane, synovial fibroblasts, endothelial cells and mast cells (48). In the gut, IL-15 produced by epithelial cells promotes T cell inflammatory responses underlying celiac disease (27, 28, 49, 50). In the skin, IL-15 is upregulated in the stroma of T cell lymphoma (51) and has been implicated in psoriasis (52, 54). IL-15 blockade protects mice from psoriasis (54), celiac disease (55) and arthritis (56). At least two anti-IL-15 antibodies are in clinical evaluation (AMG714, Mik β 1) for T cell malignancies (57) and for

autoimmunity including rheumatoid arthritis (58, 59) and psoriasis (<http://clinicaltrials.gov/ct2/show/NCT00443326>). Soluble IL-15R α have also effectively treated mouse inflammatory models (56, 60, 61).

[00315] IL-15 in alopecia areata: The IL-15 pathway may be genetically involved in AA: IL-15Ra is contained within the GWAs IL-2R linkage interval on human chromosome 10 and IL-15 is contained within the mouse AA susceptibility locus (Alaa1) on Chromosome 8 (62). Overexpression of IL-15 from a ubiquitous promoter was found to induce alopecia areata in the mouse (63). IL-15 is shown to be highly upregulated in AA lesional skin of C3H mice and humans, indicating a pathogenic role (**FIG. 23**) and implicating IL-15 in AA for the first time.

[00316] The *in vivo* requirement for IL-15 was investigated in the AA graft model. Administration of anti-IL-15R β successfully blocked AA development in grafted mice (**FIG. 24**) mAbTM- β 1 rat IgG2b, Biolegend, i.p. 200 μ g, 2x/wk). Examination of blood samples and skin biopsies from these mice in these ongoing studies confirmed that anti-IL-15R β treatment had pleiotrophic effects, abrogating the AA inflammatory response at several points. Notably anti-IL-15R β treatment prevented the accumulation of “NK-type” CD8 T cells in the circulation (**FIG. 27A**) and skin biopsies showed that treatment abrogated multiple HF AA inflammatory events including the IFN-response (**FIG. 29**) and MHCII and ICAM-1 expression. Data at 10 weeks post grafting are shown, and the results will address the effects of anti-IL-15R β treatment on AA biomarkers in the skin, blood and lymph nodes. These studies will address whether AA reversal results from treatment-related HF end-organ specific events (e.g., NKG2DL down regulation) and/or effects on systemic immune CD8 T effectors (e.g., survival/activation, or reversal of NK-like reprogramming). The data after 10 weeks of treatment are consistent with a requirement for IL-15 in the local induction of “NK-type” CD8 effectors (**FIG. 27**). The therapeutic activity of anti-IL-15R β has begun to be defined in established disease.

[00317] The downstream consequences of IL-15 will be investigated using two IL-15 overexpressing transgenic models; H-2 promoter-IL15, and K14-IL15 (64). These studies will define IL-15 proinflammatory actions on immune and HF target cells *in vivo*.

2) Targeting NKG2D

[00318] Expression of activating NK immunoreceptors is beneficial to the host as a countermeasure to viral immune evasive strategies. However, in autoimmunity aberrant expression of activating NK-family immunoreceptors on self-reactive CD8 T cells drives “natural killing” and cytokine production (principally IFN- γ) upon engagement of self-cells expressing NKG2DL or non-classical HLA molecules. Using both human and mouse systems, it was demonstrated that dermal sheath cells expanded from dissected hair follicles can be killed by relevant NK-type CD8 T effectors, either IL-15 cultured total human PBMCs (Lymphokine activated killer “LAK” cells) or CD8⁺NKG2D⁺ T cells flow sort isolated from C3H cutaneous lymph nodes (FIG. 25). Killing was both NKG2D dependent and MHC I dependent indicative of a requirement for both NKG2D co-stimulation and cognate antigen recognition. Moreover dermal sheath targets sensitivity required prior stimulation with agents known to upregulate NKG2DLs, including IFN γ and TNF or LPS. Thus for drug development strategies, blocking *either* NKG2DL or its receptor NKG2D are both rational strategies.

[00319] Blocking NKG2DLs will avoid the potential for unexpected agonistic activities of anti-NKG2D receptor antibodies. The immunostaining shows that in the mouse H60 is the predominant NKG2DL involved. In the human, it was demonstrated that multiple NKG2DLs are upregulated including MICA and ULBP3. Although just one of these ligands can be *the* driver of inflammation, without being bound by theory, the NKG2DLs have redundant functions and blocking all three will be required to abrogate the inflammatory response. To distinguish unique vs redundant functional roles of specific NKG2DL ligands, there will be access to specific NKG2DL blocking antibodies for functional ex vivo studies (human) and in vivo studies (mouse).

[00320] Anti-NKG2D: Targeting the single NKG2D receptor shared by all NKG2DL has obvious advantages. Several anti-mouse NKG2D mAbs are available for testing in the AA model and therapeutic anti-human NKG2D blockade is currently under clinical investigation in Phase II inflammatory bowel studies (Novo-Nordisk, NN8555), potentially speeding the pathway to translation in human AA (65).

[00321] The efficacy of two different anti-NKG2D mAbs (mAb CX5, rat IgG1, hybridoma) and MI6, rat IgG2b (66), will be tested, each given intraperitoneally 200 μ g, twice weekly. NKG2D blockade with mAb CX5 has successfully prevented and reversed early established T1D in NOD mice (67), blocked colitis in the prophylactic setting (68) and

together with anti-CD28 therapy improved transplant rejection (69, 70) and ameliorated collagen induced arthritis (71, 72). mAb MI6 has been successfully used to block NK mediated rejection of NKG2DL expressing tumor cells in vivo. Other anti-NKG2D mAbs have inhibited SCID models of colitis (73) and autoimmune demyelination (74).

[00322] The first anti-NKG2D in clinical investigation, NN8555, is an IgG4, which is an IgG subclass that does not engage Fc receptors.

[00323] These anti-NKG2D and anti-IL-15 blocking studies, can provide “proof-of-concept” for therapeutic targeting of the NKG2D pathway, providing the rationale for the clinical development of small molecule approaches enabling cutaneous delivery of agents targeting downstream signaling molecules. Preclinical NF- κ B-based approaches are addressed to interrupt NKG2DL upregulation in the target HF end-organ.

[00324] Identification of murine biomarkers of AA disease activity that inform clinical investigation.

[00325] The identification of immunological events that accompany treatment outcome in these pre-clinical studies, is important, both to validate the proposed mechanism of action and to provide useful biomarkers that will inform potential clinical evaluation of these same therapies in AA patients. Since in clinical evaluation, biospecimens will be limited to blood and skin samples, the focus will be on mouse biomarkers in the mouse that are easily sampled from these sites. Approaches using GWAS and transcriptional profiling, for example, as well as approaches using immunostains and FACs of T cell subsets, for example, have been taken to identify several pathogenic biomarkers in the skin and blood of AA mice. The studies in alopecic C3H/HeJ mice have already identified several skin biomarkers upregulated in AA; NKG2DL, IL-15 (FIG. 23). Spectratype data (FIG. 22) has provided striking evidence that these circulating NKG2D⁺ CD8 T effectors are *bona fide* autoantigen-driven effector T cells. Temporal assessment of these serologic, cutaneous and cellular biomarkers in mice, with and without treatment, will enable one to identify dynamic, mechanistically important, prescient inflammatory biomarkers that predict therapeutic outcome and inform clinical biomarker discovery/utilization.

[00326] Cellular biomarkers of circulating immune effectors in AA: “NK-type” CD8 T cells are expanded in the skin, blood and lymph node in AA mice and are a valuable tool to

investigate pathogenesis. Moreover, the cellular subset appears directly relevant to the human disease.

[00327] i) *Spectratype-based immunomonitoring*: NK-type CD8 T cells will be identified in AA mice in the circulation by flow cytometry of 50 μ l of whole blood (FIG. 20, 27). The total numbers and spectratype of this CD8⁺NKG2D⁺ sorted T cell subset, will be assessed every three weeks after grafting to monitor the evolution of clonal dominance/epitope spreading within a single mouse. For instance in anti-IL-15R β treated mice, after 6 weeks of treatment, complete disappearance of these cells is observed in the blood (FIG. 27). Spectratype analysis will be used to confirm that immunophenotypic loss of CD8⁺NKG2D⁺ T cells is due to treatment-related elimination of the circulating alopecic T cell clones. This non-invasive immunomonitoring approach will be used to follow mice on all treatments.

[00328] ii) *Development of a NKG2D+CD8+ biomarker panel*: The abundance of NKG2D⁺CD8⁺ T cells in the cutaneous LNs of AA mice (up to 5 million cells per mouse) provides a unique opportunity to develop functional biomarkers specific for this T cell subset. Transcriptional profiles will be compared between (otherwise phenotypically matched) NKG2D⁺ vs. NKG2D⁻ effector memory LN T cells (e.g., CD8⁺CD25⁻ CD44⁺CXCR3⁺NKG2D⁺ vs. CD8⁺CD25⁻CD44⁺CXCR3⁺NKG2D⁻) to identify specific genes upregulated in NKG2D-bearing memory CD8 T cells. Second level comparative analysis will be performed after IL-15 stimulation of these same cells, to mimic their exposure to this activating cytokine in the skin, providing a gene expression profile of activation-induced transcripts. Both sets of gene lists will be used to query other transcriptional profiles obtained from total skin and blood RNA from AA mice and humans. In this manner, a biomarker panel that detects the presence/activity of CD8⁺NKG2D⁺ T cells in the blood and tissue will be developed. In essence, this panel of CD8⁺NKG2D⁺ associated transcripts can provide an “expression signature” to be deployed as a PCR-based strategy to identify human alopecic cells in the blood and skin. This approach has the potential to be vastly more sensitive and specific for disease activity than current flow cytometric/immunostain techniques. One approach to human biomarker development, capitalizes on the abundance and ready availability of highly enriched (sorted) LN NKG2D+CD8+ T cells in the mouse, to cross-reference and identify transcripts for the creation of a biomarker panel in human tissues signifying NK-type CD8 T cells involvement.

[00329] Cytokines and chemokines in the serum are a reflection of the inflammatory milieu in the target organ. Our data in the human demonstrate elevations of several cytokines and chemokine some of which correlate with disease activity (for instance IL-8), extending the observations of prior studies^{106, 107, 109}. Consistent with our overall findings implicating IL-15 and IFN- γ in AA pathogenesis, we see that IFN- γ is upregulated in human AA sera. Functionally relevant inflammatory cytokine/chemokine biomarkers will be identified in the sera from AA mice using a multianalyte approach (Luminex platform, see ISI Core). The advantage of biomarker development in the mouse, besides genetic homogeneity, is the relative ease of longitudinal studies to identify biomarkers that occur early or late in disease progression and conversely with treatment response. At the RNA level, C3H mouse skin IFN microarrays demonstrated that both Type I IFNs (IFN α_{1-3} , ϵ) and type II IFN (IFN- γ) were upregulated as well several IFN-associated chemokines. Cytokine/chemokines production will be examined in sera using custom multianalyte Luminex assay (R&D), comprehensively addressing serum levels of 9 chemokines (CCL2-5, CCL11, CXCL5, CXCL9, CXCL10, CXCL11) and 14 cytokines (IFN α , IFN γ , IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-15, IL-17, TNF, GM-CSF). We will include antibody pairs to detect serum levels of NKG2DLs¹¹⁷ and IL-15R α .

[00330] Preclinical validation of targeted approaches using human biospecimens

[00331] Treatments that demonstrate “proof-of-concept” in the AA mouse model will be further interrogated using human AA biospecimens for target validation in man. These studies will provide useful biomarkers for gauging treatment response/failure, facilitating bench-to-bedside development. Taking the example of the IL15 pathway, clinical evaluation of anti-IL-15 mAbs will be greatly expedited with advanced knowledge of the relevance of IL-15 in each individual. Simply put; how much IL-15 is being produced locally/systemically; how intense is the response to IL-15 locally/systemically; which aspects of this IL-15 response can be monitored on therapy for predictive insights. The biomarkers identified in C3H mice will provide important leads to be validated in human *ex vivo* specimens obtained from cross-sectional and longitudinal studies in patients.

[00332] IL-15 as an AA biomarker in humans: IL-15 levels are increased in the serum of AA patients and the immunostaining demonstrated that IL-15/IL-15R α is upregulated in microdissected HFs obtained from 4 out of 4 AA patients (**FIG. 23**). The staining is localized primarily to the outer root sheath which would be available for cross-presentation to

infiltrating dermal T cells. In whole skin sections, IL-15/IL-15R α was also found in many keratinocytes in the interfollicular epidermis as well as in scattered cells in the dermis, consistent with its known production by epithelial cells and monocyte/dendritic cells. Importantly DCs are known to induce NK and T cell activity through IL-15 production (121-124) and can be pivotal to priming alopecic CD8 cytotoxic T cells. Immunostaining with DC markers (Langerin, CD1c, HLA-DR) and macrophage markers (CD68, HLA-DR) will identify whether these cells are additional IL-15 sources in the dermis. pSTAT5 immunostains will be examined in infiltrating T cells expected to be responsive to local IL-15 production. IL-15/IL-15R α staining will be addressed in a cross-sectional cohort of AA subjects with two biopsies per subject to assess intra- and inter-patient variability and sampling issues. The biopsies are routinely obtained from perilesional areas exhibiting a positive “pull-test”. In the mouse studies, inflammatory lesions around HFs are most robust at this “leading edge”.

[00333] An IL-15 response signature will be developed using whole skin RNA. IL-15 signatures will be developed and refined using transcriptional profile data integrated from several sources, including the mouse studies herein (e.g., IL-15 response profile of CD8⁺NKG2D⁺ cells, profiles of mouse skin after injection of IL-15, skin RNA profiles after anti-IL-15 treatment) to add to published IL-15 T cell response signatures (125). Candidate IL-15 response genes will be tested for correlation with IL-15 and pSTAT5 immunostaining results from the same individuals and then assessed for superior intra-patient sampling variation. These tools will be incorporated into an IL-15 index activity that will be evaluated below in cross-sectional and longitudinal studies.

[00334] Validating IL-15 activity as a disease biomarker in the clinic: If IL-15 is a causal factor in AA pathogenesis, without being bound by theory, its activity will correlate with disease intensity and other relevant clinicopathological parameters. Using a cross-sectional design and subjects seen in the Mackay-Wiggan clinic, including “baseline” biospecimens obtained from AA subjects upon entry into clinical study, an IL-15 global activity index will be compiled for each individual (integrated from serum IL-15 levels, tissue IL-15/IL-15R α immunostaining and IL-15 skin expression signatures). To establish links between IL-15 activity and disease intensity, correlative evidence will be sought between the IL-15 index and disease extent (percent scalp involvement or SALT Score) and histopathological measurements (e.g., the intensity of skin infiltration with CD8⁺ T cells, the level of

expression of activating NKG2D on CD8 T effectors in the blood). Multivariate analysis integrating quantitative/semi-quantitative measures will be used.

[00335] To examine the utility of the IL-15 index longitudinally as a predictive marker for disease progression/treatment response/spontaneous remission, biospecimens will be analyzed in its two placebo-controlled trials. Whether high IL-15 activity scores at baseline are poor prognostic indicators will be noted and steroid resistant patients will be identified. Indeed there are data suggestive of IL-15 overcoming the anti-inflammatory effects of steroids (126-131).

[00336] Using AA T cell “crawl-outs” to demonstrate IL-15 dependence of alopecic T cells: IL-15 is an attractive target clinically for the induction of AA reversal because IL-15 is a key survival factor required for the survival of otherwise short-lived effector memory cells. Moreover, although other cytokines (IL-7) can promote T cell survival elsewhere, local IL-15 may be an obligate factor for effector T cell survival in the skin, further justifying topical rather than systemic therapy. Additionally avoidance of systemic exposure to anti-IL-15 agents will limit adverse events. Identification of a potent, specific small molecule inhibitor of the IL-15 pathway can lead the way to effective topical delivery, which will be more favorable therapy, economically and medically.

[00337] Yields of $0.3\text{-}3.0 \times 10^6$ CD8⁺NKG2D^{hi} CD8 T cells are obtained after a 3 week expansion from dispase-treated minced 2mm³ AA skin samples. To examine the effects of IL-15 on survival, after three weeks of culture, T cells will be shifted to conditions containing IL-2 alone or with increasing amounts of IL-15 to establish a dose/response relationship for IL-15 enhancement of survival/proliferation, cytokine (IFN γ) production and NKG2D-mediated killing of dermal sheath cells. This *ex vivo* assay using primary human AA cutaneous T cells can be deployed as a screen to identify additional immunoreceptor/signaling pathways that can also be targeted. Thus using blocking antibody approaches, future studies will use this *ex vivo* system to identify roles for other contributing human NK family activating and inhibitory immunoreceptors (for instance NKG2A, NKG2C, NKp46). Positive results demonstrating proof of concept in the human bioassay would launch the initiation of studies using blocking antibodies as treatment in the mouse model.

[00338] Human Subjects

[00339] Skin biopsies from the scalp will be collected from a minimum of 100 Alopecia Areata patients and approximately 100 controls. The subject population will consist of AA patients and unaffected control subjects receiving hair transplantation. Approval for collection of these samples underwent expedited review/exemption since they are discarded tissue. A protocol for collection of scalp biopsies from AA patients is in preparation. A minimum of 100 AA subjects will be recruited, but collection of greater than 100 will be beneficial and increase the power of this approach.

[00340] In order to achieve maximum homogeneity among samples for generating the expression interactome, hair follicles will be collected exclusively from middle aged Caucasian male subjects with AA, since the matching control donors are most commonly from this group. Therefore, despite the fact that AA shows no ethnic or gender predilection, the AA patients will be selected to match in age and gender to the controls for the purpose of this study (middle aged Caucasian males). Patients with active disease (ie: with some remaining hair follicles) will also be selected so that they can be efficiently microdissected.

[00341] Vertebrate Animals

[00342] Young C3H/HeJ mice (2-3 months) and retired breeders showing visible hair loss will be acquired from Jackson Labs. The animals will be kept under standard conditions in the animal facilities.

[00343] The mice will be administered drugs or antibodies by either systemic injections or topically. For topical applications, the mice skin will be shaved on the dorsal surface with electric clippers. One week after shaving, mice will receive topical application of the chemicals dissolved in acetone or 10% v/v in propylene glycol as vehicles. Injections will be given either intraperitoneally or subcutaneously. The dosage as well as frequency of administration will be followed. The mice will be monitored daily for signs of distress throughout the duration of treatment.

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EXAMPLE 5

[00344] Alopecic effector T cells were identified in the lymph node and blood of alopecic mice using spectratyping of flow sorted cells. A blood-based assay was developed to monitor these alopecic T cells during treatment. Cytotoxic assays have been developed for evaluating functional components of the hair follicle (HF) interactions with CTLs, in both human and mice.

[00345] Using human biospecimens obtained from AA subjects, it was demonstrated that circulating T cells express high levels of NKG2D and primary T cells obtained from the skin are dominated by a CD8⁺NKG2D⁺ IFN- γ producing cells establishing parallels between the mouse and human disease.

[00346] In the target HF cells, H60 has been defined as an upregulated NKG2DL in alopecic mouse HFs and ULBP3/MICA as their counterparts in the human HF. Finally it is shown that IL-15/IL15R α are upregulated in mouse and human AA HFs, defining a second precipitating event triggering inflammatory responses in the HF end-organ. ✓

Immunophenotyping and Skin Immunobiology

[00347] To interrogate pathways in biospecimens requires analysis of heterogeneous cell populations in the skin and in the blood. In the clinical investigation of human autoimmunity, alopecia areata provides a unique opportunity because of the accessibility of the end-organ enabling study/isolation of the pathogenic immune effectors in their relevant microenvironment. Analytic tools and personnel required will be provided for optimal study of these precious end-organ skin biospecimens, and their cellular/serological counterparts in the blood obtained from the same individual.

[00348] Preparation and analysis of biospecimens:

[00349] The following will be provided to assist in the analysis of blood: i) Multianalyte cytokine/chemokine analysis from serum samples; ii) Flow cytometric immunophenotyping, including cytokine analysis of human PBMCs from AA subjects; and iii) Flow sorting for downstream applications using T cell subsets (RNA profiling, spectratyping).

[00350] The following will be provided to assist in the analysis of skin: i) Preparation/analysis of viable cellular fractions of both primary hair follicle and T cell components for functional (cytotoxicity) and analytic studies (T cell cloning, spectratyping); ii) Immunostaining and transcriptional profiling of whole skin; and iii) Flow cytometric immunophenotyping of dermal lymphocytes from human and mouse AA skin.

[00351] Scientific Experiments

[00352] Pathogenic HF-specific cellular subsets in the peripheral blood of AA subjects will be identified. To this end, spectratype analysis will be used as a tool to identify circulating human alopecic T cells populations, as was done for the mouse model, by matching the spectratype found in total skin T cells with the spectratypes of specific sorted peripheral blood T cell subsets in the peripheral blood obtained from the same patients.

[00353] Th1-predominance in human AA will be established and the rationale and biomarker platform for Th1-targeted therapies will be developed. Therapeutic targeting of disease-specific Th-pathways has succeeded in other human skin inflammatory diseases, most notably in psoriasis a prototypical Th17 disease. There is substantial data in the AA animal model for AA as a predominant Th1 disease. The Th-profile of infiltrating dermal AA T cells and relevant circulating T cell subsets in the blood will be addressed using a multi-prong approach including multianalyte, RNA profiling, immunostaining and intracellular flow cytometric analysis from the skin and blood.

[00354] An integrative approach to translational research begins with basic studies identifying targetable pathways in AA, then testing these pathways therapeutically in preclinical models, longitudinal biomarker assessment in clinical trials and pursuing population-based research based on genetic studies. The pre-clinical therapeutic effects of interventions will be evaluated in the grafted AA mouse models, treatment effects on the inflammatory responses will be assessed in the skin and blood and human biospecimens will be provided for validation of human relevance in ex vivo studies.

[00355] Monitoring the pathogenic interactions between immune cells and target cells in the skin in humans requires sophisticated processing and analysis of primary human tissues and blood. Since these materials are precious and cell yields are small, it is essential to bring the greatest analytic capacity to bear on each human tissue sample.

[00356] Flow cytometry has become the primary tool for the identification of cell populations according to specific parameters, and is therefore employed by an ever-growing number of biomedical scientists.

[00357] The immunobiology of the skin presents unique characteristics and challenges; the special architecture of the tissue including its barrier functions, its associated unique immune cell populations and its close proximity/interactions with microbial flora, and the practical difficulties of isolating infiltrating lymphocytes, are all part of the complexities needed to be understood in the approach to immunological diseases of the skin. Services that provide assistance with tissue processing and staining, as well as cell isolation, and essential to insuring high quality consistent results are available.

[00358] Flow cytometry is a tool for clinical investigators and a working knowledge of its use is required for all translational immunologists. Four flow cytometers, including two

workhorse instruments (FACs Canto and FACs Caliber) a 6-laser LSR II and a 4-laser BD Influx can be used.

[00359] The LSR II and BD Influx have been designed to have comparable lasers/detectors to facilitate transition from analysis to preparative sorting. The LSR II is equipped with 6 lasers, capable of detecting in total 19 colors simultaneously providing versatility to detect both fluorescent proteins (mBanana, GFP, BFP, RFP/dsRed, mCherry mRaspberry) and fluorescent dyes/chemical fluorochromes. LSR II can analyze diverse cell types within heterogeneous cell populations from tissues using a wide array of organic and inorganic fluorochromes. These additional fluorescent parameters allow detection of coordinated functional events in specific cell types in mixed populations (e.g. intracellular IFN-gamma, IL-10 production and phosphoprotein detection in T cell populations from cutaneous tissues and draining lymph nodes). The basic LSR II instrument is equipped with three lasers; blue (488nm), red (633nm), violet (405nm). The custom designed LSR II contains in addition, three additional lasers permitting up to 20-color detection and a 96-well plate reader. "Leave one out" 10-color panel designs for T cell subsets (for example, Tregs), B cells and monocyte have been designed to take advantage of the 100W yellow-green 594 laser which has exquisite sensitivity for "red" Alexa 594. Thus, investigators may adopt our "leave-one out" design to sensitively examine expression for a specific marker of interest by staining with Alexa 594 conjugated antibodies.

[00360] Quality: Panel development for immunophenotyping lymphocyte populations in human peripheral blood and other experiments will be conducted. Because of the unique capacity of the 6 laser system of the LSR II, a common "easy-to use" set of colors that can be used without the need for compensation was established. The ability to do a 5/6-color experiment without the need for fluorescent compensation gives increased data resolution as the fluorochromes as far apart from each other in the spectrum, and excited independently by each laser line. This allows for the ability of populations to clearly separate, something that is rare when compensation is applied. The current selected fluorochrome combination is DAPI (gated on the DAPI negative cells) Pacific Blue, FITC, PE, Alexa Fluor 594 and APC, however other combinations of fluors are currently being tested and used. This 5-fluorochrome set serves as the base platform for panels, to which additional fluorochromes can be added. In a proof-of-concept experiment, the use of 5 fluorochemicals was shown simultaneously without the need for compensation. C57BL/6 splenocytes were stained with

CD8 Pacific Blue, CD3 FITC, CD45R (B220) PE, CD11b Bio+Streptavidin Alexa Fluor 594, and CD4 APC.

[00361] Maintenance and Quality control for Skin Immunobiology Core: The procedure the core uses for the isolation of lymphocytes from skin biopsies has been adapted from a method developed by Clark and Kupper (2) in which biopsies are culture on 3-dimensional tantalum-coated cellfoam matrices to promote migration of T cells from the biopsies. Of each isolated cell populations a minor fraction will be used for CD45/CD3 staining to assess the number of leucocytes / T lymphocytes by flow cytometry prior to further studies or cryopreservation. For re-use, the matrices will be soaked for 30 min in 10% bleach, then rinsed in water and transferred into a solution of Enzyte enzyme cleaner (Decon Labs). The matrices will be left in this solution on a hot plate with stirrer for 24 hrs, rinsed with distilled water and let dry before autoclaving.

[00362] Immunomonitoring:

[00363] a. Multianalyte cytokine & chemokine analysis from serum samples.

[00364] Two approaches can be utilized: a) The multiplex bead based immunoassays systems (e.g. CBA/FlowCytomix) provides multiple analytes that allow 10 or more cytokines/chemokines to be assessed on a single read of a small volume (50 µl) sample. The LSR II 96 well high throughput capacity provides ease of use combined with efficiency. Software ("snap-to" gating) is available in house for data analysis. A multiplex bead-based assay system will also be developed to quantify soluble human and murine NKG2DL in the sera, for which specific antibody sets are commercially available. b) Multiplex analysis using the Luminex platform can be used in the CTSA for analysis of commercially available mouse and human cytokine and chemokine arrays (FIG. 27B, FIGS. 29-30**).**

[00365] Flow cytometric immunophenotyping

[00366] It has been shown using multiparameter flow that CD8+NKG2D+ cells dominate the dermal leukocyte population harvested from the skin of mice with spontaneous alopecia (FIG. 31**). To address the human situation, flow panels have been developed to define quantitative alterations in any of 30 PBMC subsets focusing on NK and CD4 and CD8 T cell populations using 3 FACS tubes (T cell subset tube, NK- marker tube for CD4, CD8 and NK cells and a third non-T cell tube). Lineage Markers: CD3, CD4, CD8α, CD8β, CD14, CD19,**

CD20, CD56, CD64. T cell subset Markers: CD45Ra, CD45Ro, CD56, CD62L, CCR7, CD127, FoxP3, Helios. Cutaneous Chemokine Receptor Analysis: CCR4, CCR8, CCR10, CLA, E and P-selectin. NK immunoreceptor family members: Inhibitory: KIR/CD158, CD94/NKG2A, LILR/ILT, LAIR1 NKR-P1 Activatory: NKp46, NKp30, NKp44, NKG2C, NKG2D. In the initial evaluation of PBMCs from four AA subjects, increased NKG2D expression was observed on CD56+ CD8+ T cells and NK cells, but not on CD4+ T cells (FIG. 32).

[00367] Cytokine analysis: Activation of freshly isolated buffy coat peripheral T cells with PMA/ionomycin will drive cytokine production by circulating memory T cells. After a 4 hour incubation with brefeldin, cells are cell surface marker stained, fixed and permeabilized prior to intracellular staining for IL-2, IFN- γ , TNF, IL-4, IL-17, and FOXP3/helios. These cytokines/transcription factors together with the surface markers CD3, CD4, CD8, CD25, CD56, CD62L, NKG2D, CD45Ra, CD45Ro, CLA/CCR4 will delineate the fraction of Th1/Th2/Th17/Tregs in the total and cutaneous CD4/CD8 naïve and memory T cell compartment and their expression of NKG2D. Without being bound by theory, using the LSRII machine, which enables multi-parametric testing from a single tube, 2-4 million PBMCs will be more than sufficient for this analysis. This will leave enough PBMCs (>20 million cells) from a 30 ml blood draw to provide for other testing.

[00368] Flow sorting for downstream applications using T cell subsets

[00369] The goal is to provide potential alopecic T cells for downstream functional analysis. For example, the Core will provide flow sorted T cell subsets for RNA profiling, for instance, transcriptional profiling of NK-type T cells. For biomarker studies, it is critical to hone in on the appropriate cellular subset, as this will greatly improve the resolution of the analysis, which will otherwise be diluted by the presence of RNA from the mixed heterogenous PBMC population,

[00370] Studies in the AA mouse indicate that the alopecic T cells (FIG. 27B, FIGS. 29-30), are recirculating and can be readily found and isolated from the blood and cutaneous lymph nodes. More than 5 million CD8+NKG2D+ T cells can be isolated by flow sorting from the cutaneous lymph nodes of a single mouse. This is an impressive number of fresh primary “pathogenic” T cells for downstream study and is a substantial improvement on the cell yields one can expect to recover from “crawl-outs” from alopecic skin. The

identification of pathogenic T cell subsets in the blood of AA subjects human AA subjects is the major scientific goal. Flow isolated T cell subsets will be provided for downstream applications including biomarker studies, functional studies and spectratyping to provide evidence for immuopathogenic relevance of specific T cells subsets.

[00371] Spectratype Analysis of TCR repertoire

[00372] Spectratyping or TCR β -chain length distribution analysis of RNA from tissues or phenotypically separated lymphocyte subsets provides a qualitative portrait of the TCR clonotype utilization/repertoire of the infiltrating T cells. In recent years this technique has been used by others (5-7) as a highly sensitive and accurate method to delineate the proportion of clonally expanded T cells in a sample. Oligoclonality, evidence of expansion of subsets of T cells with restricted TCRs is indicative of antigenic drive in inflammatory processes and can be used as a first step to identify pathogenic T cell clones. Since each TCR rearrangement varies in CDR3 length in multiples of three nucleotides, heterogeneity of CDR3 length within a population of T cells can be used as a measure of TCR diversity. The principle of this technique is to PCR amplify the cDNA obtained from a T cell population using an upstream specific V β family member primers and a downstream constant region primer that together span the CDR3 region made by combinatorial and junctional VDJ joining. The PCR products are then fluorochrome-labelled in a primer extension ("run-off") reaction using a fluorescently labeled constant region primer. The products are then run on the ABI PRISM 3700 DNA analyzer. With Gene Mapper software the size of the peaks corresponding to discrete CDR3 lengths can be displayed and analyzed (**FIGS. 22 and 33**). The data are exported and compared to reference polyclonal repertoires, and measures of oligoclonality such as the Hamming distance are calculated. The area of the peaks in each histogram reflects the expression frequency of each CDR3 length within each family. A predominance of clonal expansions in the skin indicates a primary role for dermal antigens in driving disease and will enable focused investigation of pathogenic T cells. In contrast, a polyclonal repertoire of unexpanded clones will indicate recruitment by non-clonally specific chemokine receptors. Comparisons of the TCR repertoire in non-lesional skin and blood of the same individuals will indicate whether clonally expanded skin resident T cell populations might also be found in the circulation. Similarly sorted T cell subsets can be used for source RNA material for repertoire analysis. For instance **FIGS. 22 and 33** demonstrate that in C3H mice an oligoclonal population is found in the lesional skin, a repertoire that is also over-

represented in NKG2D positive but not negative CD8 lymph node populations. These data confirm that CD8+NKG2D+ T cells contain most (but not all) of the oligoclonal T cell populations found in the skin. Note that some TCR/clones are found in skin but not in draining cutaneous lymph nodes, e.g., V β 10. These likely include CD4+ T cell populations found in AA skin but not present in the sorted CD8+ LN fraction. This approach provides evidence for “antigen-drive”, and combined with flow sorting as shown here, identifies pathogenic T cell subsets amongst populations of cells.

[00373] This evidence of clonal sharing or oligoclonality will be investigated and validated in mouse and human samples at the level of clonotype resolution by preparing bacterial libraries of the PCR products using topoisomerase-based cloning, and sequencing the TCR β -chain of resulting clones in a high throughput manner using 96-well based techniques as described (5-7). Usually 48 or 96 clones are selected and sequenced for each PCR product. The sequences are exported and aligned in Geneious and the fine structure of VDJ element usage and joining is determined using Vquest. This allows identification of shared clones and lymphocyte trafficking in different regions, as well as precise enumeration of the clonotypic composition of a sample. Furthermore, clonotypic TCRs can be used for gene transduction studies to generate T cells and retrogenic mice expressing alopecic TCRs. This effort can also lead to development of “humanized” transgenic TCR models of alopecia using transgenically expressed human alopecic TCRs in HLA02 transgenic mice.

[00374] Transcriptional biomarkers of circulating immune cells

[00375] A transcriptional signature marking aggressive, drug refractory Lupus can be identified within the CD8 T cell compartment, but not when using RNA of total PBMCs (8). Thus combining flow sorting with transcriptional profiling greatly powers the analytic capacity. In alopecia areata the pathogenic cellular subset appears to be CD8+NKG2Dhi T cells that envelop the hair follicle and may be of pathogenic relevance broadly in autoimmunity (9) including both celiac disease (4, 10), Type I diabetes (11) and rheumatoid arthritis (12-14). Transcriptional profiling of these cells in celiac disease (4, 10) previously described an NK-like transcriptional programming of these cells that will be similarly assessed in sort-purified circulating and skin-derived CD8 T cells. It will be important to specifically address the transcriptional profiles of these cellular populations to identify biological pathways central to these cells.

[00376] Epigenetically regulated autoimmune genes using Illumina-based genomic approaches were identified and DNA was obtained from sorted autoimmune human T cells from patients with Type I diabetes, alopecia areata and celiac disease. Hypermethylation of the promoter regions that downregulate the expression of important immunoregulatory genes including CD3, PD-1 and FasL was identified.

[00377] Cellular Immunology

[00378] Flow cytometry analysis of activated PBMCs can be used for intracellular cytokine analysis and or signaling activation of phosphorylated proteins. Classic antigen specific, mitogenic or mixed lymphocyte responses can be appreciated with CFSE-stained T cells or with thymidine incorporation. A cell harvester and Microbeta/Trilux plate reader (for thymidine based proliferation and chromium release cytotoxicity assays), ELISA plate readers/washers, ELISPOT readers can also be used in conjunction with this analysis.

[00379] Skin immunobiology

[00380] Preparation of primary hair follicle and T cell components for functional (cytotoxicity), preparative (T cell cloning) and analytic studies (spectratyping, RNA profiling). Biopsy specimens from AA patients on study are precious, with immunostaining and transcriptional profiling studies taking the highest priority given the feasibility and informative power of these two approaches. Routinely human scalp skin is procured from control individuals (hair transplant donors) to establish primary cultures of individual cellular populations within the skin and hair follicle.

[00381] a) Culture of T cells and hair follicle components, hair follicle organ culture and skin organ culture.

i) HF targets: Human scalp skin procured from control or AA affected individuals will be used to establish primary cultures of individual cellular populations within the skin and hair follicle (**FIG. 34**) to study the regulation of NKG2DL in primary HF populations and to evaluate HF populations as targets in CTL assays. Interfollicular skin will be dispase treated to separate the epidermal and dermal components and enzymatically processed to establish primary cultures of keratinocytes and fibroblasts. The hair follicles will be microdissected to separate the mesenchymal components and further used to culture dermal sheath cells (DSC) and dermal papilla cells (DPC). Serum free hair follicle organ culture established in the

laboratory modeled after protocols from Kondo and Philpott et al (15) (**FIGS. 25A and FIG. 36**) will be used to routinely culture individual follicles from control individuals, as well as lesional (when possible) and control skin from AA patients. The follicles are cultured in serum free growth conditions and show normal anagen growth for 7-10 days, followed by catagen entry and disintegration of the matrix (16). For cytotoxicity assays, the follicles will be transferred to media which sustains cytotoxic T-Cells or NK cells (MyeloCult, Stemcell Technologies) for 4-8 hrs. The skin explants maintain normal skin architecture, as well as the immune microenvironment, and will be used for functional studies (17).

ii) T cell effectors: Isolation of sufficient numbers of T cells from enzymatically digested/dispersed human skin can be difficult; however cultured T cells weren expanded using the dermal crawl-out approach developed by Clark (2). Skin explants have been used to expand the resident T-cell population of the skin by culturing the explants on cell foam matrices for 3 weeks in IMDM containing 20% FCS, IL-2 and IL-15. This technique yields $0.3\text{-}3.0 \times 10^6$ T cells per 4mm biopsy enabling immunophenotyping, T cell cloning, Th-profiling, and other downstream applications (e.g., transcriptional profiling, cytotoxicity and other functional assays). For AA patients on intervention trials, if sufficient T cells are obtained after three weeks of expansion ($>1 \times 10^6$) they will be divided, with half the population used for RNA isolation/profiling and the other half used for flow immunophenotyping/Th profiling. If the yields are less than 1×10^6 total cells, studies will be limited to RNA profiling. For AA patients that are not on study, there will be more latitude for using crawl-out T cells and autologous HF targets for alternative functional studies, including for instance cytolytic assays and studies with antibody blockade/small molecules. As an example of the ability to analyse rare clinical populations of T cells, **FIG. 35** illustrates a T cell ‘crawl-out’ assay, in which cultured human dermis obtained from punch skin biopsies are used as source material for flow cytometric analysis.

[00382] Note that T cells obtained from AA skin biopsies are oligoclonal IFN- γ producing CD8+NKG2D+ T cells in striking contrast to the expected polyclonal CD4 population seen in crawl-outs from normal skin (2, 18).

[00383] b.) Interaction of the skin and the immune components in Alopecia Areata models

[00384] Established protocols are available for assessing skin-immune interactions for the studies herein. Primary cultured cells, as well as organ-cultured follicles, can be used as

targets using cytotoxic T cells from control or patient peripheral blood lymphocytes, or those derived from the skin (**FIG. 25A and FIG. 36**). The immune effector cells can be co-cultured with CFSE-labeled target cells/hair follicles and the cytotoxicity will be measured colorimetrically by LDH release, or alternatively, the dead cells will be stained with 7-AAD and the cell numbers counted by flow cytometry (see **FIG. 25A and FIGS. 35-36**). These assays can be used to determine molecular requirements for the interaction of immune effectors and HF targets, including cytotoxicity. For instance as shown here, cytotoxicity required NKG2D engagement and prior sensitization of targets with cytokines/TLR ligands known to upregulate NKG2DL transcription and surface expression.

[00385] Evaluation of skin biomarkers of pathogenesis: Immunostaining and transcriptional profiling

[00386] AA shows high correlation with several autoimmune disorders, making the hair follicle a highly accessible organ in which to study basic mechanisms of autoimmunity. There has been substantial interest in identifying early surrogate biological markers of pathogenesis. As such, AA represents a model system for biomarker development which may have relevance to a broad range of diseases. The following biomarkers are currently used to monitor AA development and response to treatment both in humans and in mice.

[00387] a) Immunohistochemical Staining for lymphocytic Infiltration. AA is associated with the presence of intrafollicular, and perifollicular immune infiltrate. For immunohistochemistry, tissue will be either fixed in 10% formalin in PBS for 8 hours at room temperature and stored in 70% ethanol for paraffin sections or embedded directly in Cryomatrix (Shandon, Waltham, MA) on dry ice for frozen sections which are stored at -80°C.

[00388] Paraffin-embedded tissue blocks are cut into 8- μ m sections, and hematoxylin and eosin (H&E) staining is carried out for histological studies. Frozen tissue is also sectioned to a thickness of 8 μ m and fixed in 4% paraformaldehyde for immunofluorescence staining. Sections are stained with fluorescence-labeled secondary antibodies and immunofluorescence imaging carried out using Zeiss Axioskop microscope. Basic immunohistological/fluorescent staining will include staining with primary antibodies to CD3, CD4, CD8 as well as IL-15 and NKG2DL family members. As an example of the immunostains, evidence that MHC I

and ICAM-1 are massively upregulated in alopecic HFs which are associated with a dense CD8 dominated infiltrate is provided (FIG. 37).

[00389] Upregulation of IL-15/IL-15R α has been identified in the HF as a second tissue specific immunostimulatory event with probable pathogenic relevance to driving Nk-type CD8 effector differentiation and AA development in both the human and mouse (FIG. 39).

[00390] b) Isolation of total skin RNA and Quantitative PCR for inflammatory markers.

The human and mouse skin will be tested for elevation of inflammatory gene transcripts already defined, including IFN-response genes (FIG. 38), NKG2DL, and gene signatures to be defined, including IL-15 and "NK-type" CD8 T cell transcripts. Total RNA will be extracted from skin using an RNeasy purification kit (Qiagen). DNase-treated total RNA will be reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA).

[00391] RT-PCR shall be performed using SYBR Green Master Mix and an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). GAPDH and β -actin will be used as internal normalization control genes.

[00392] One of the goals of the studies herein is to establish the phenotype of circulating AA effector populations by matching spectratypes of sorted peripheral blood AA T cell subsets with those found in total skin T cells from the same patient. Flow cytometric sorting techniques will be combined with spectratype analysis to identify pathogenic T cells in the circulation of AA patients. This same approach was used in the mouse model to show that CD8+NKG2D+ lymph node cells contain the majority of the T cell clones found in alopecic skin. In AA subjects, the TCR repertoire will be compared in lesional and non-lesional skin and blood of the same individuals to identify whether clonally expanded skin resident T cell populations are also found in the circulation. By progressive refinement of the sort criteria to isolate source RNA material from circulating T cell subsets one should be able to (at least partially) match the TCR repertoire found in total skin from the same patient and in this way identify the immunophenotypic markers of pathogenic CD4 and CD8 T cells. Identification of pathogenic T cell subsets will be enormously valuable for monitoring clinical studies, refining biomarker development and would provide unique cellular materials for bedside-to-bench studies of pathogenesis.

[00393] Another goal of the studies herein is to establish the Th profile of AA T cells infiltrating the dermis and circulating in the peripheral blood. Pathogenic AA T cell Effector

Differentiation/Cytokine Profile: Therapeutic targeting of specific Th-pathways has succeeded in other human skin inflammatory diseases, most notably in psoriasis a prototypical Th17 disease. There is substantial data in the AA animal model for AA as a predominant Th1 disease (19-23). To establish Th1-predominance in humans and develop the rationale and biomarker platform for Th1-targeted therapies, the Th-profile of infiltrating dermal AA T cells and relevant circulating T cell subsets in the blood will be addressed using a multi-prong approach including multianalyte, RNA profiling, immunostaining and intracellular flow cytometric analysis from the skin and blood. Refining the immunophenotypic markers for AA CD4 and CD8 T cells will of course allow detection of Th-profiles of the potential alopecic-specific T cells amongst the polyclonal circulating population.

[00394] Although transcriptional profiling of AA lesions from a small series of subjects are indicative of Th1-type skewing (24), without being bound by theory, AA is not “one disease”. The common occurrence in AA subjects of co-morbid immune conditions that reflect both an underlying Th2 or Th1/17 bias that predisposes to diseases with common pathogenic pathways, including hypersensitivity (25) (dermatitis) and other autoimmune conditions (26-33) (see studies described herein) suggests that there may be a Th2-type AA as well as a Th1 or Th17 type of AA. Thus it is important to identify functional T cell responses in PBMCs from the periphery and from AA lesional tissue explants from a large number of patients that might identify subsets of AA with different Th profiles and to correlate these immune function biomarker parameters with the presence or absence of the relevant SNPs.

[00395] The T cell immunophenotype of peripheral T cells and lesional T cells will be assessed. 50 AA patients and 50 normal controls will be studied from the GWAS, with attached clinical history. Assessment of Th-profiles of total circulating T cells is straight forward but multi-parametric flow will be applied to T cell subsets for more granular assessment, as aided ultimately by definition of immunophenotypic markers of AA pathogenic populations. Isolation of sufficient numbers of T cells from enzymatically digested/dispersed human skin can be difficult, and maybe altered by culture conditions. Correlation will be performed with transcriptional profiling using whole skin approaches and microdissected skin samples to describe the cytokine profile within the follicular sheath infiltrate as well as from infiltrates in the interfollicular epithelium.

[00396] Human Subjects

[00397] Skin biopsies from the scalp will be collected from normal subjects consist of AA patients and unaffected control subjects receiving hair transplantation. Additional biopsies will be obtained from subjects on clinical trials.

[00398] The recruited AA subjects will sign informed consent and will have the conditions of their participation explained. The sources of research material will be scalp skin biopsies from AA patients and controls. RNA from small tissue samples, including microdissected hair follicle compartments are routinely obtained.

[00399] Tissue biopsies from AA patients and controls will be provided.

[00400] Vertebrate Animals

[00401] Young C3H/HeJ mice (2-3 months) and retired breeders showing visible hair loss will be acquired from Jackson Labs. The mice strains will be housed in their respective animal housing suites. Mice will be given free access to water and pellet diet (5010 rodent diet, LabDiet, PMI Nutrition International)

[00402] Primary cell culture for individual skin and follicle components as well as hair/skin organ cultures will be established for wild-type and transgenic or knock-out mice. These studies also involve hair follicle and skin tissue characterization. The mice will be euthanised by CO₂ asphyxiation followed by cervical dislocation, a veterinary-approved and widely recommended method. It is the quickest and most humane method that is not associated with any pain or stress for the animals. Mice will be shaved on the dorsal surface with electric clipper and then skin tissue will be dissected out. The isolation scheme of individual cellular components of the skin and hair follicle as well the T-cells isolation from skin explants is described in **FIGS. 31, 34-35**. Skin Samples will also be embedded in OCT and paraffin. In addition to skin, spleen and thymus will also be dissected from the animals and further used for flow cytometry. Serum from the animals will also be collected by tail bleeding and used for cytokine profiling.

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EXAMPLE 6

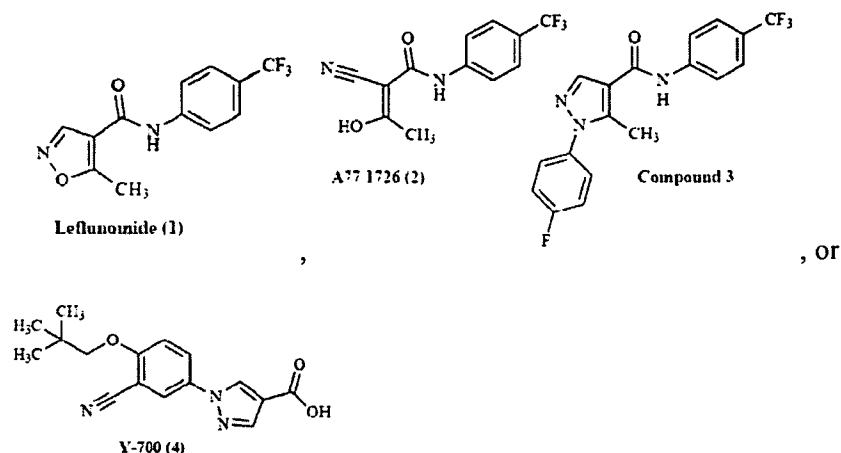
[00404] Immunostaining of both IL-15 and IL-15RA was observed in the hair follicle outer root sheath in both human and mouse active AA lesions. Likewise, IL-15B staining on the was shown on infiltrating CD8+T cells surrounding affected hair follicles.

[00405] In vivo pre-clinical studies of blocking IL-15 was performed using an antibody against CD122 (IL-15beta) in the grafted animal model of the disease, C3H-HeJ mice. 0/5 mice developed alopecia after treatment with IL-15 blocking antibody, whereas 4/5 placebo treated mice developed AA.

[00406] Normalization of an inflammatory gene expression signature by microarray analysis, and elimination of CD8+NKG2D+/NKG2A/C/E+ cells from the blood of treated animals was also observed.

What is claimed:

1. A method of treating a hair-loss disorder in a mammalian subject in need thereof, the method comprising administering to the subject an IL-15 inhibitor.
2. A method for inducing hair growth in a subject, the method comprising:
 - (a) administering to the subject an effective amount of a IL-15 inhibitor, a NKG2D inhibitor, or a combination thereof, thereby controlling hair growth in the subject.
3. A method of treating a hair-loss disorder in a mammalian subject in need thereof, the method comprising administering to the subject a NKG2D inhibitor, an IL-15 inhibitor, or a combination thereof.
4. The method of claim 1, 2 or 3, wherein the inhibitor comprises an antibody that specifically binds to a protein comprising SEQ ID NO: 1, 3, 5 or 7.
5. The method of claim 2, wherein the subject is afflicted with a hair-loss disorder.
6. The method of claim 1, 3, or 5, wherein the hair-loss disorder comprises androgenetic alopecia, telogen effluvium, alopecia areata, telogen effluvium, tinea capitis, alopecia totalis, hypotrichosis, hereditary hypotrichosis simplex, or alopecia universalis.
7. The method of claims 1, 2, or 3, further comprising the step (b) determining whether the inhibitor administered induced hair growth in the subject afflicted with a hair loss disorder as compared to the subject's hair growth prior to treatment with the inhibitor.
8. The method of claims 1, 2, or 3, wherein the inhibitor is an antisense RNA that specifically inhibits expression of the gene that encodes the NKG2D or IL-15 protein; a siRNA that specifically targets the gene that encodes the NKG2D or IL-15 protein; or a phenylpyazoleanilide small molecule.
9. The method of claim 8, wherein the phenylpyazoleanilide small molecule is



10. The method of claim 8, wherein the siRNA is directed to a human nucleic acid sequence comprising SEQ ID NOS: 2, 4, 6 or 8.
11. The method of claim 8, wherein the siRNA directed to a NKG2D gene is any one of the sequences listed in Table 13.
12. The method of claim 8, wherein the siRNA directed to a IL-15 gene is any one of the sequences listed in Table 11.
13. The method of claim 8, wherein the siRNA directed to a IL-15RA gene is any one of the sequences listed in Table 12.

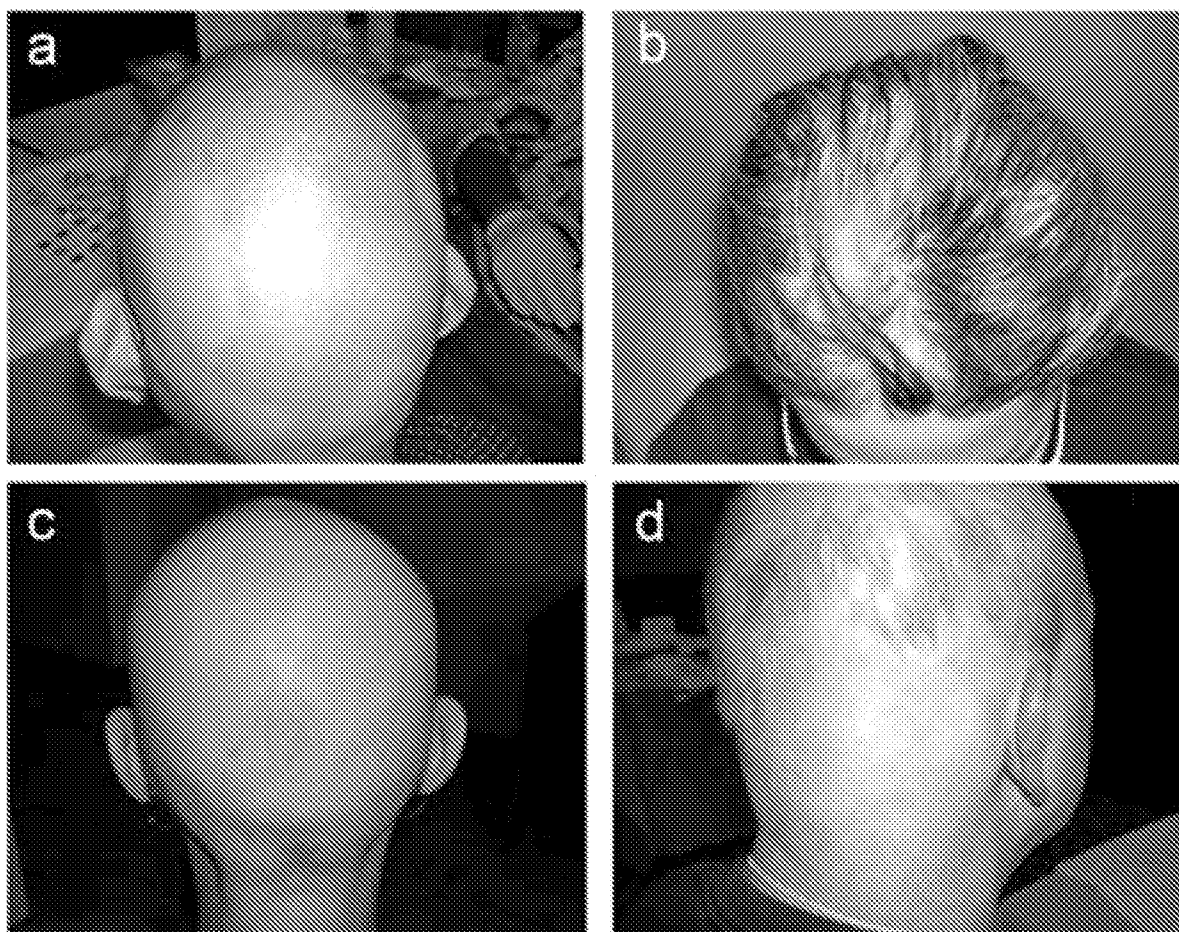


FIG. 1

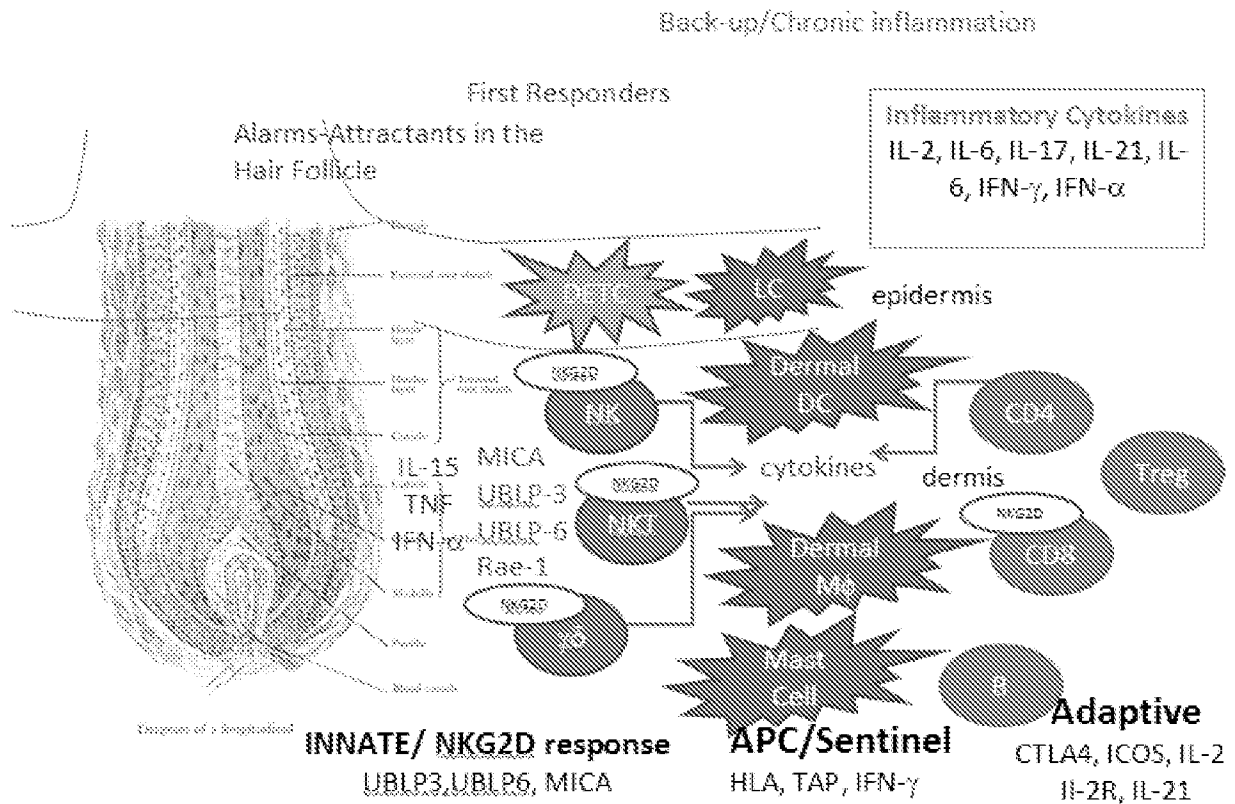


FIG. 2

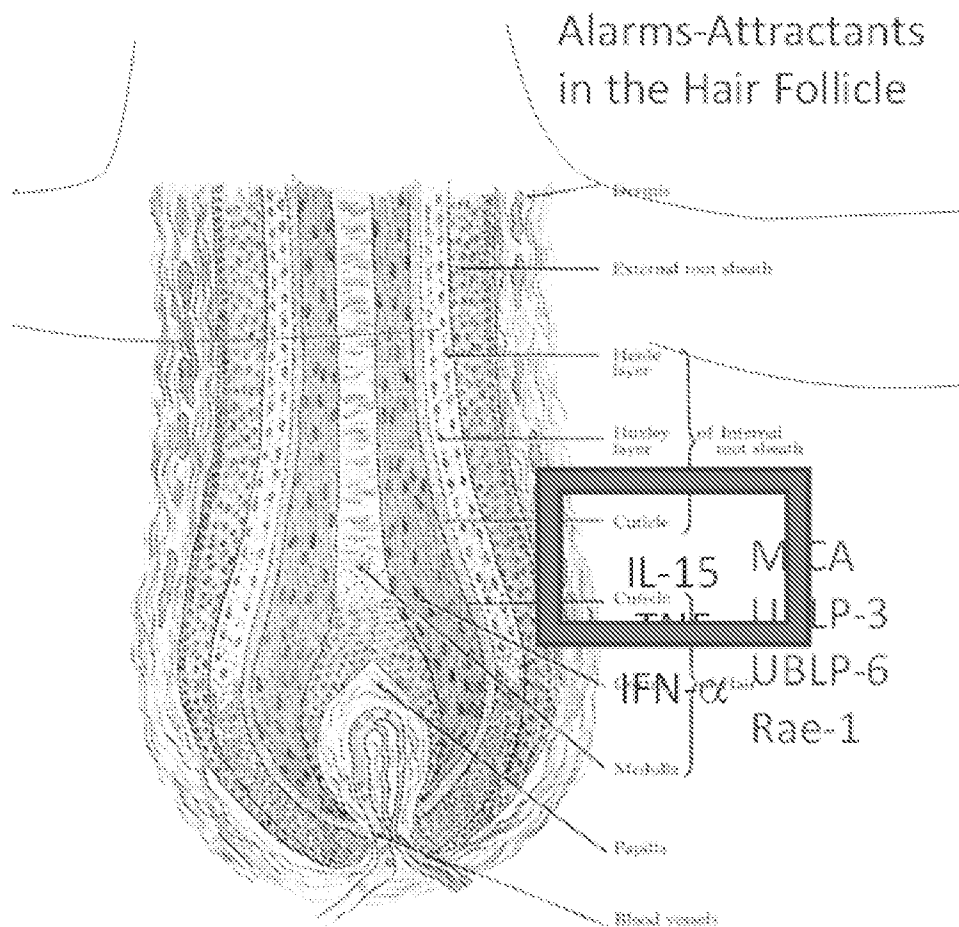


FIG. 3A

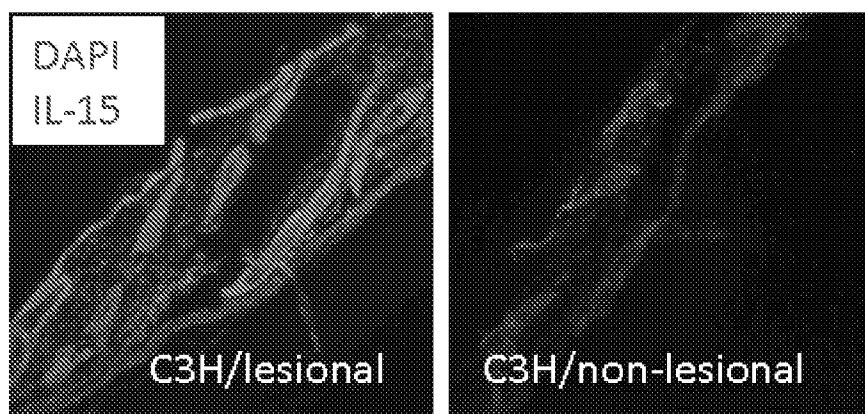


FIG. 3B

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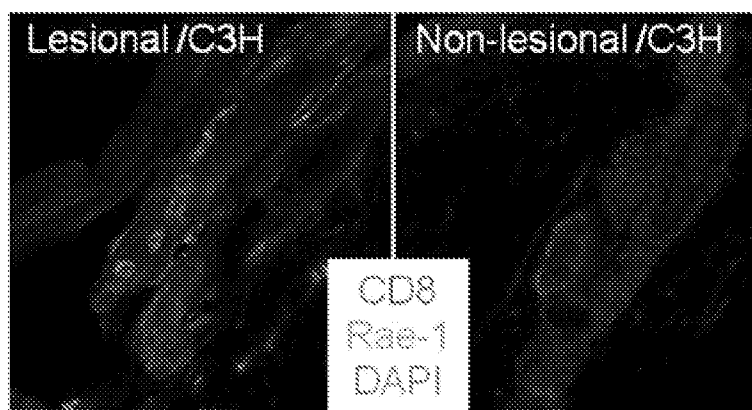


FIG. 3C

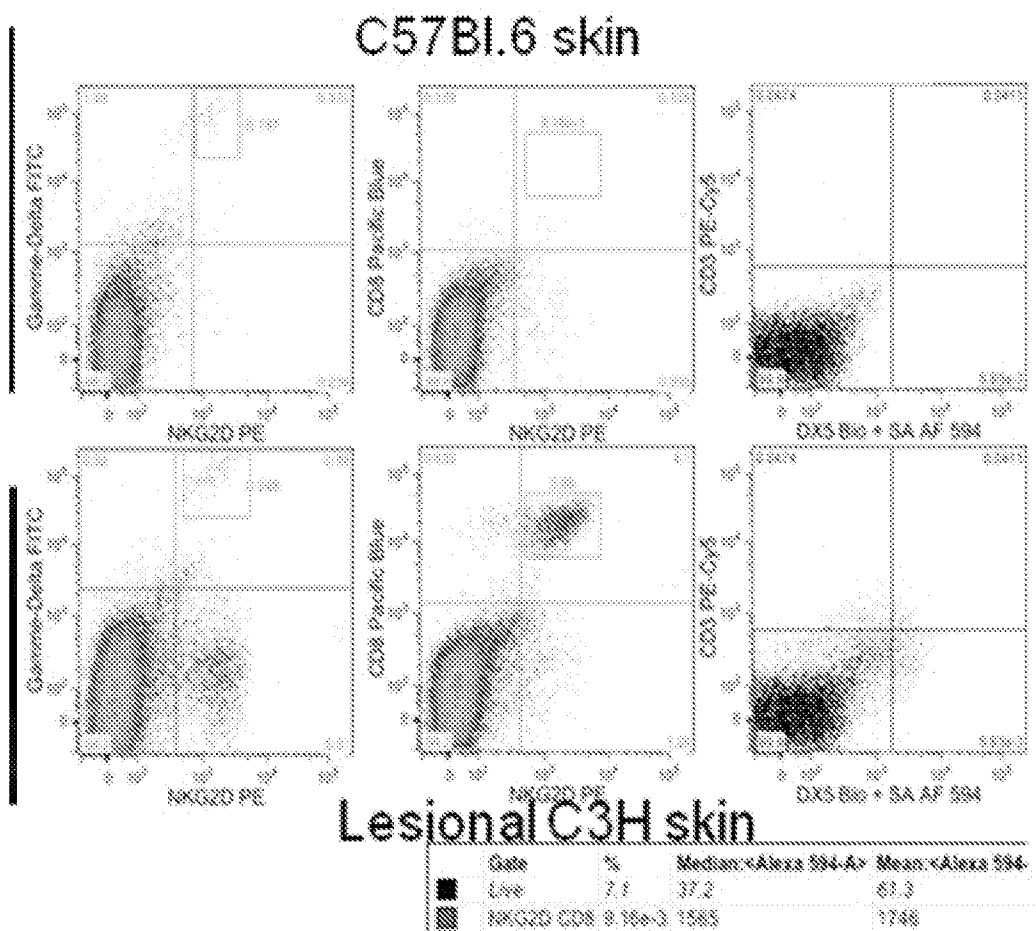


FIG. 4A

Lesional C3H skin

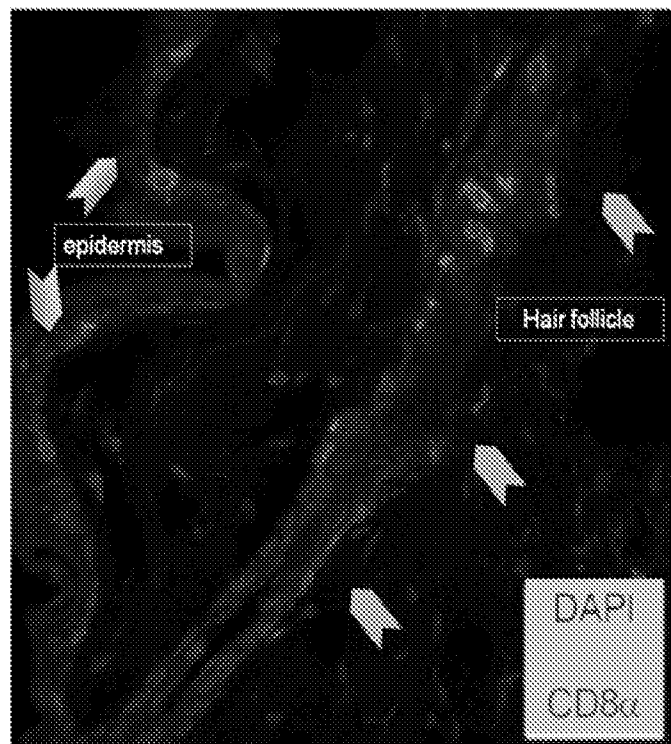


FIG. 4B



FIG. 5A

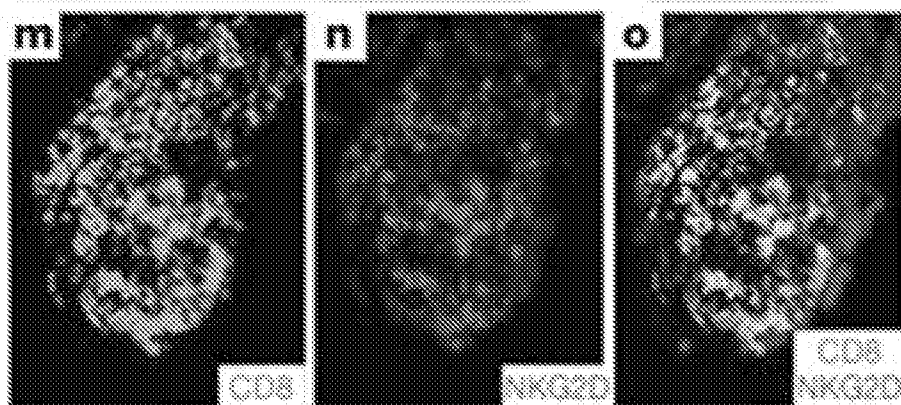


FIG. 5B

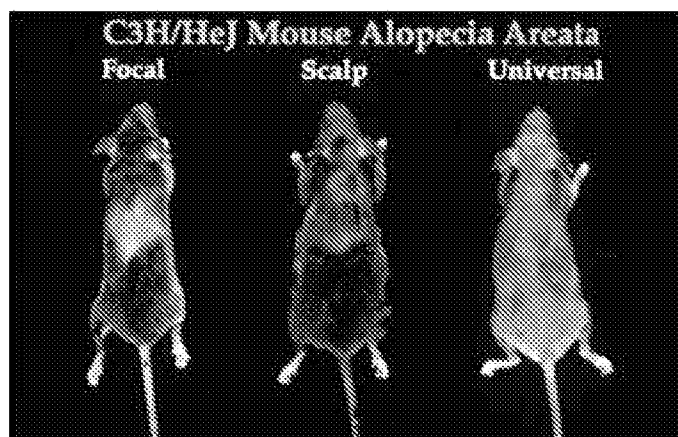
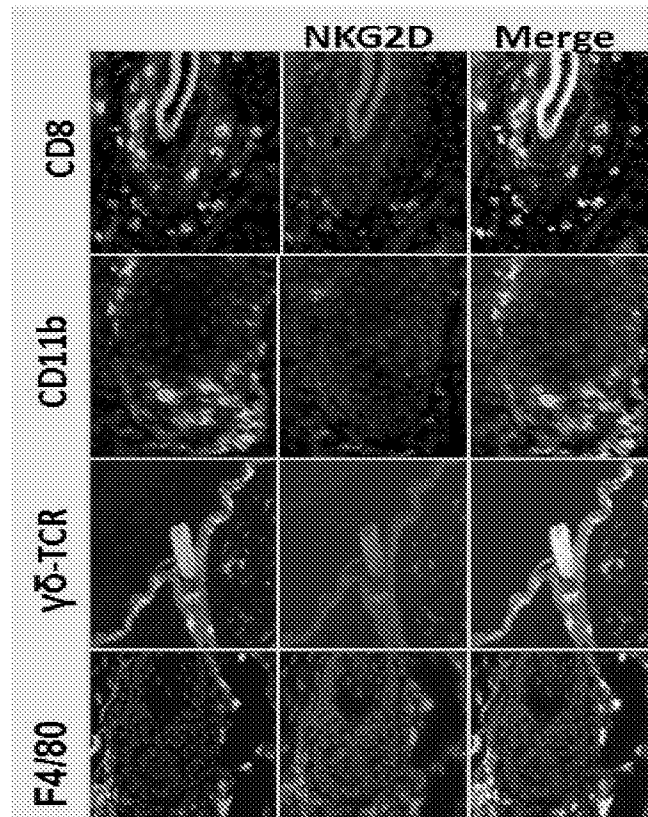
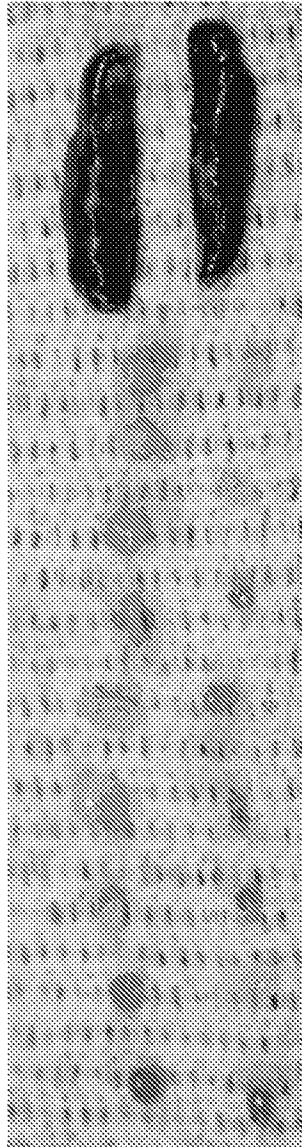


FIG. 5C

**FIG. 5D**



Alopecic
C3H
Unaffected
C3H

Figure 7A

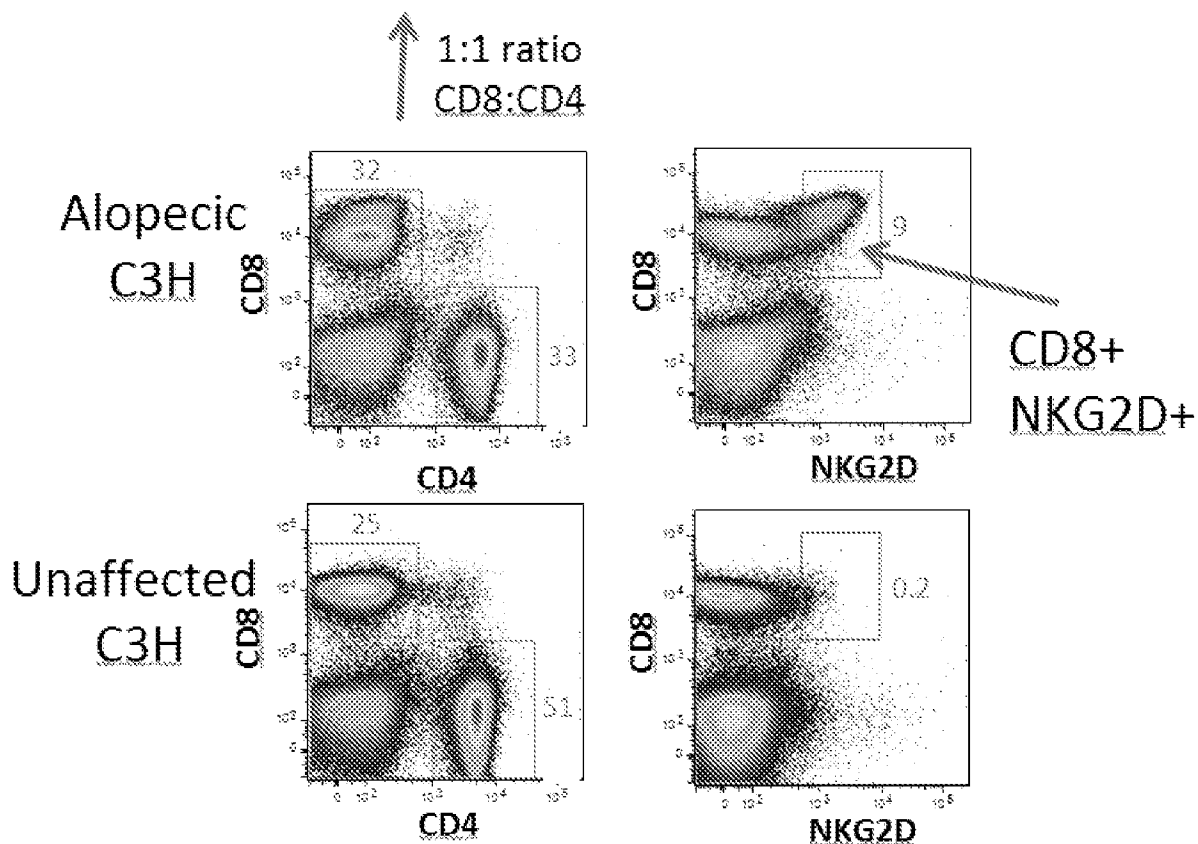


FIG. 7B

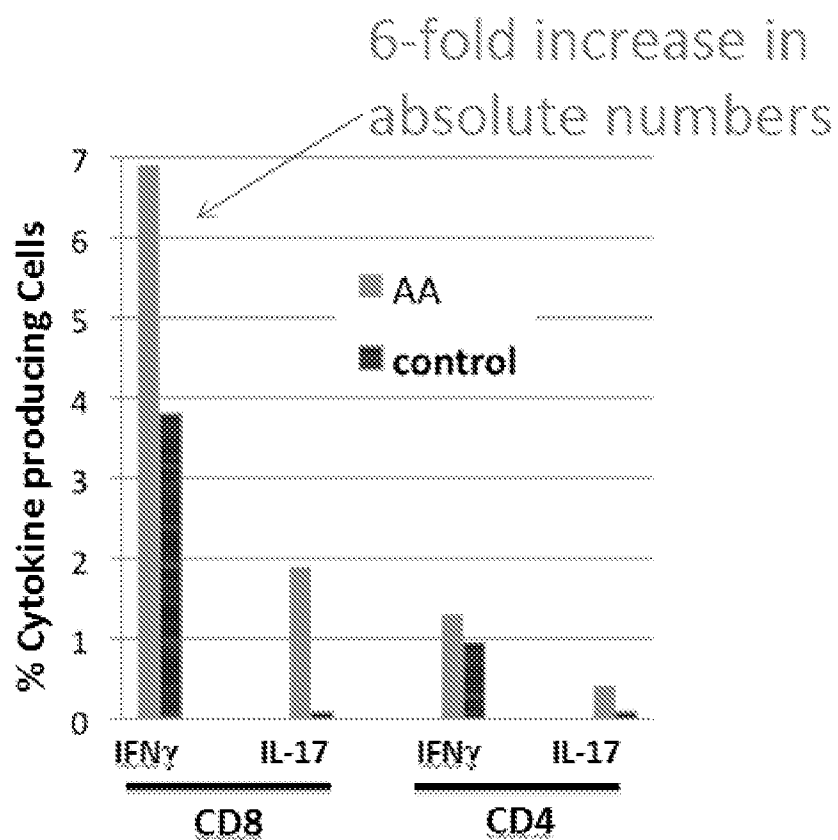


FIG. 7C

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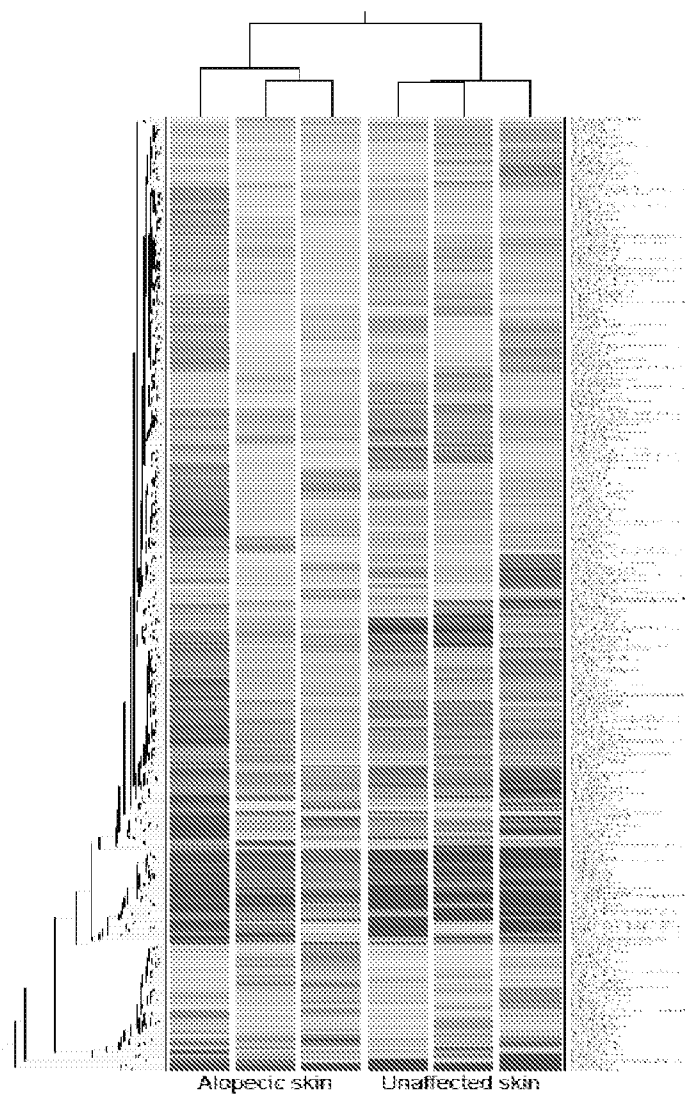


FIG. 8A

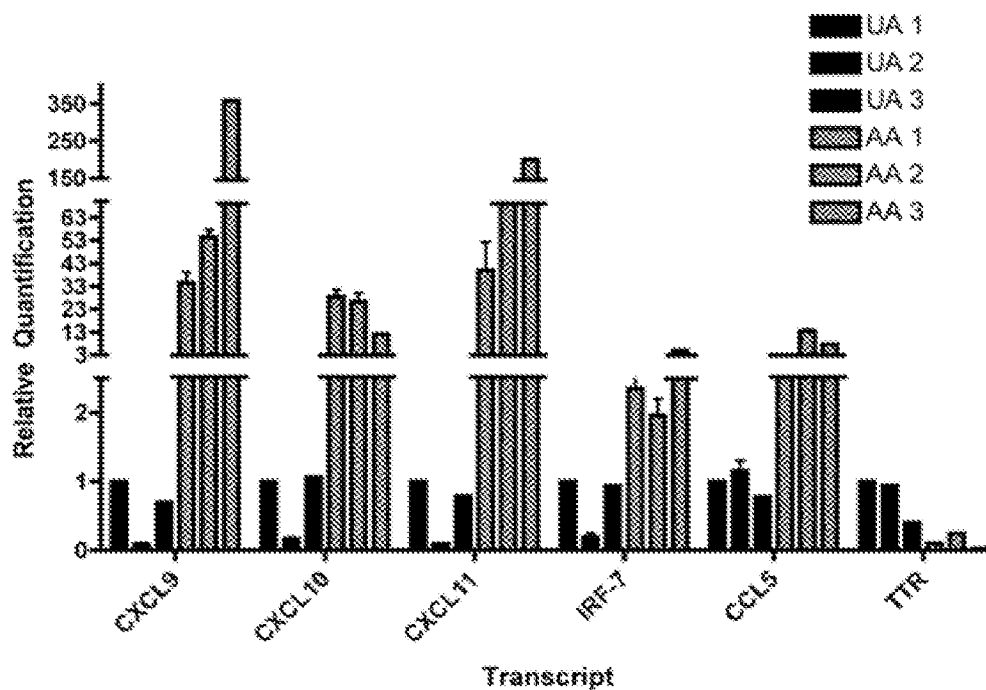


FIG. 8B

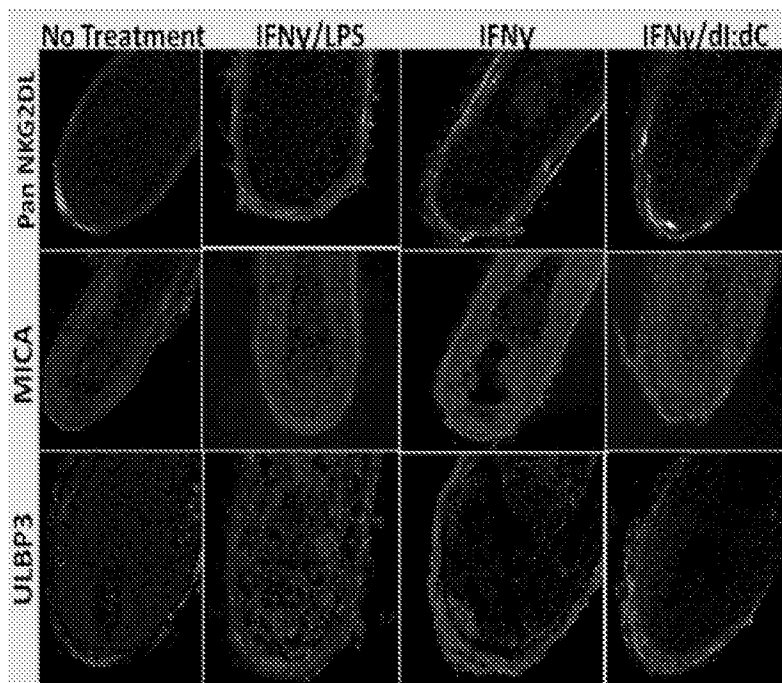


FIG. 9A

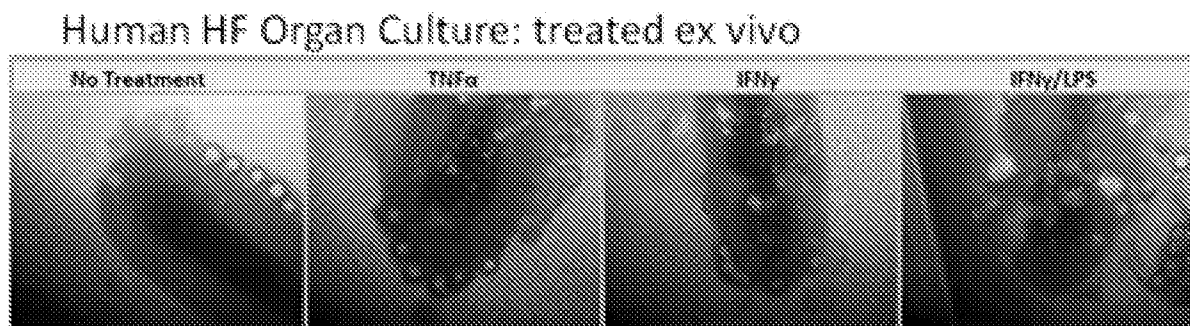


FIG. 9B

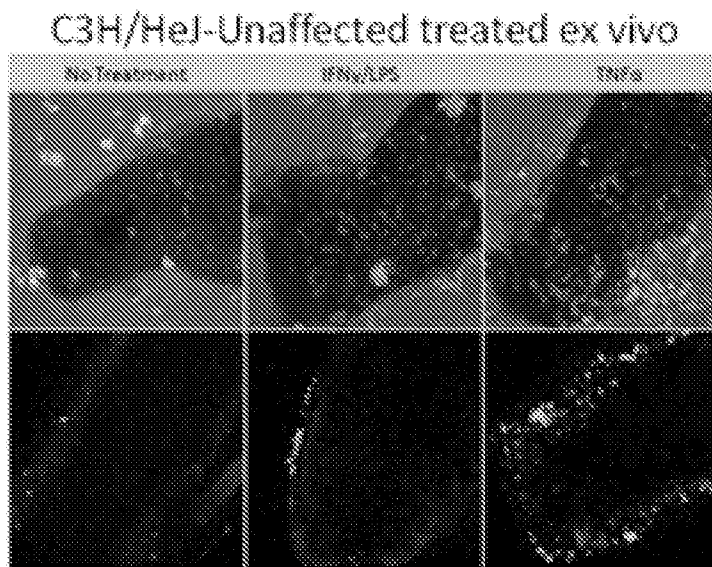


FIG. 9C

C3H/HeJ
Affected vs. Unaffected

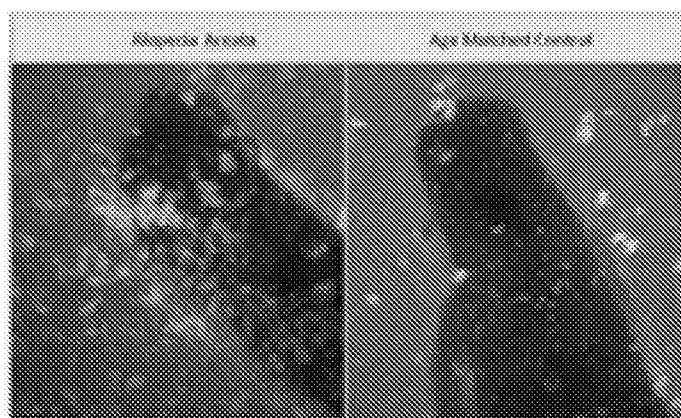


FIG. 9D

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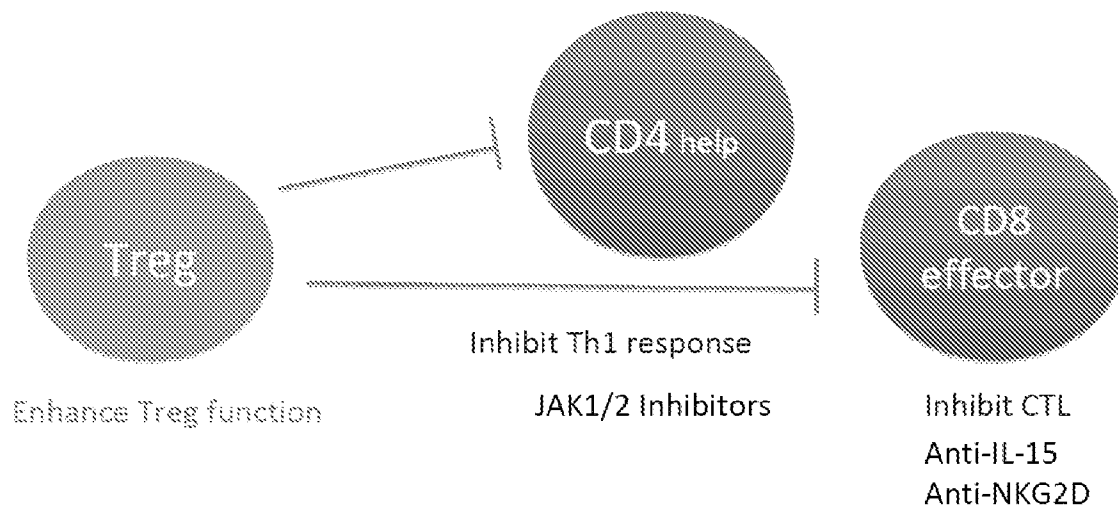


FIG. 10

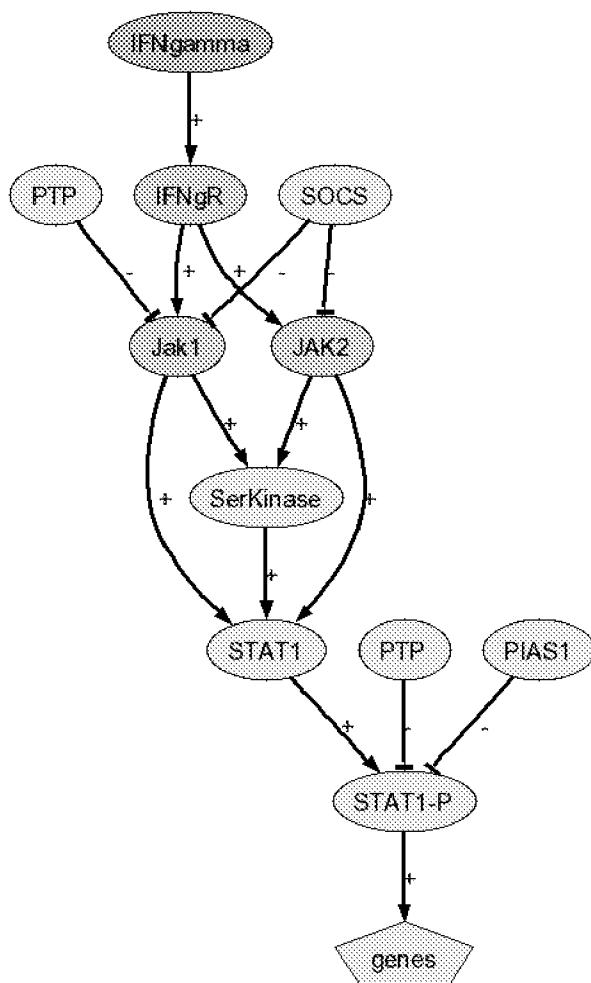


FIG. 11

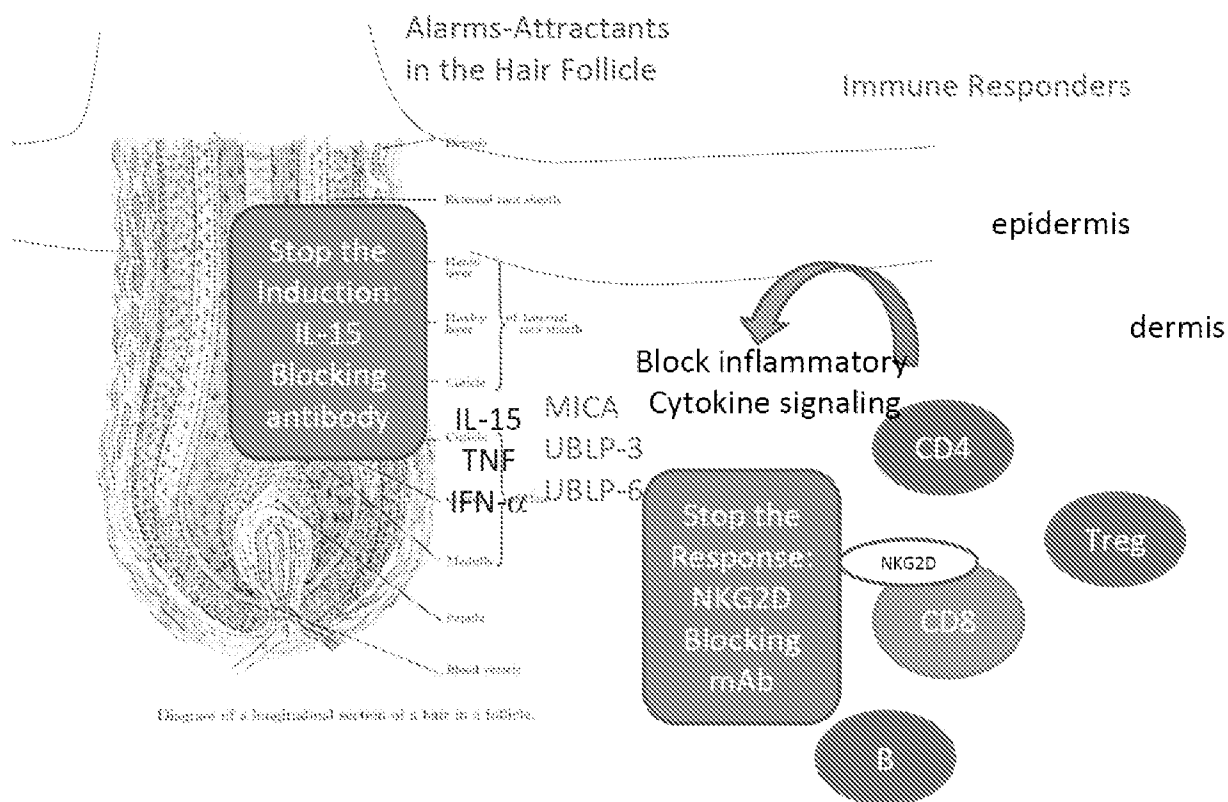


FIG. 12

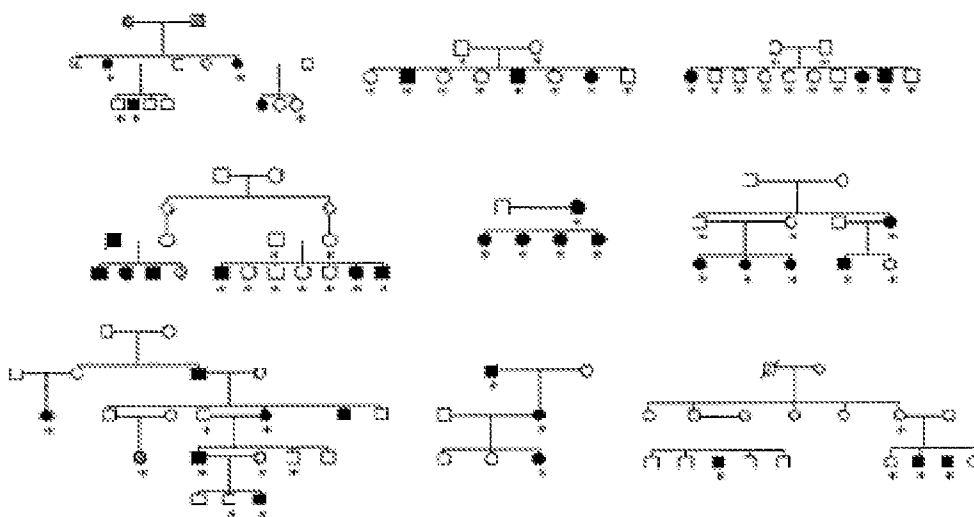


FIG. 13A

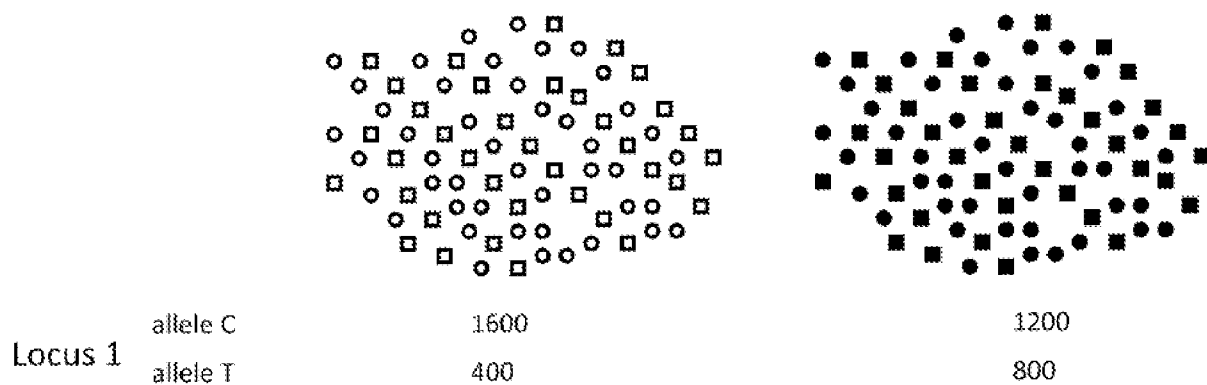


FIG. 13B

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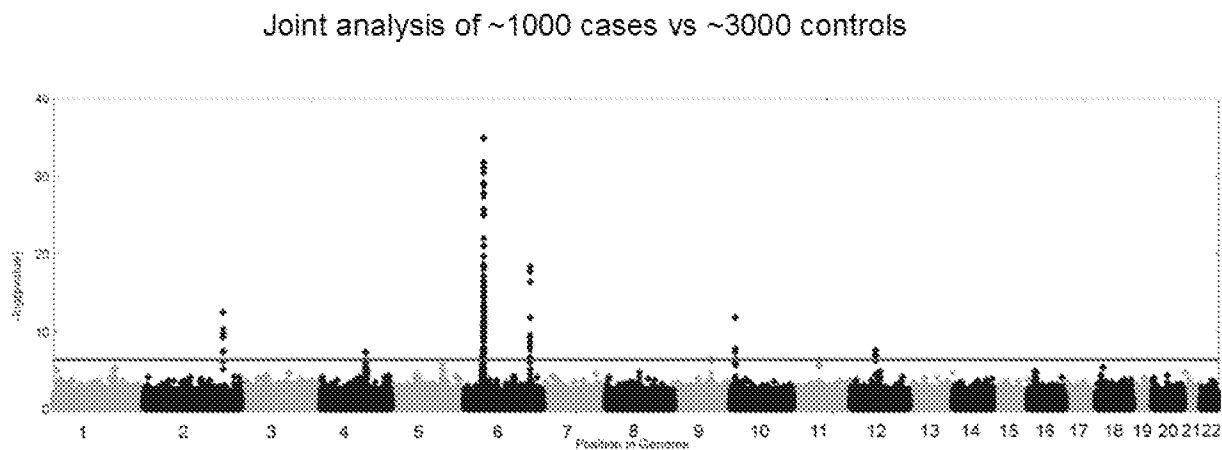
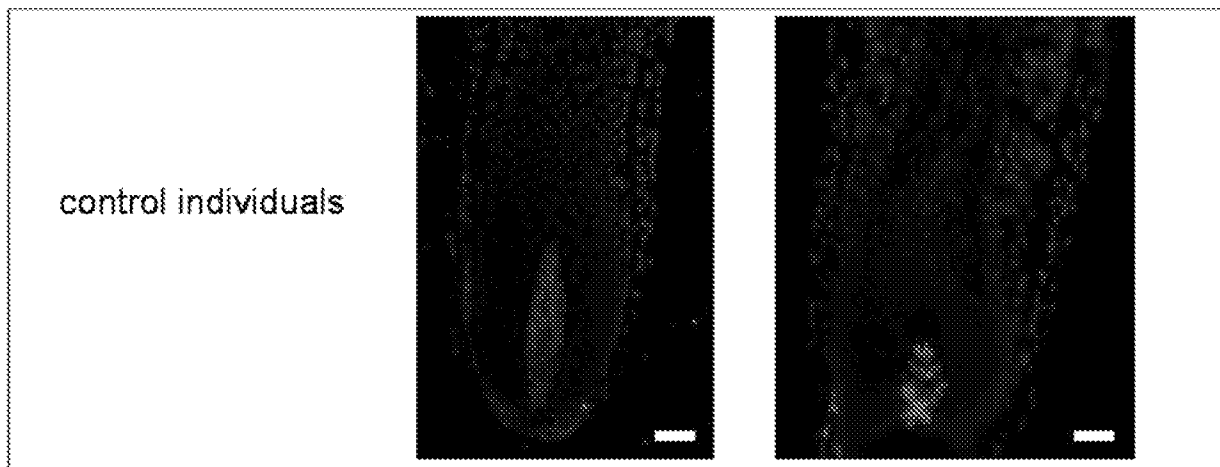
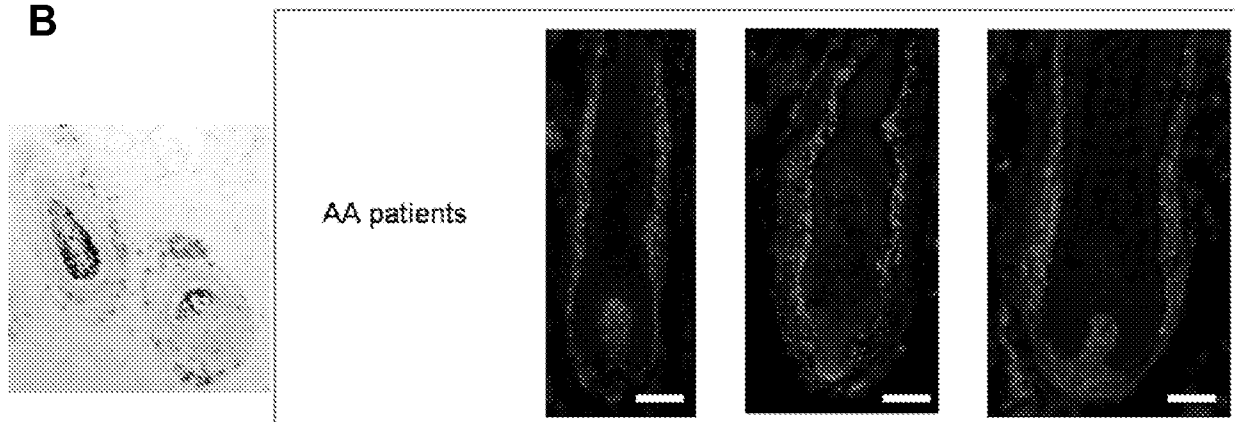


FIG. 14

A



B



FIGS. 15A-B

AA
“Pre-2010”
Swarm of Bees

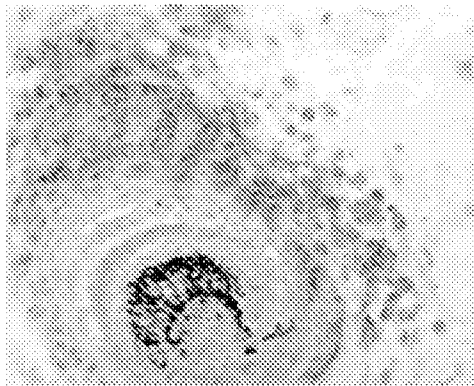


FIG. 16A

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AA

“2010”: Bees are identified

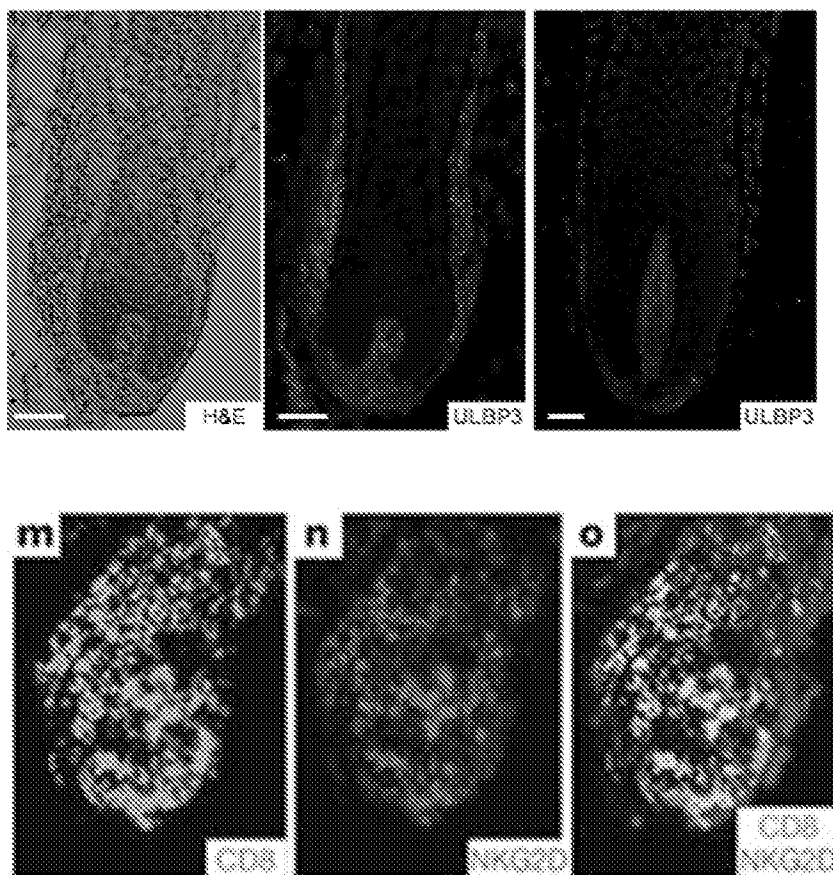


FIG. 16B

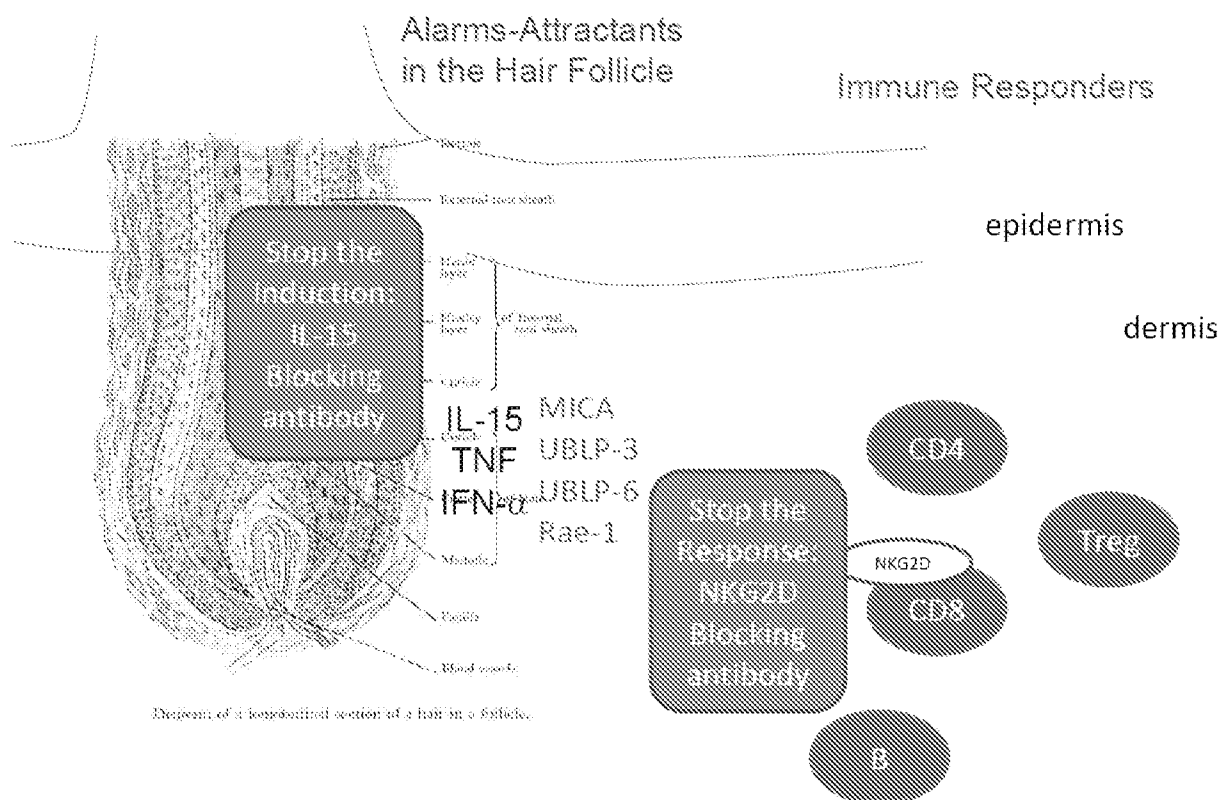


FIG. 17

Targeted Therapies in Alopecia Areata

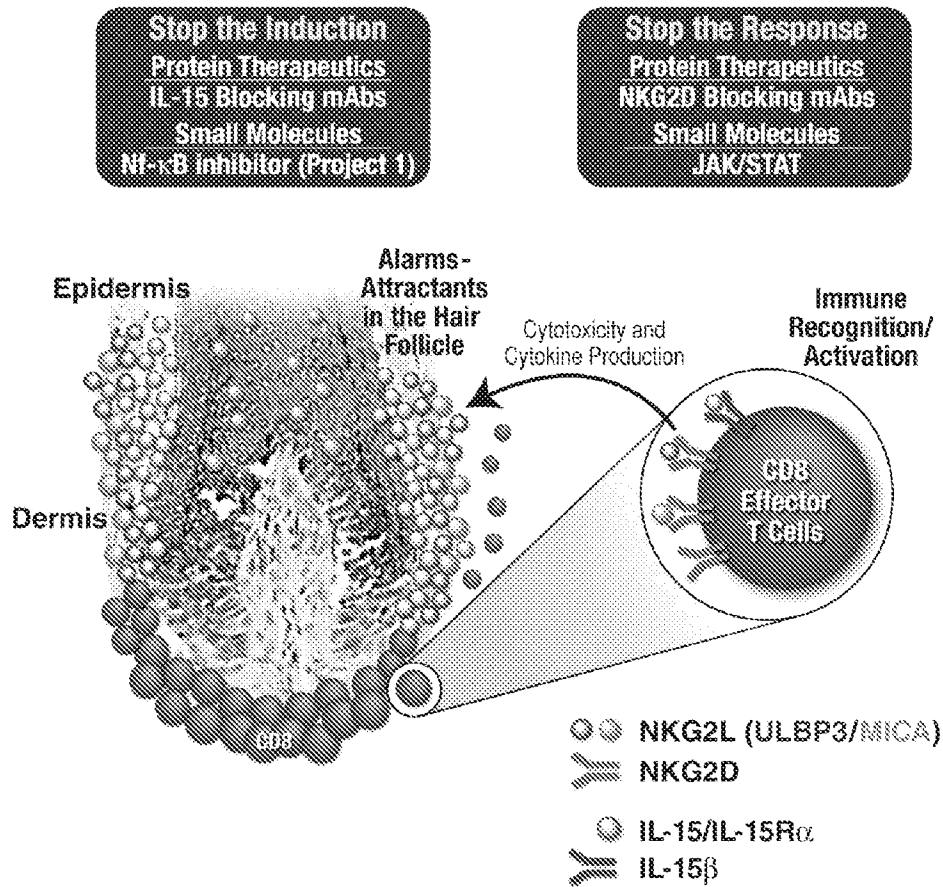


FIG. 18

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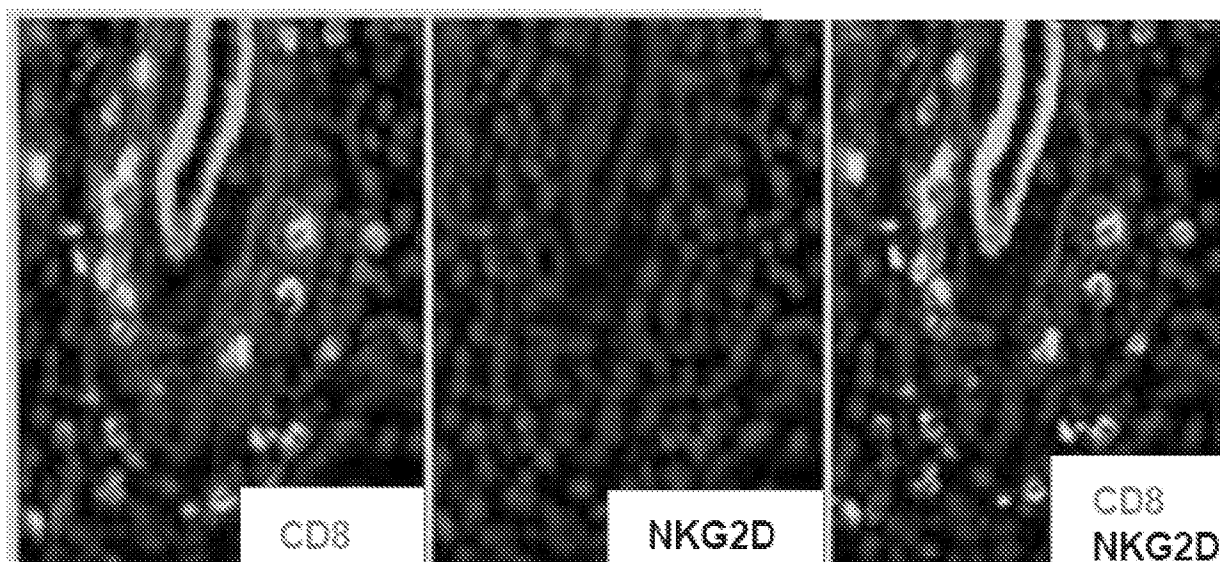


FIG. 19

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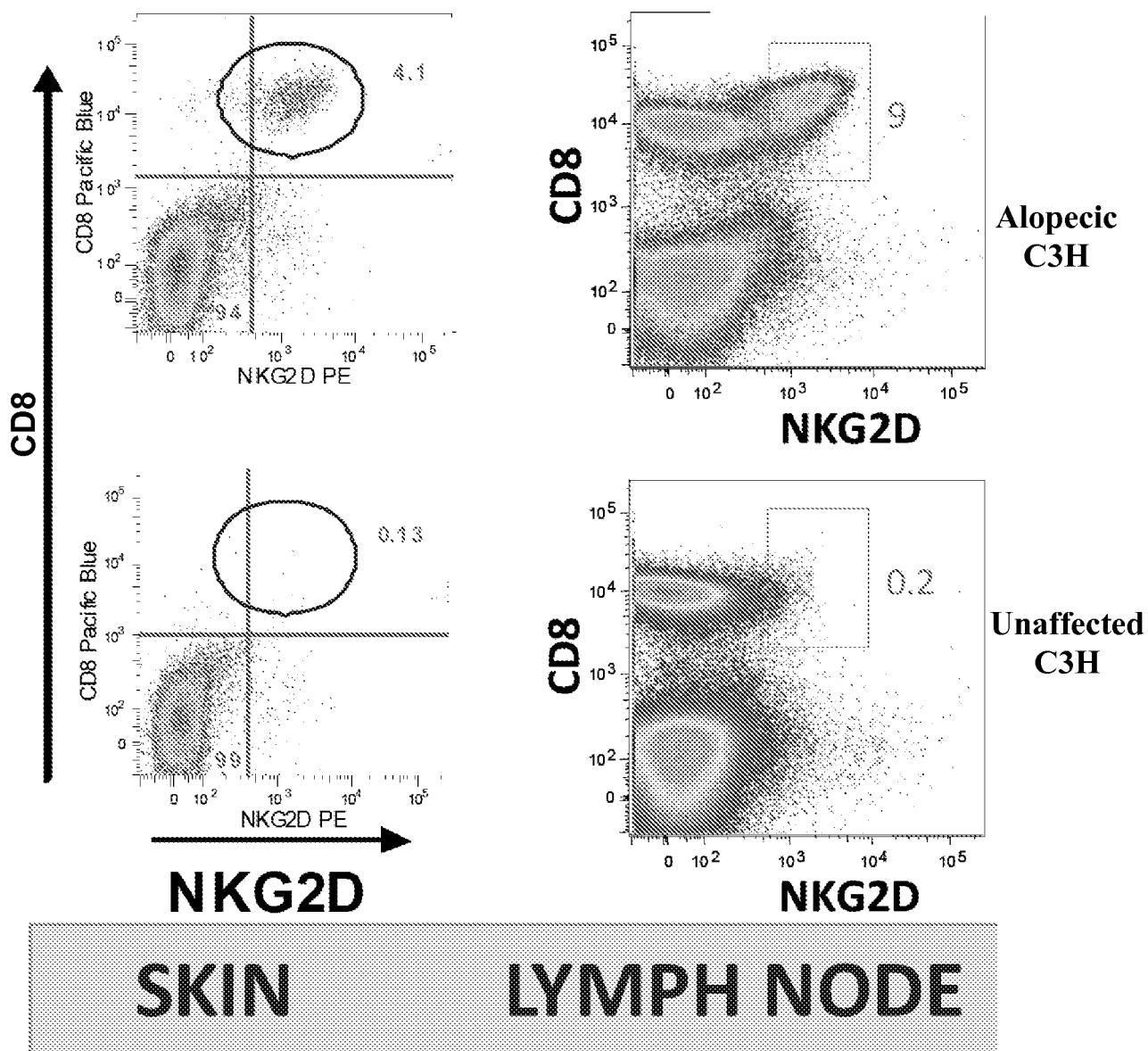


FIG. 20

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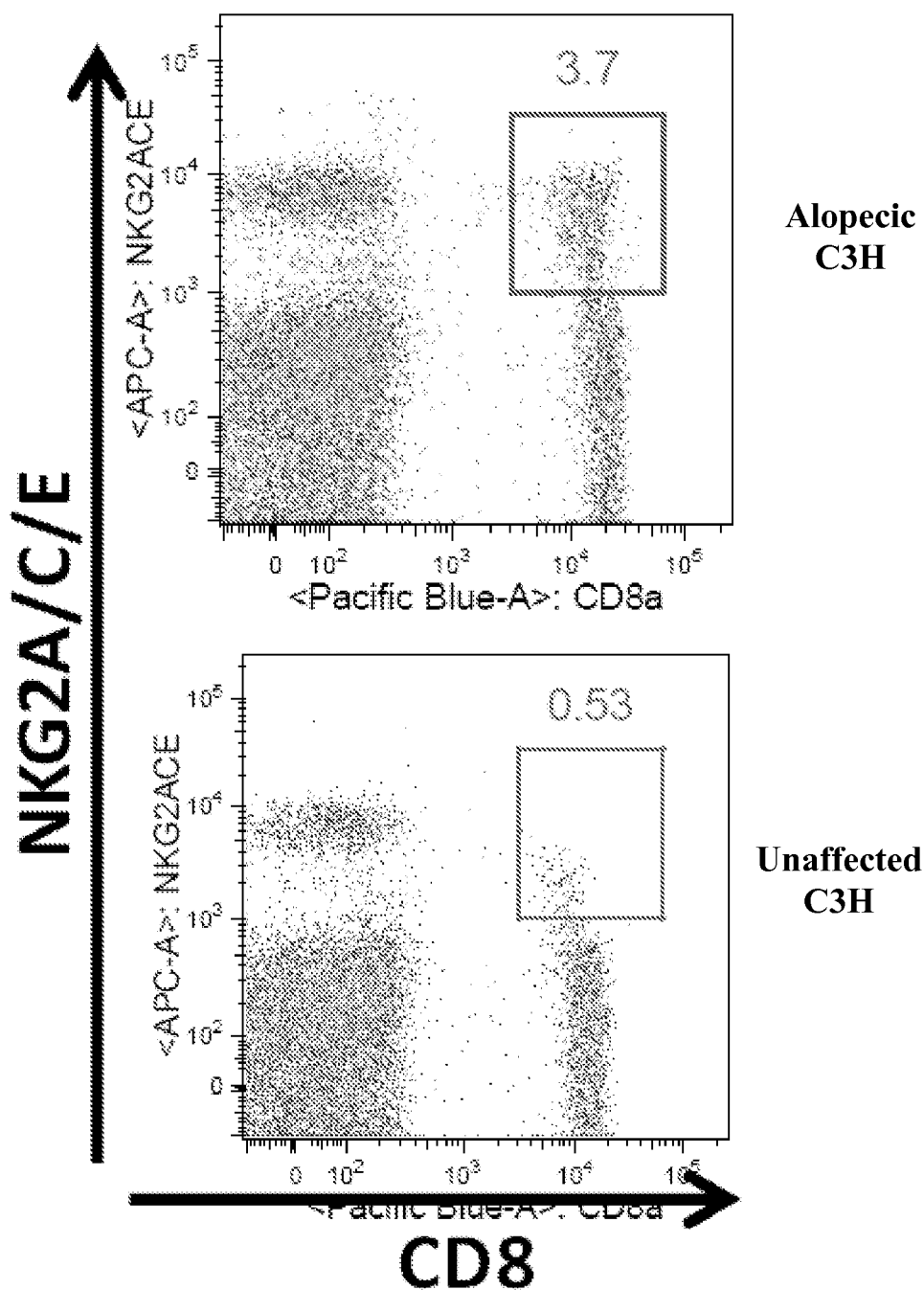


FIG. 20 – Cont.

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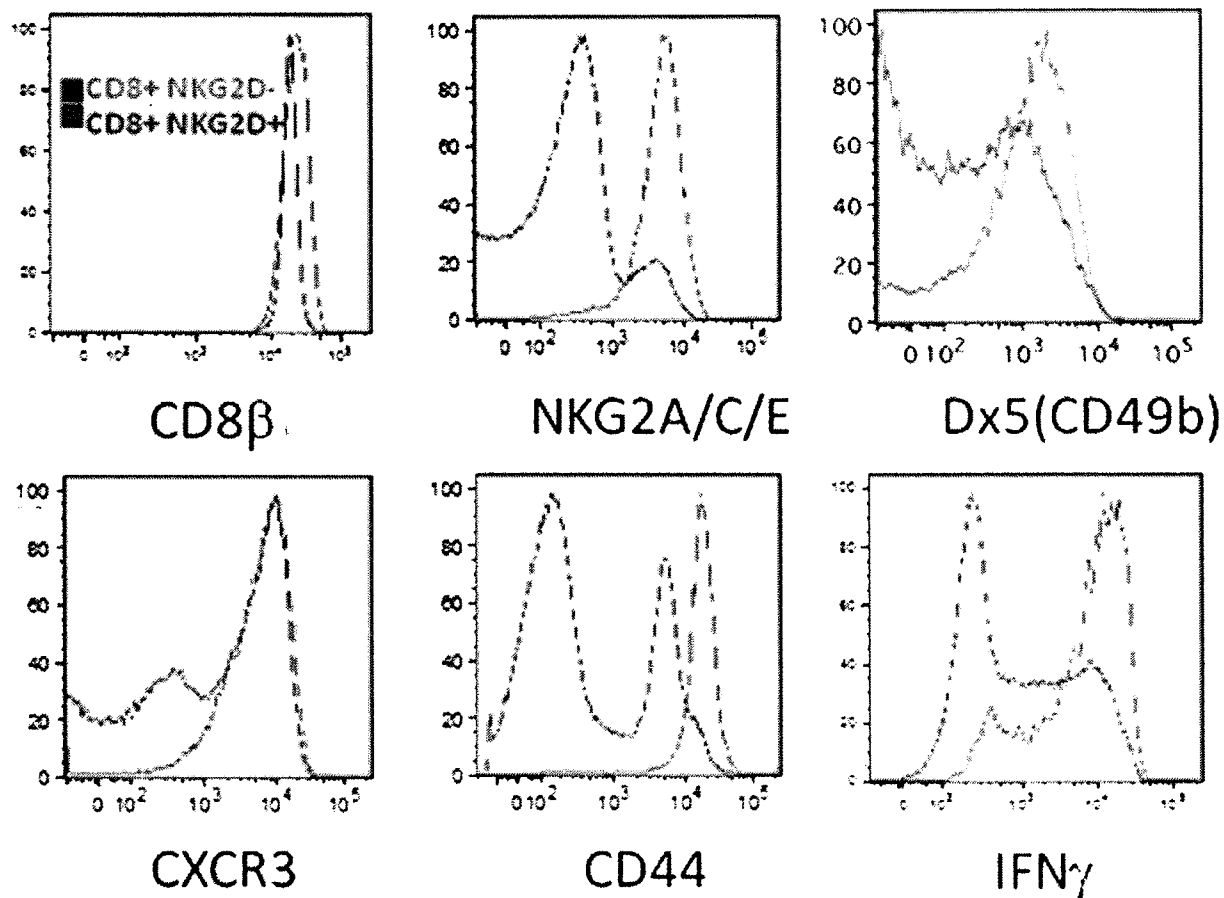


FIG. 21

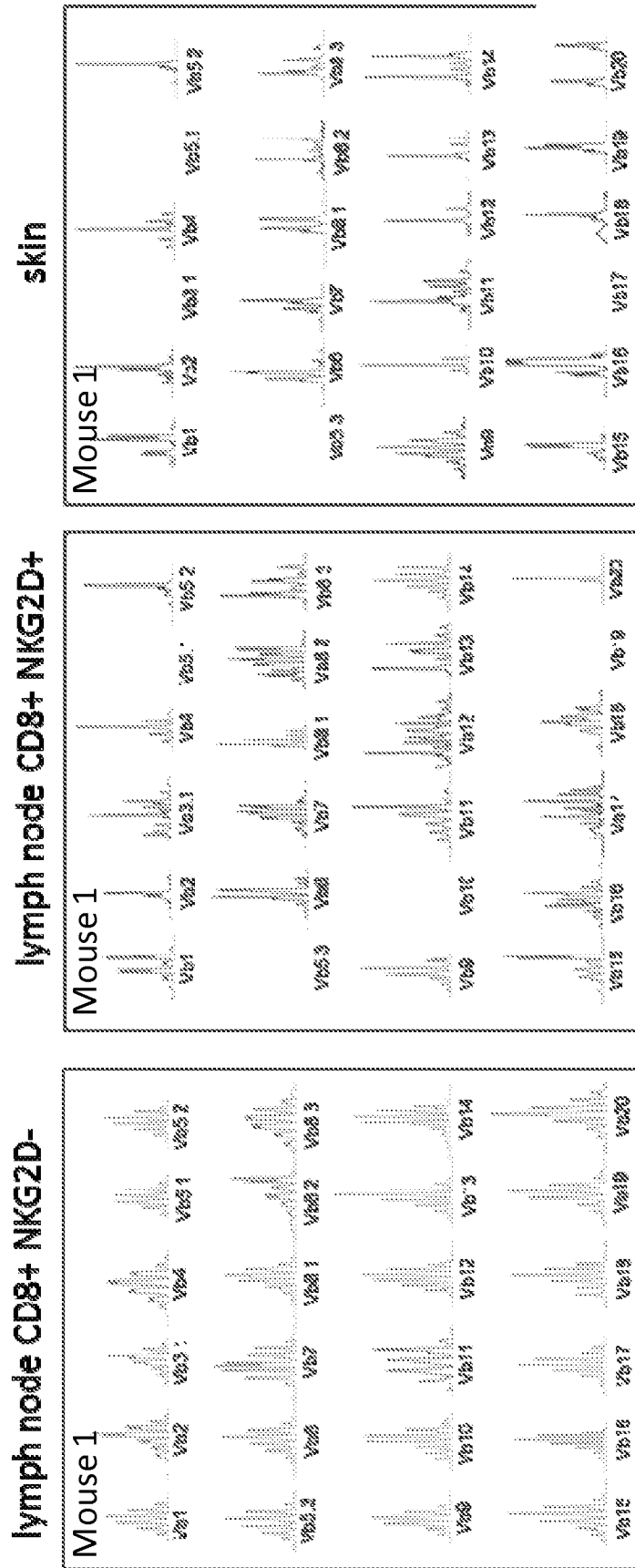


FIG. 22

IL-15R β + CD8 T cells infiltrate IL-15/IL15R α
expressing HF in AA mice

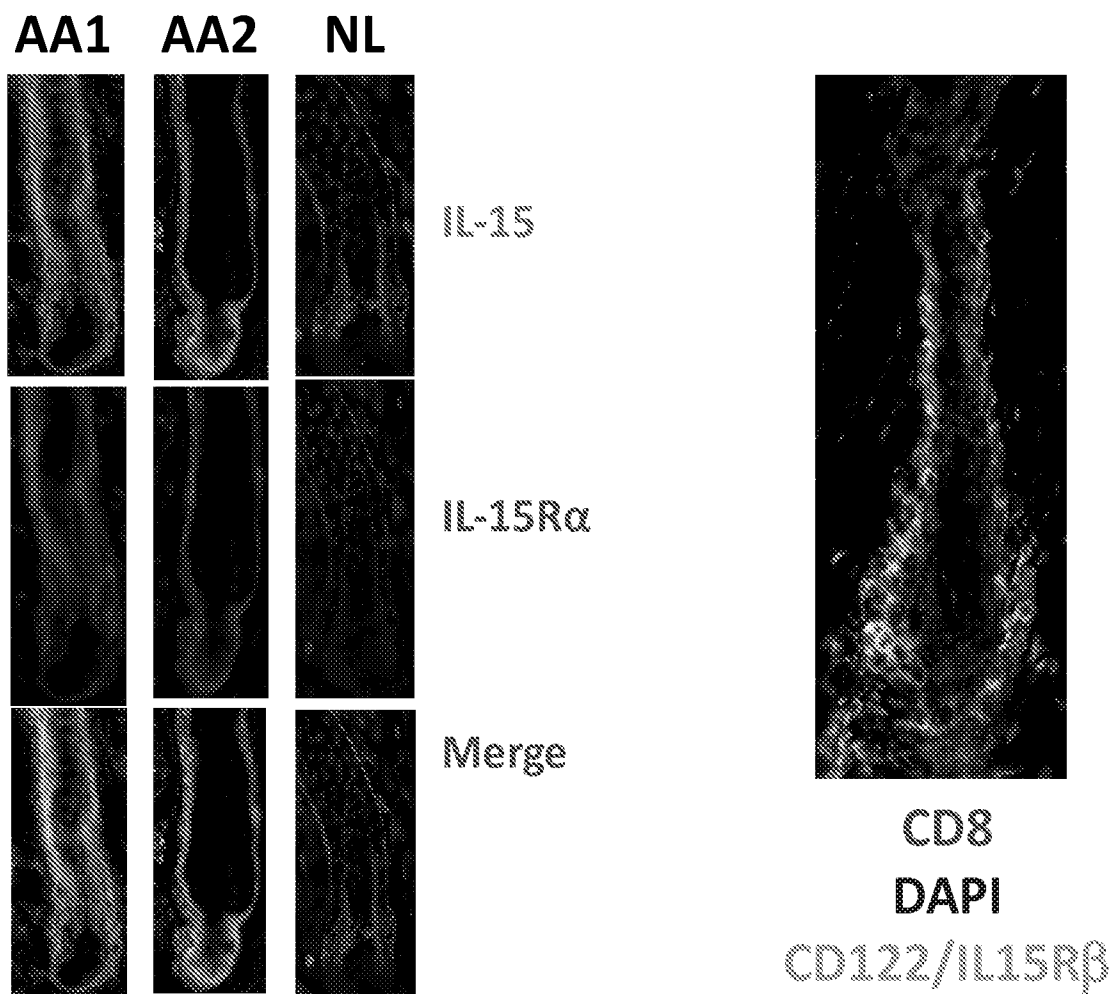
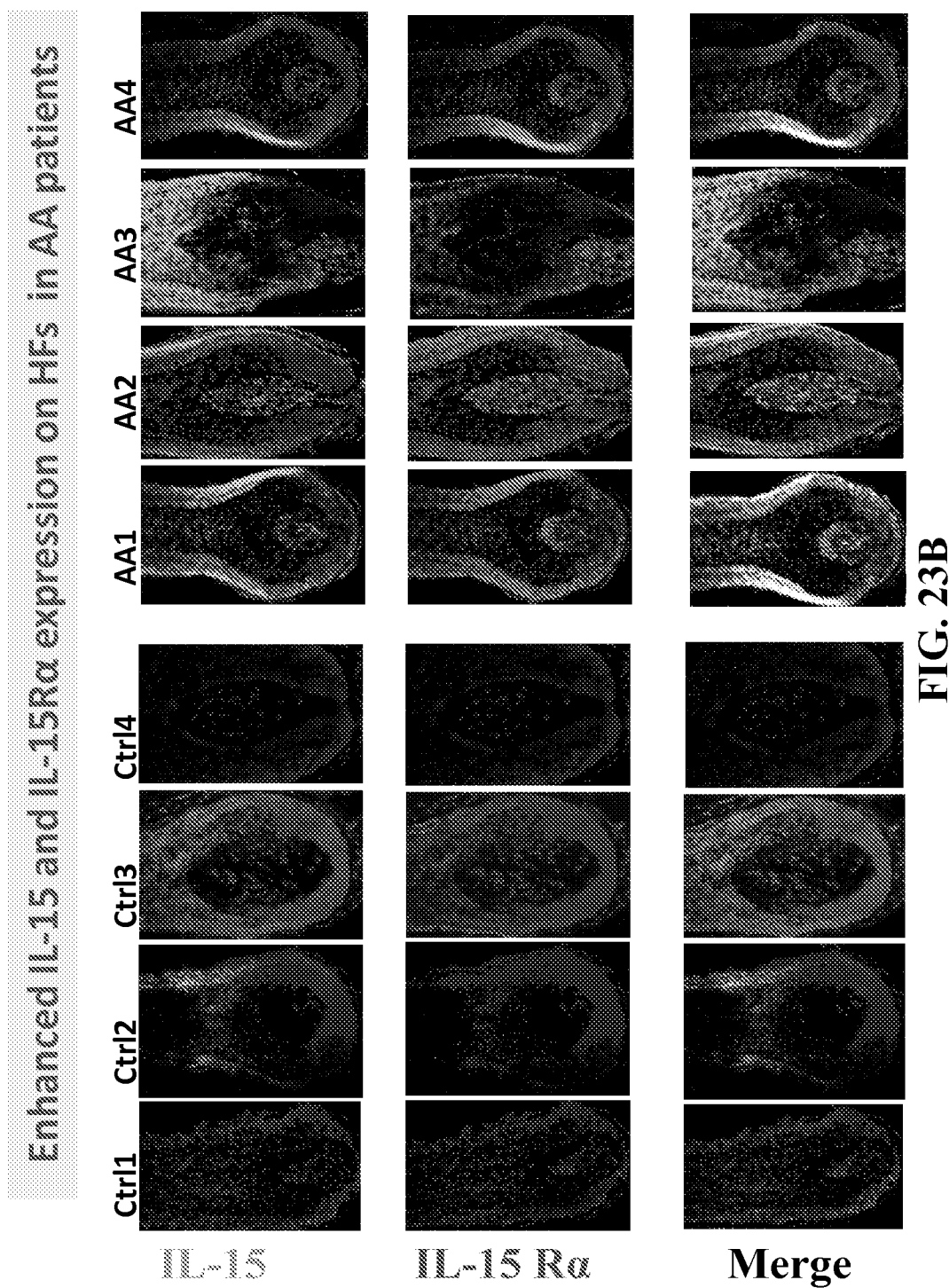


FIG. 23A



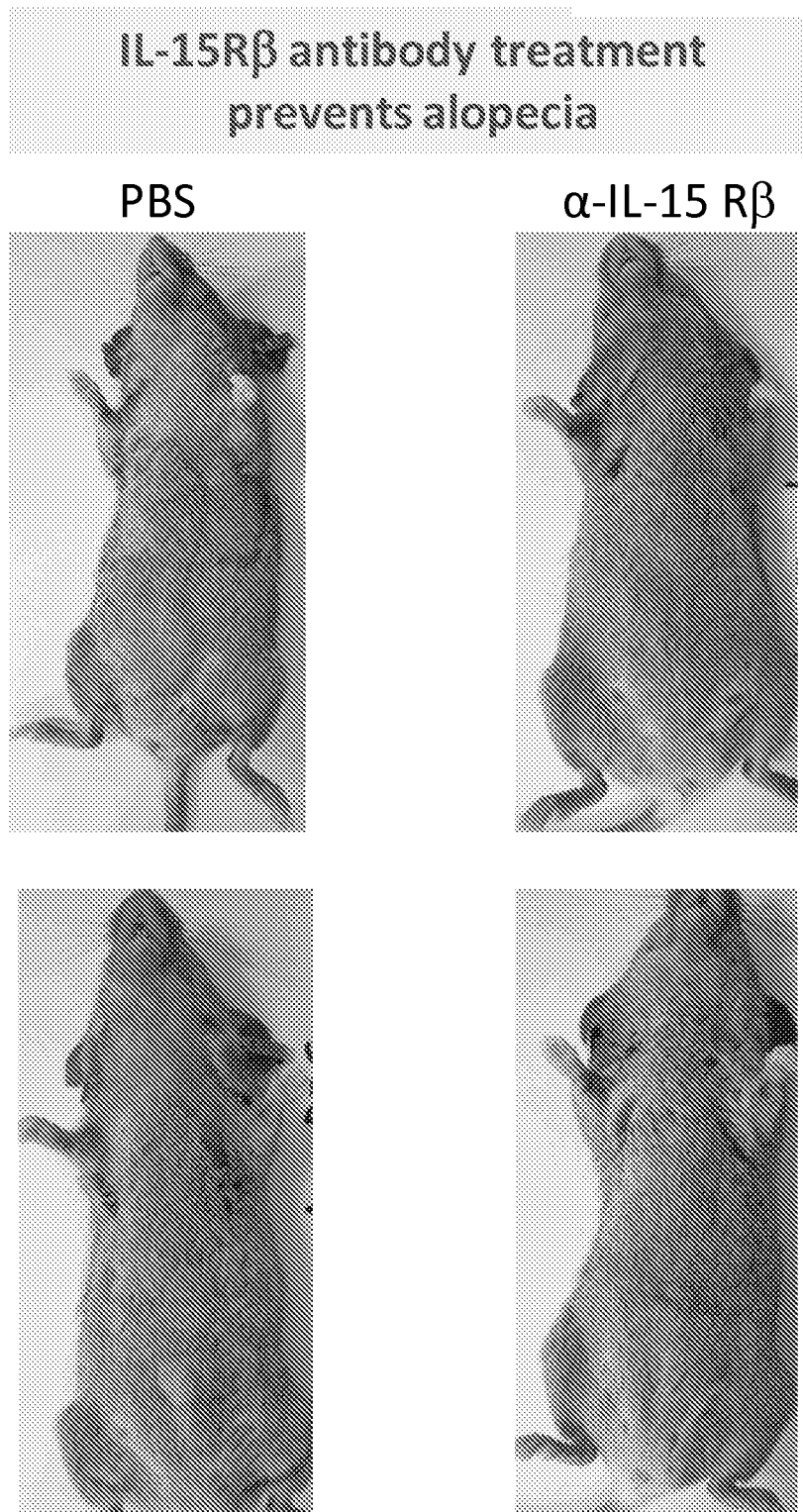


FIG. 24

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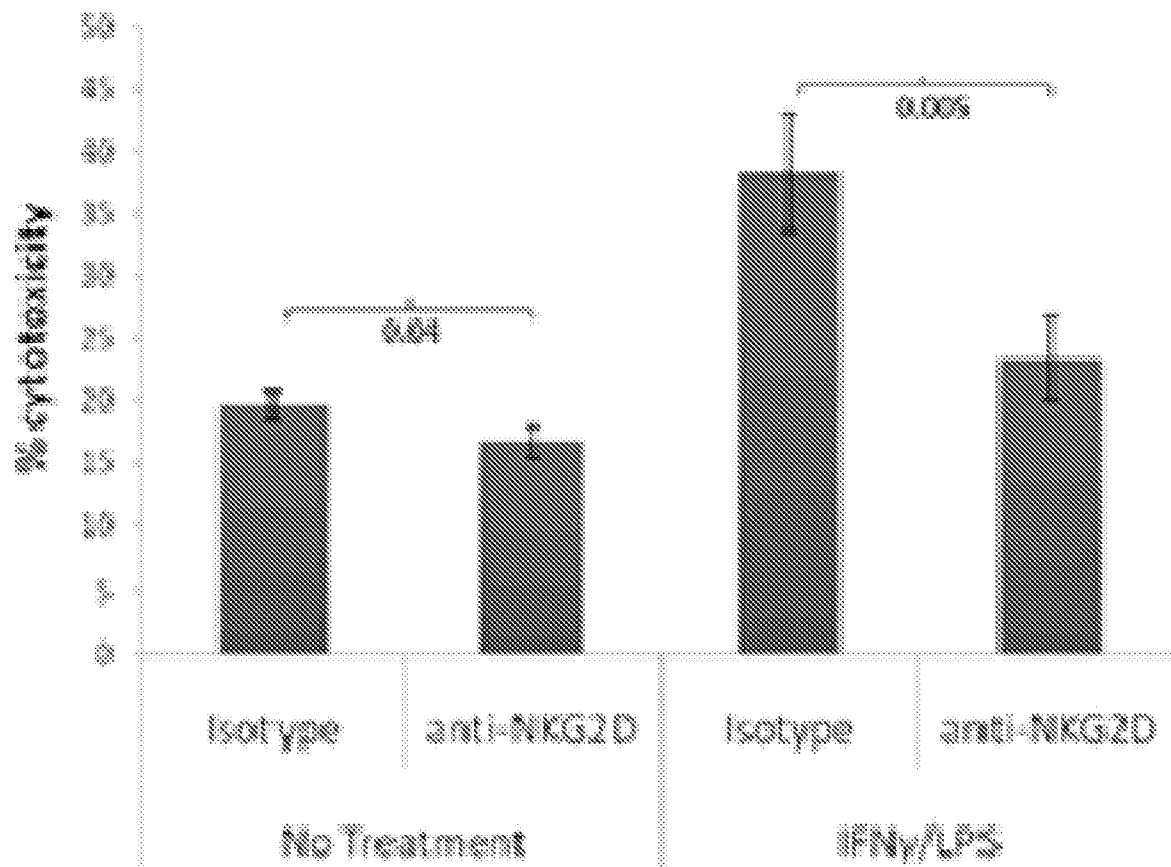


FIG. 25A

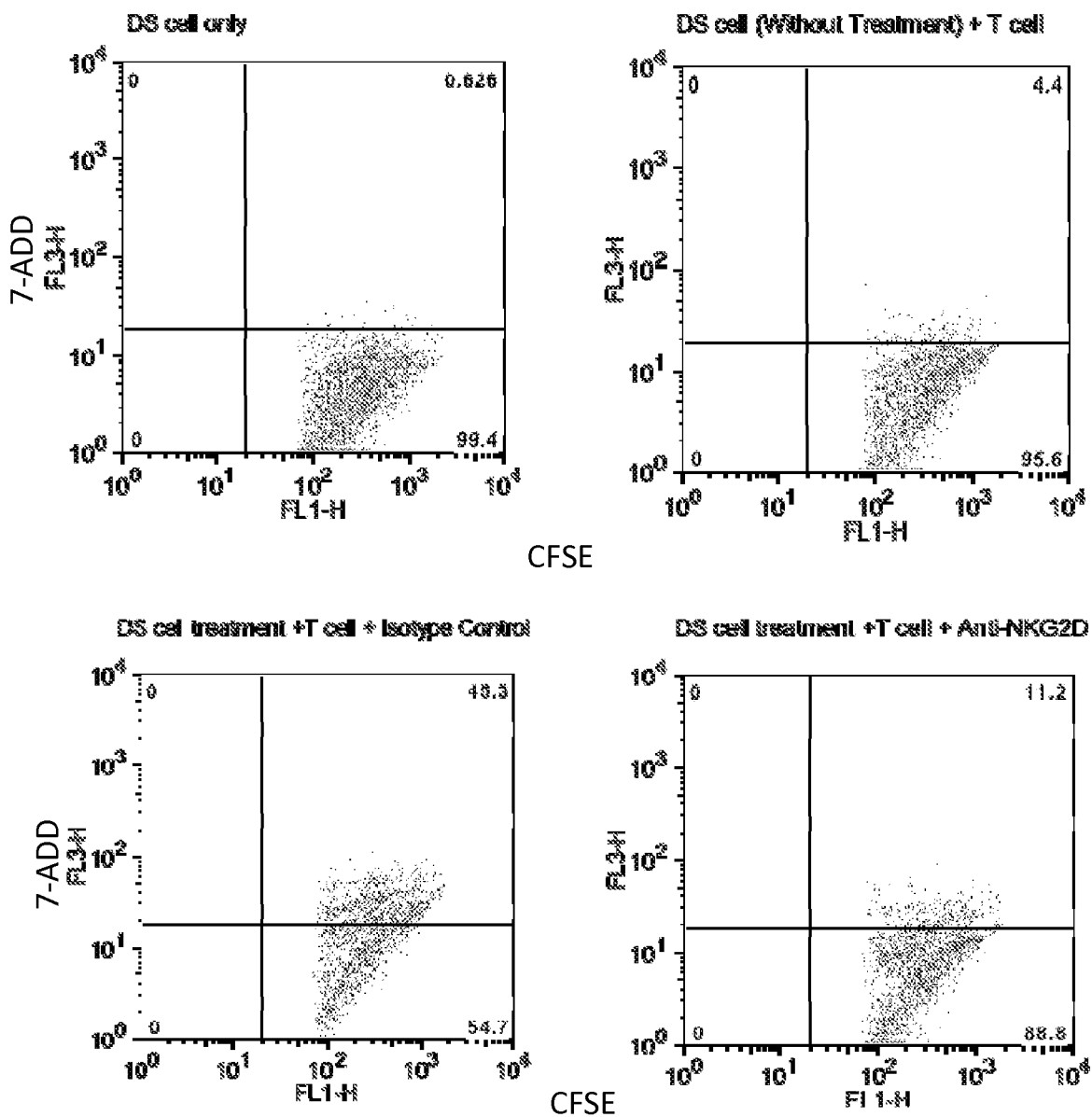


FIG. 25B

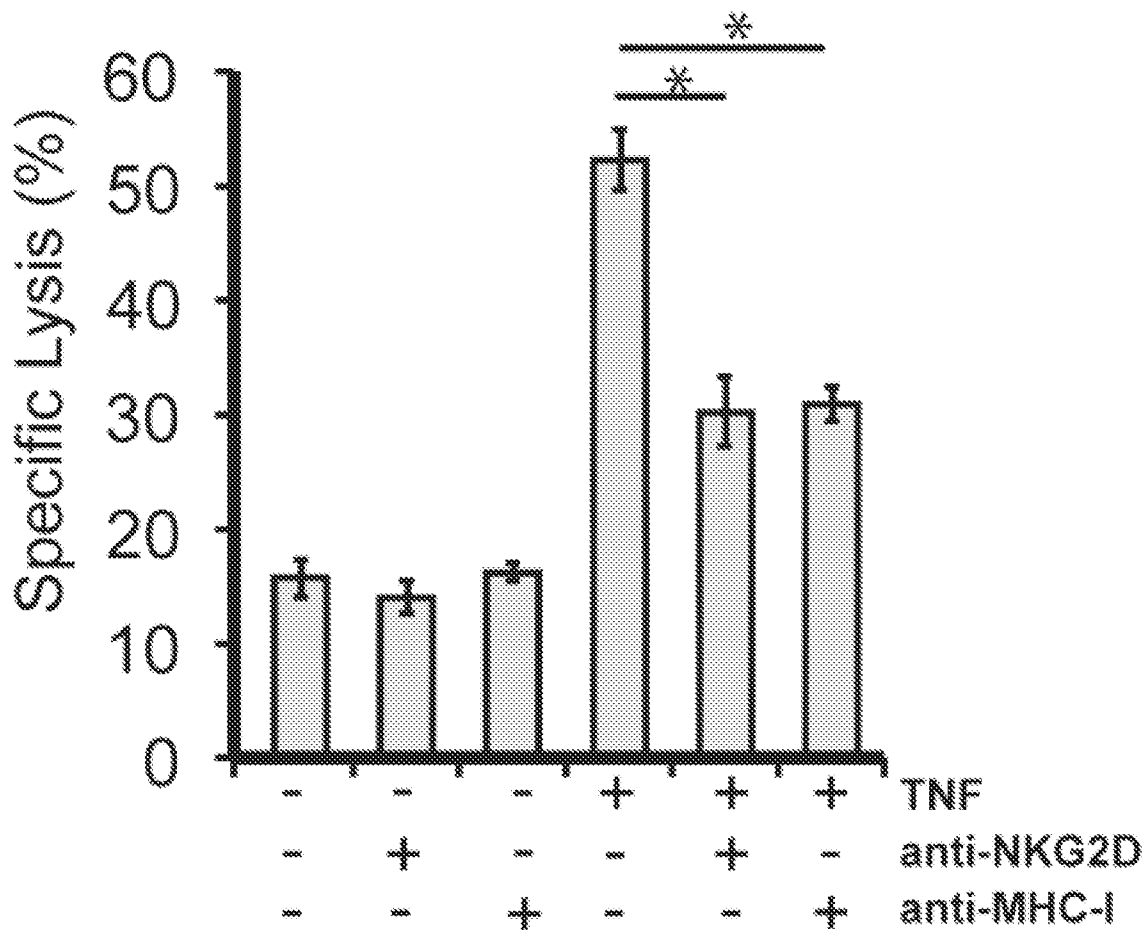


FIG. 25C

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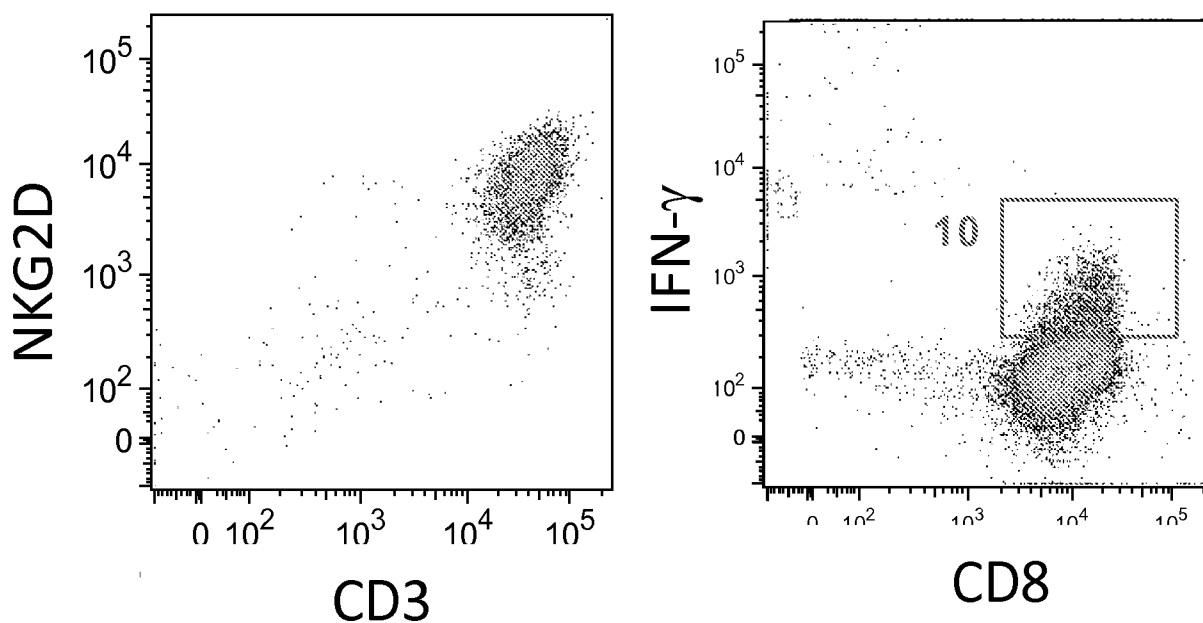


FIG. 26A

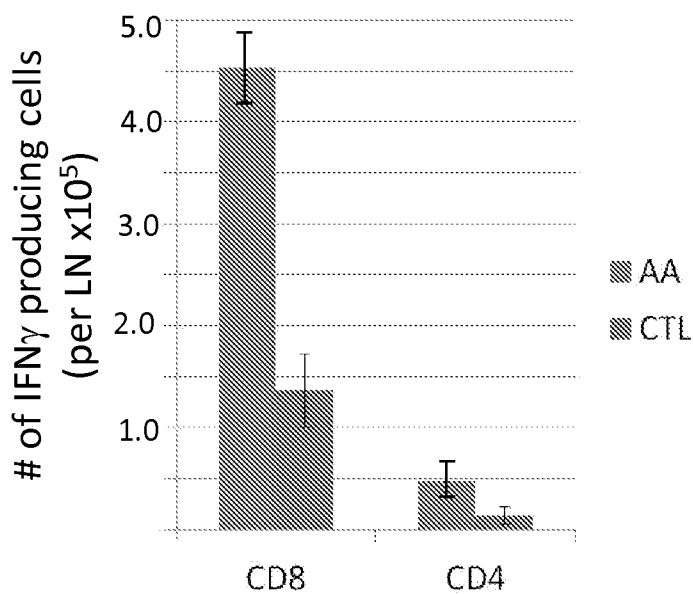


FIG. 26B

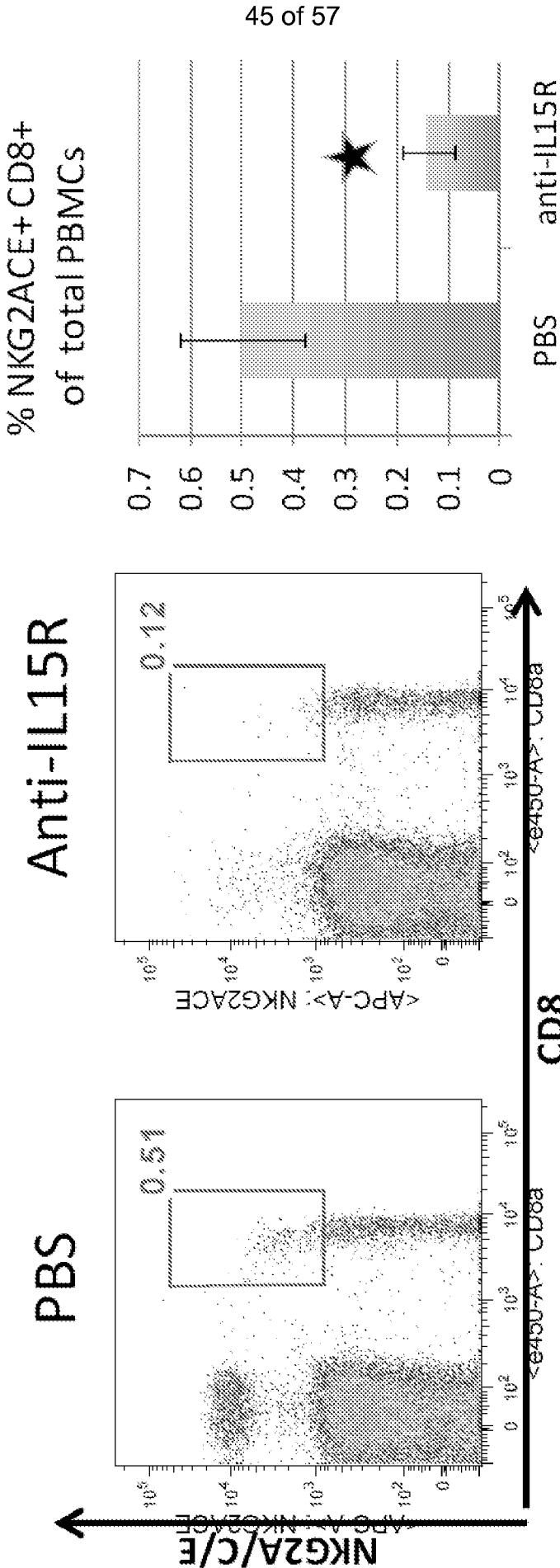


FIG. 27A

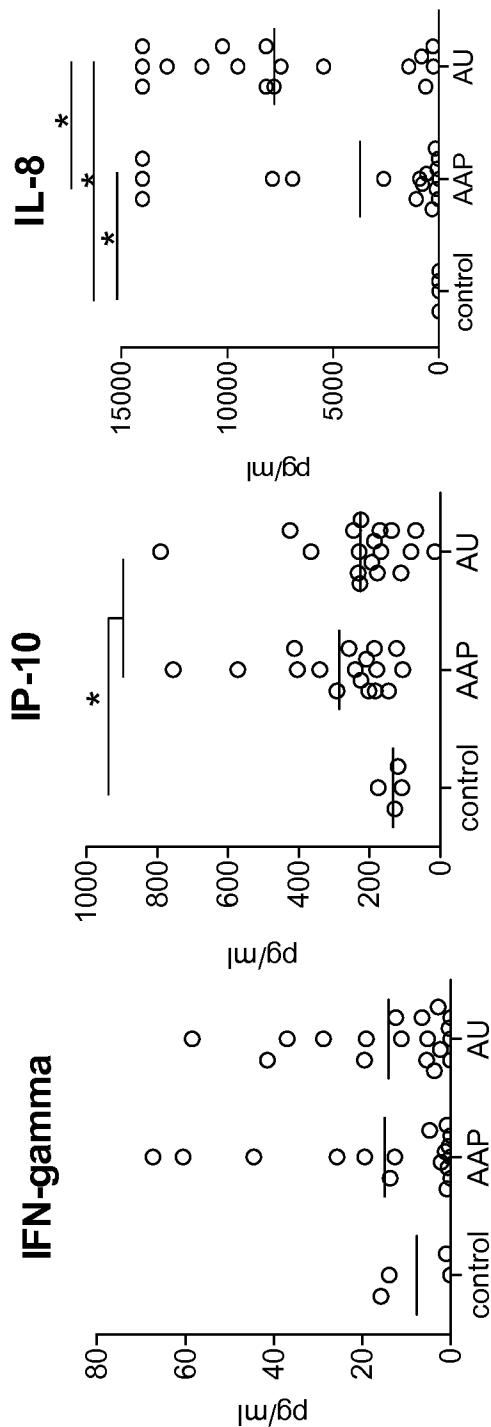


FIG. 27B

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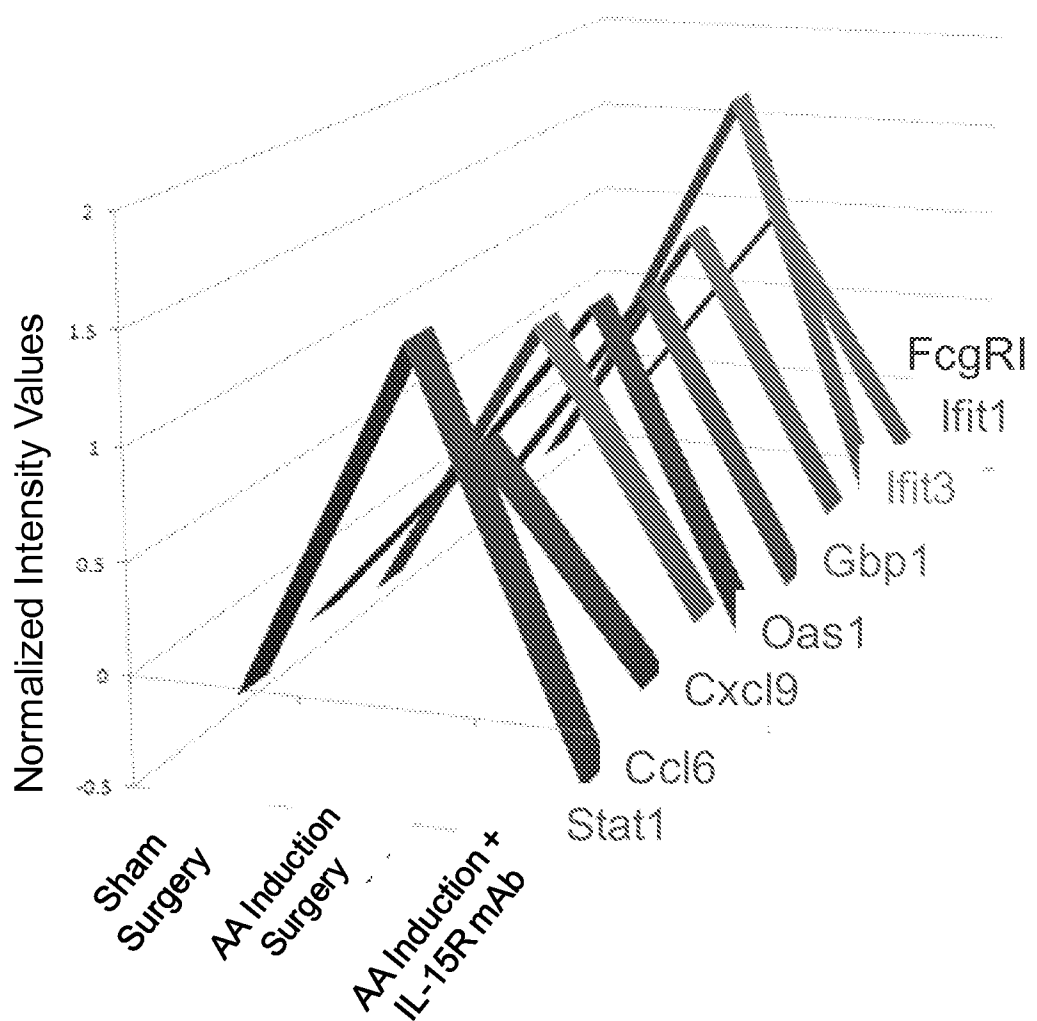


FIG. 28

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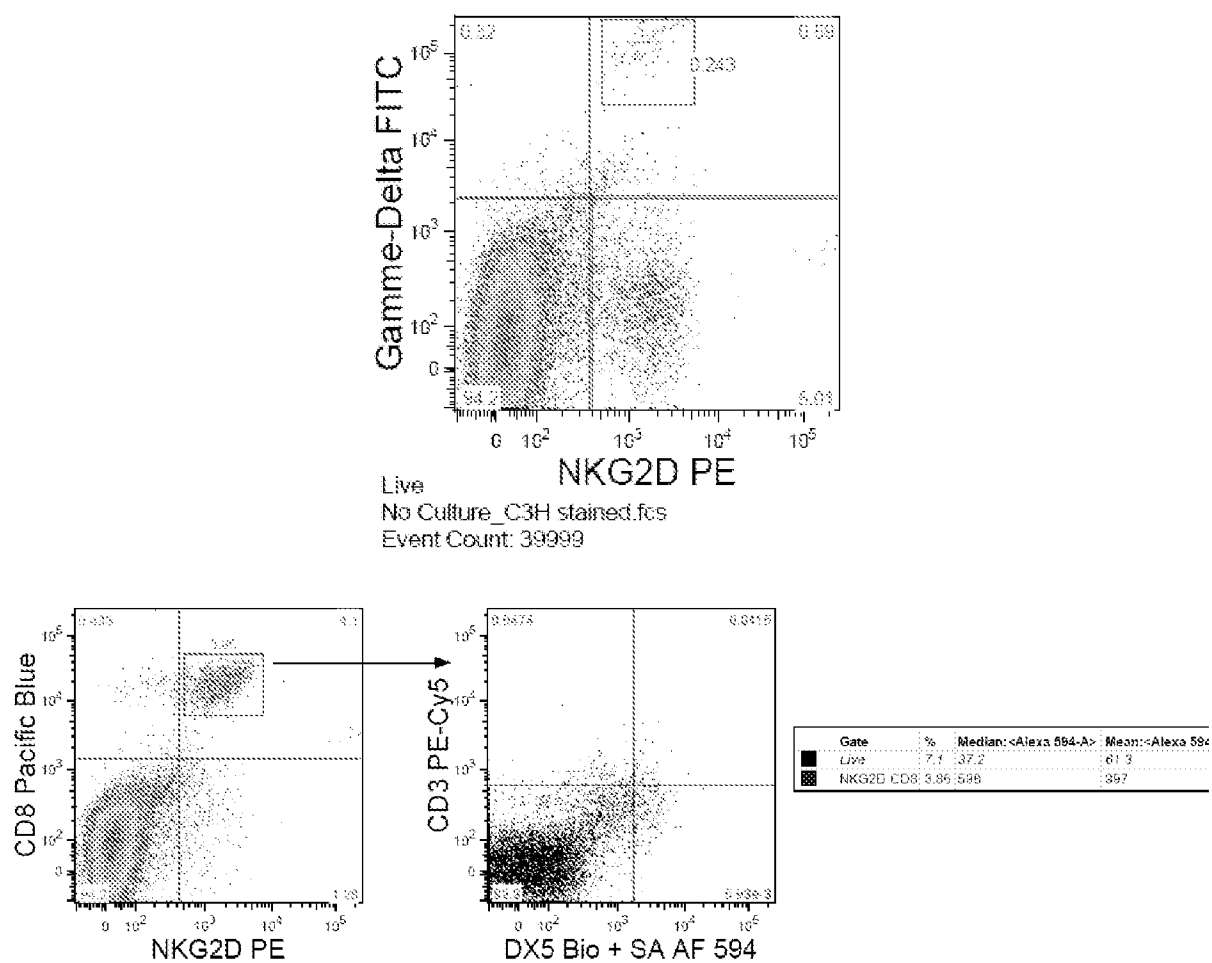


FIG. 31

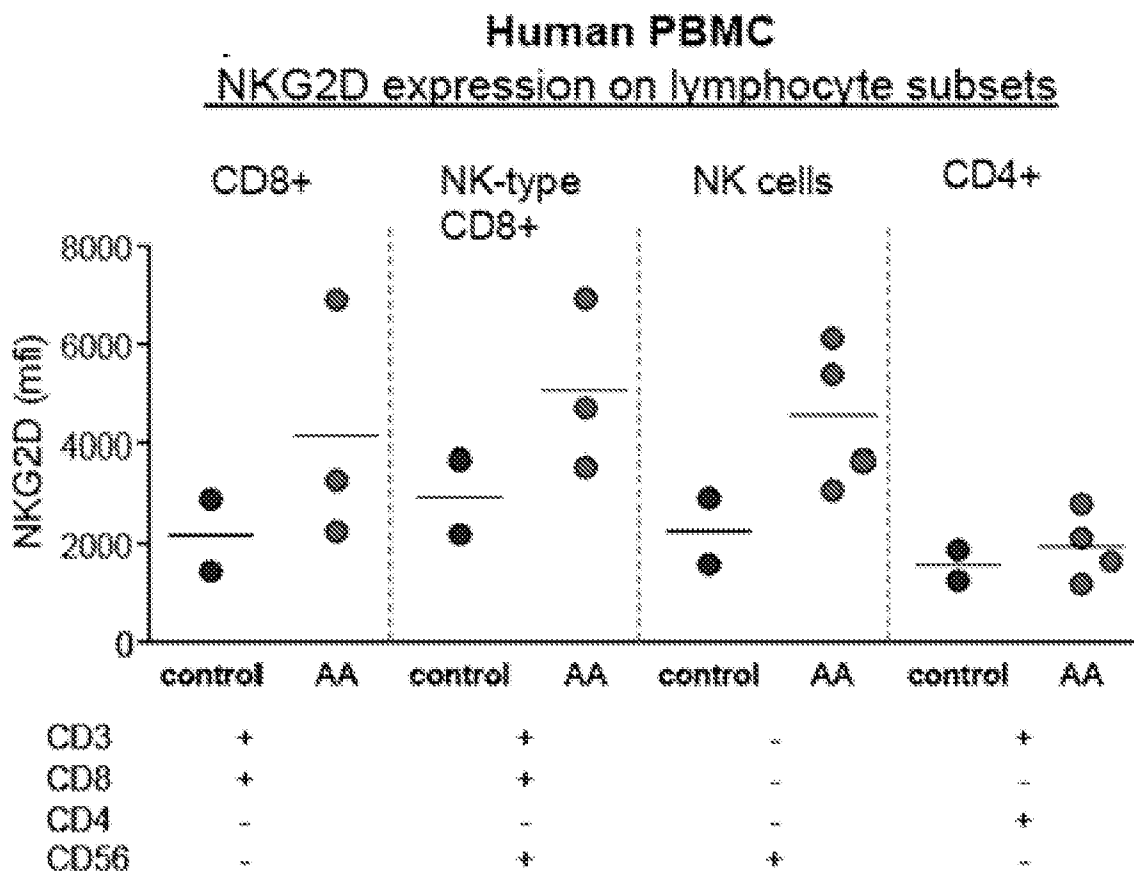


FIG. 32

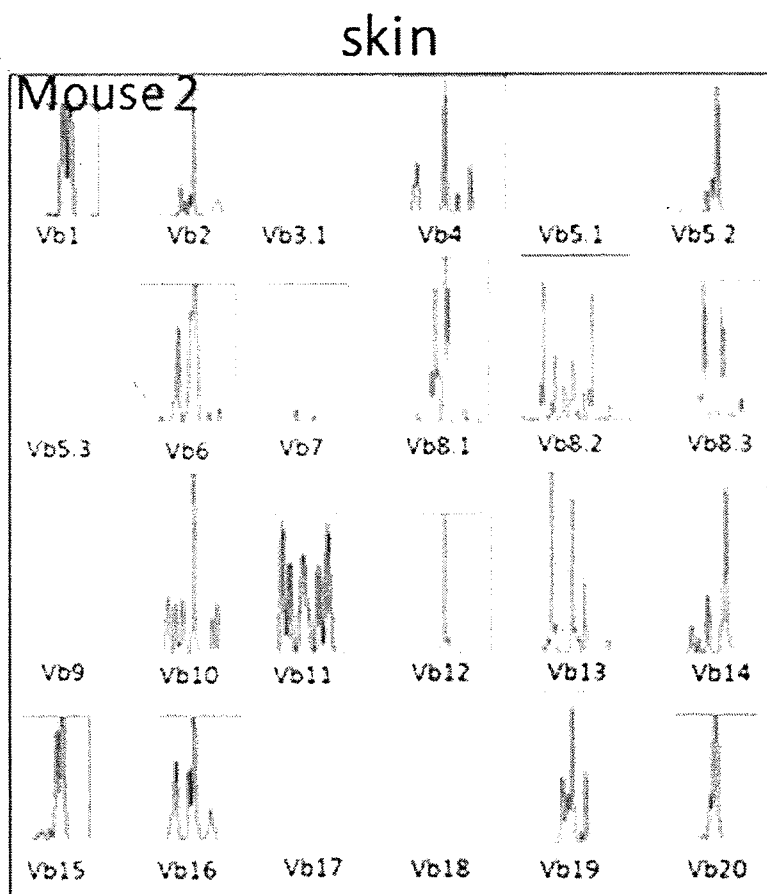


FIG. 33

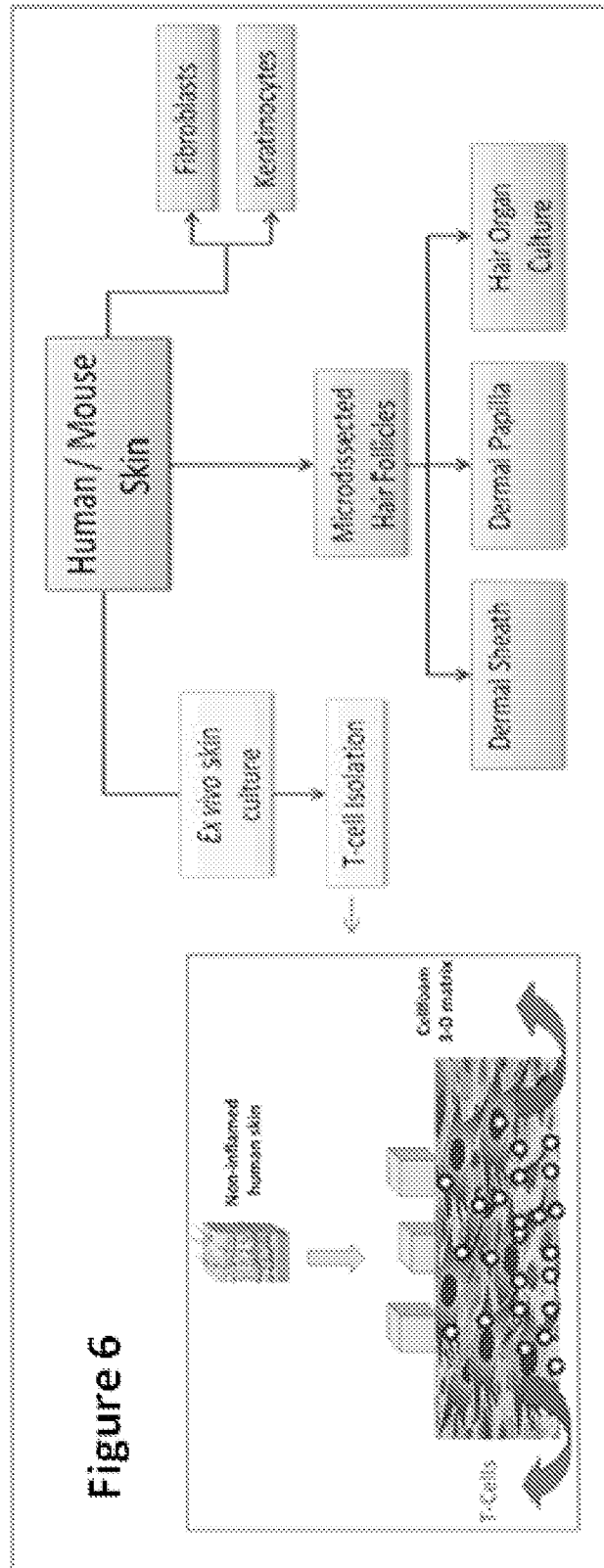
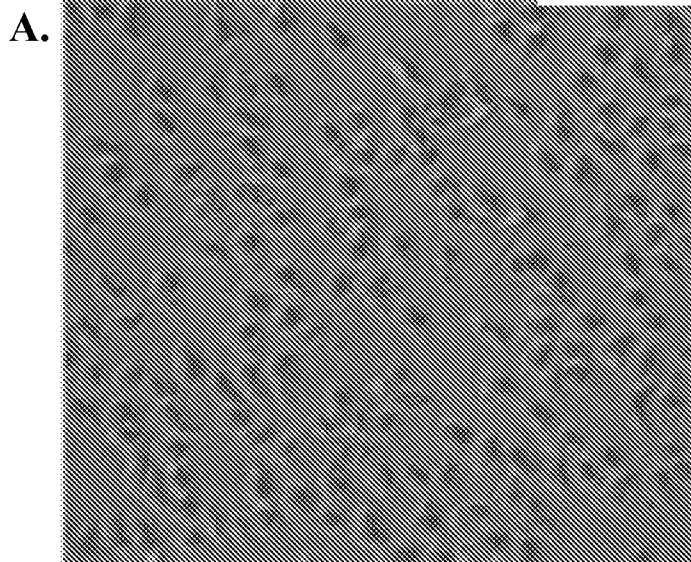
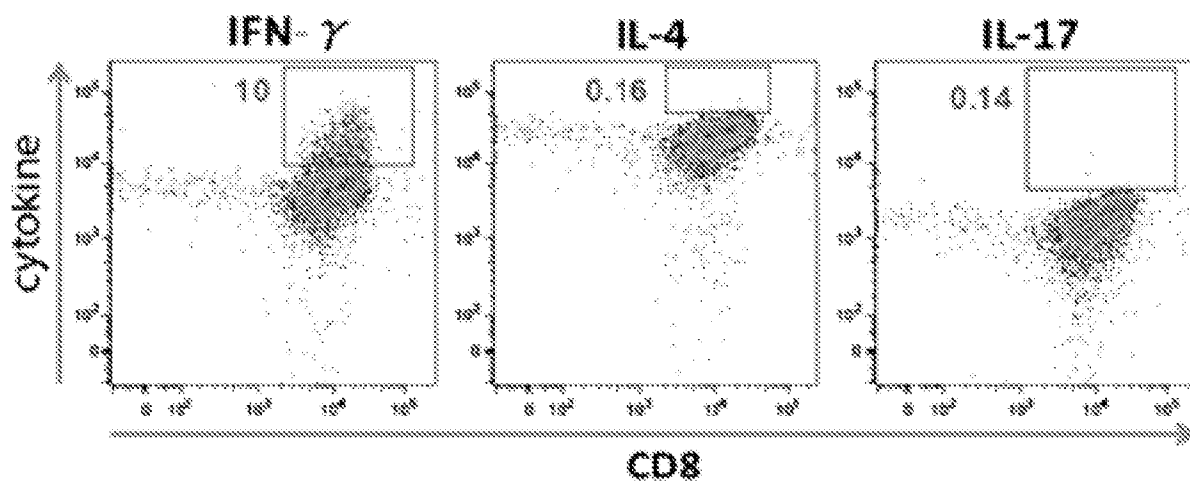


FIG. 34

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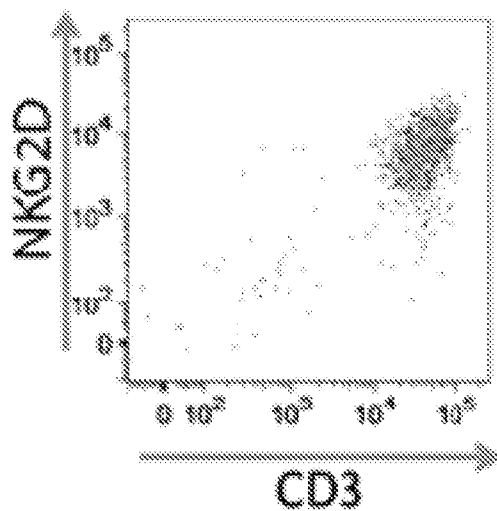
B. Human skin T cells stimulated with PMA/Ionomycin



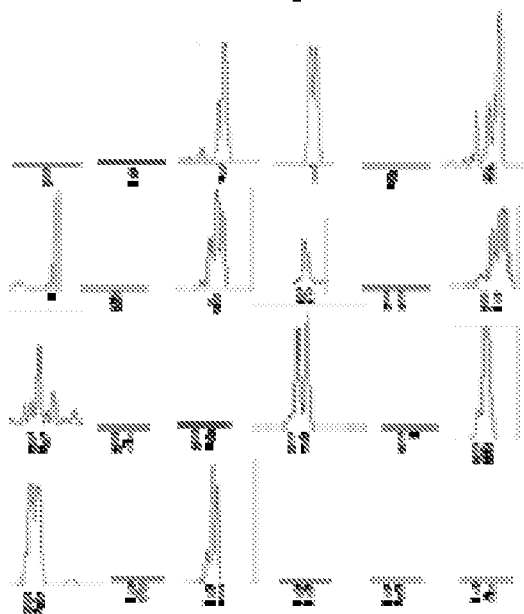
FIGS. 35A-B

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**C. NKG2D expression
on skin T cells**



**D. A. Spectratyping of cultured
T cells from an AA patient:**



FIGS. 35C-D

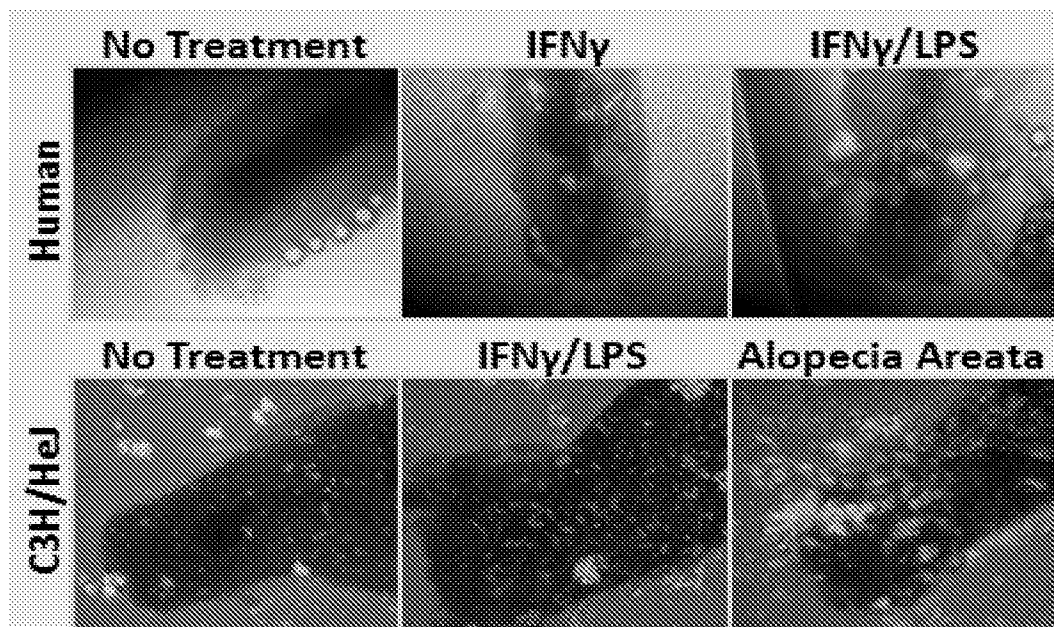


FIG. 36

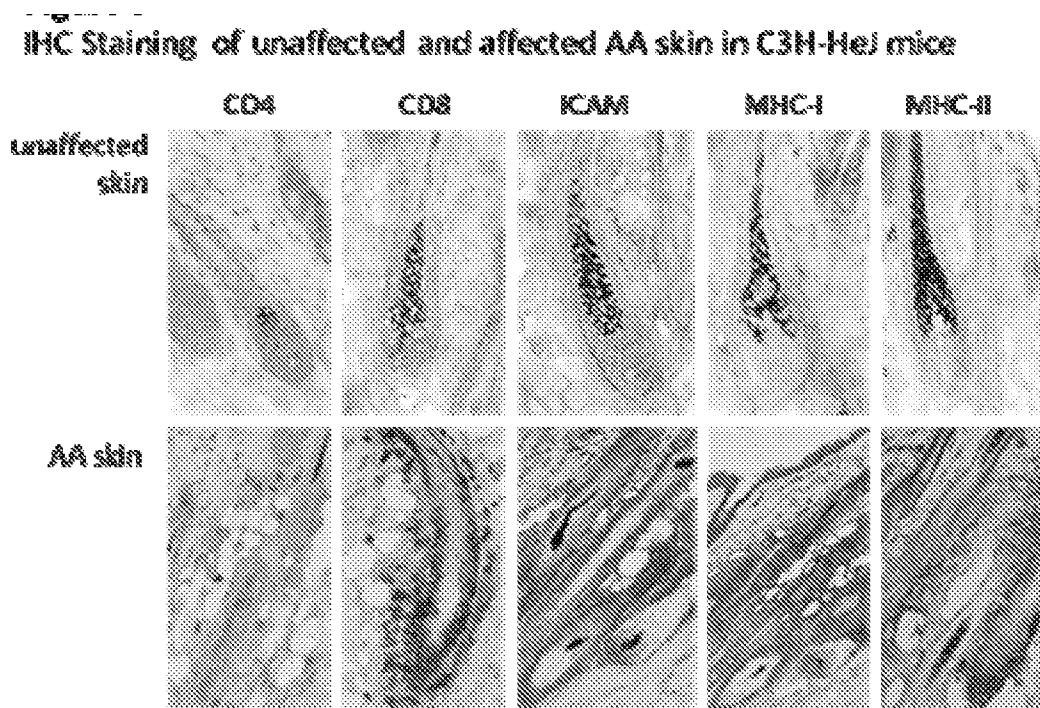


FIG. 37

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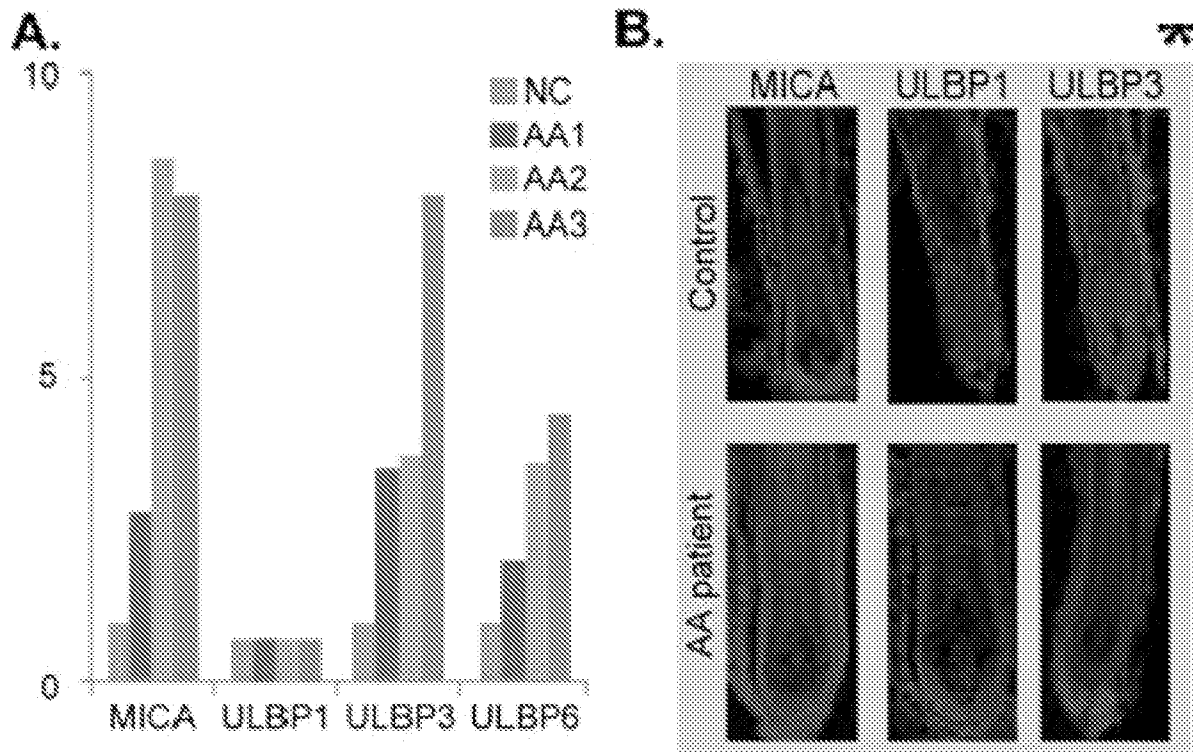


FIG. 38