ABSTRACT

Alopecia areata (AA) is a chronic inflammatory disease of hair follicles manifesting as patchy areas of hair loss on the scalp and body. Development of AA is associated with peri- and intra-follicular inflammation of anagen stage hair follicles, primarily by CD4+ and CD8+ cells. We hypothesized that if cell-mediated cytotoxicity against hair follicles is to be a component of the hair loss disease mechanism, increased expression of genes and products typical of cytotoxic cells, as well as increased apoptosis activity within affected hair follicles, would be expected to occur in the lesional skin compared to the normal skin. Furthermore, we studied gene expression levels of multiple cytokines and characteristic chemokines, using the C3H/HeJ mouse model of AA. mRNA expression levels of granzyme A, granzyme B, perforin, Fas, Fas ligand, TNF-α, TNF-αR1 and R2, TRAIL, TRAILR, TRAMP, Th1-, Th2-, and Th17-associated cytokines, as well as multiple chemokines were compared between the skin, draining lymph nodes, thymus and spleens of normal and AA-affected mice using quantitative reverse transcriptase PCR. FasL, granzyme A, granzyme B, pro- and anti-inflammatory cytokines were all highly up-regulated in the skin of AA-affected mice. Immunohistochemical studies of the skin revealed that, although greater numbers of granzyme B and FasL expressing cells were present in AA affected skin, the cells were morphologically diffusely distributed and not exclusively located within the focal peri- and intrafollicular infiltrate. The majority of these cells were further characterized as mast cells, which were also found in substantially greater numbers in the skin of mice with AA compared to their normal haired controls. Almost no perforin expressing cells were identified in AA affected mouse skin and TUNEL staining suggested relatively limited apoptosis activity in hair follicle keratinocytes. In conclusion, while granzymes and FasL may play important roles in disease development, the profiles and patterns of expression are not consistent with direct cell-mediated cytotoxic action against the follicular epithelium in chronic mouse AA. Potentially, hair growth inhibiting cytokines may play a more dominant role in AA development than previously thought. Furthermore, mast cells, with their increased presence around hair follicles in the AA affected mouse skin and their ability to express granzyme B and FasL, are suggested as potential key players in the pathogenesis of AA.
TABLE OF CONTENTS

Abstract ...........................................................................................................ii
Table of Contents ...........................................................................................iii
List of Tables ...................................................................................................v
List of Figures ..................................................................................................vi
Acknowledgements .........................................................................................vii
Dedication ..........................................................................................................viii
Co-Authorship Statement ................................................................................ix

CHAPTER 1 Alopecia areata ................................................................. 1
   Etiology and Epidemiology ............................................................... 1
   Clinical presentation ......................................................................... 3
   Histopathology and pathodynamics .............................................. 4
   AA and collapse of immune privilege in hair follicles .................. 6
   Animal models for AA ................................................................. 7
      Severe combined immunodeficiency (SCID) mice .................... 8
      Dundee experimental bald rats (DEBR) .................................. 8
      C3H/HeJ mouse model for AA ............................................... 9
   AA and the immune response ......................................................... 12
   Tables and figures ............................................................................ 16
   References ......................................................................................... 18

CHAPTER 2 Cell-mediated cytotoxicity .............................................. 25
   Introduction ....................................................................................... 25
   Material and methods ..................................................................... 28
   Results .............................................................................................. 33
   Discussion .......................................................................................... 36
LIST OF TABLES

Table 2.1 .........................................................................................................................44
Table 3.1 .........................................................................................................................75
Table 4.1 .........................................................................................................................99
LIST OF FIGURES

Figure 1.1 Clinical findings in AA .................................................................16
Figure 1.2 The experimental induction of hair loss in C3H/HeJ mice ..................17
Figure 2.1 Granule exocytosis pathway gene expression in murine alopecia areata .......45
Figure 2.2 Immunohistochemical studies of granzyme B and perforin ...................46
Figure 2.3 The average number of granzyme B expressing cells per square millimeter of the skin ...............................................................47
Figure 2.4 The Fas-FasL pathway gene expression in murine alopecia areata ............48
Figure 2.5 The expression patterns of FasL+, Fas+, and CD8+ cells in the skin of C3H/HeJ mice .................................................................49
Figure 2.6 Genes expression of the TNF family of receptors and ligands in murine alopecia areata .................................................................50
Figure 2.7 Apoptosis activity in the skin of C3H/HeJ mice ..................................51
Figure 2.8 Mast cells in the skin of C3H/HeJ mice ...........................................52
Figure 2.9 Mast cells in AA ............................................................................53
Figure 3.1 NK cell surface marker gene expression in murine alopecia areata ..........76
Figure 3.2 Immunohistological staining of NK cells in the skin of C3H/HeJ mice ......77
Figure 4.1 Th1-associated and other proinflammatory cytokines in AA mice ...........100
Figure 4.2 Th2-associated cytokine gene expression in AA mice .........................101
Figure 4.3 Th17-associated cytokines ................................................................102
Figure 4.4 Chemokines ..................................................................................103
Acknowledgments

I would like to express my deep gratitude to Dr. Kevin McElwee under supervision of whom I had the pleasure to study. Had it not been for his great support, encouragement, suggestions, and scientific thinking, this work would have never been accomplished. I am also much obliged to Dr. Jerry Shapiro for his support throughout these years. Together, they provided me with an opportunity to conduct this research project at the Hair Research Laboratory, University of British Columbia, for which I am sincerely thankful.

I thank my supervisory committee members, Dr. Jan Dutz and Dr. Youwen Zhou, whose directions and constructive inputs greatly contributed to making this thesis work possible. I would also like to thank Dr. Mei Yu, Blanche Lo, and all the past and present members of the Hair Research Laboratory. Finally, I wish to express my thanks to Dr. Aziz Ghahary, students and members of the BC Professional Fire Fighters’ Burn & Wound Healing Research Laboratory, Dr. Vince Duronio, Patrick Carew, Dr. Harvey Lui, UBC Faculty of Graduate Studies, and Department of Dermatology and Skin Science.
Dedication

This work is dedicated to

His Holiness Molana Salaheddin Ali Nader Shah Angha
“Pir Oveyssi”

And to my parents, for their ceaseless love and support
Co-Authorship statement

Following is the list of co-authors and their contributions to this research project:
Dr. Mei Yu—protocol development and statistical analysis development
Nooshin Sadeghi—protocol execution assistance
Blanche Lo—protocol execution assistance
Dr. Jerry Shapiro—clinical advisor and study design and development
Dr. Kevin McElwee—research advisor and study design and development
**Chapter 1. Alopecia areata**

**Etiology and Epidemiology**

Alopecia areata accounts for up to 2% of new dermatological outpatient attentions in the UK and USA (Dawber R et al. 1998), and a National Health and Nutrition Examination Survey study estimated its prevalence at 0.1% to 0.2% during the early 1970s, with a lifetime risk of 1.7% (Safavi K 1992). One recent study revealed that approximately 25% of visits for all types of alopecia in the United States are for AA, and found the highest number of visits in the 30- to 59-year age group, with a predominance of female and black patients (McMichael AJ et al. 2007). In up to 60% of cases, AA has been reportedly associated with atopy, especially atopic dermatitis (Tan E et al. 2002, Shellow WV et al. 1992, and Sharma VK et al. 1998). Concurrent autoimmune diseases, especially autoimmune thyroiditis, have been reported in 8 to 28% of patients with AA (Tan E et al. 2002, and Cunliffe WJ et al. 1969). Patients with more severe subtypes, namely alopecia totalis (AT) or universalis (AU), tend to have a younger age of onset, and are more likely to have associated atopic disease or autoimmune conditions (Goh C et al. 2006).

AA is a heterogeneous condition, and can not be attributed to any single cause. It is believed that AA is a polygenic condition, with genetic factors involved in disease susceptibility and prognosis and environmental factors playing roles in triggering disease initiation and exacerbation.

A large number of studies have pointed to genetic contributions to the disease. The incidence of a family history of AA has been estimated as from 4.27% up to 32.9% (van der Steen P et al. 1992, Sharma VK et al. 1006, Wang SJ et al. 1994, and Goh C et al. 2006). A concordance rate of 55% has been reported for AA amongst monozygotic twins (Jackow C et al. 1998). A strong association between AA and trisomy 21 (Down’s
syndrome) has also been shown in a number of studies (Du Vivier A et al. 1975, and Carter DM et al. 1976).

A genome-wide scan of extended families has suggested at least four susceptibility loci on chromosomes 6, 10, 16 and 18 (Martinez-Mir A et al. 2007). Chromosome 6 exhibited two regions of disease association, one at 6p that corresponds to the HLA locus; subsequently, it was shown that major histocompatibility complex (MHC) genes on chromosome 6p21 encoding human leukocyte antigens (HLAs) are major determining loci for T-cell-mediated diseases including AA (Nair RP et al. 2000, and Martinez-Mir A et al. 2007).

In sporadic AA, HLA-DQB1*03 alleles were present in 92% of patients with AT/AU and in 80% of all patients with AA (Barahmani N et al. 2008). Certain HLA associations may provide relative protection from AA, as has been proposed for HLA-DRw52a (Duvic M et al. 1991) and HLA-DRB1*03 (Broniarczyk-Dyla G et al. 2002). In addition to HLA molecules, MHC class I chain-related gene A (MICA), a stress-inducible antigen, is also associated with several autoimmune diseases including AA (Barahmani N et al. 2006). These human studies are complemented by a genome-wide screen using the C3H/HeJ mouse model of AA (Sundberg JP et al. 2004). The strongest risk was associated with a region on murine chromosome 17, which corresponds to the MHC locus, the equivalent of the HLA region in humans.

Alleles of genes coding for cytokines, cytokine antagonists, and their receptors have also been associated with AA. These include polymorphisms in IL-1 receptor antagonists, macrophage migration inhibitory factor (MIF), and Notch4, which maps to the MHC class II region. These polymorphisms are associated with early onset of more severe disease (Gilhar A et al. 2006).

Several environmental factors have been proposed to be involved in initiating and/or prognosis of AA. Acute or chronic psycho-emotional stress has been reportedly associated with the onset and/or development of AA in up to 65% of patients (Manolache
L et al. 2007, Sehgal VN et al. 2007, Tan E et al. 2002, and García-Hernández MJ et al. 1999). Murine stress models have recently enabled scientists to further explore the brain-skin connection and the inhibitory roles of stress on hair growth. Stress-induced upregulation of the neuropeptide substance P (SP) appears to prematurely precipitate anagen hair follicles into the catagen stage. Moreover, when injected into the skin, SP results in mast cell degranulation and perifollicular neurogenic inflammation, which in turn causes hair growth inhibition and premature catagen induction (Peters EM et al. 2006, Arck PC et al. 2005, Arck PC et al. 2006, Siebenhaar F et al. 2007, and Peters EM et al. 2004). Intriguingly, SP may also induce collapse of hair follicle immune privilege by upregulating MHC class I antigens in organ-cultured normal human scalp hair follicles (Peters EM et al. 2007), and provides a possible explanation on how stress may trigger or aggravate AA.

The frequency and severity of AA appears to be associated with a greater frequency of allergies compared with the general population. Atopy is reported in 10 to 52.4% of patients with AA, and in up to 75% of patients it is associated with a more extensive and prolonged hair loss (Goh C et al. 2006, Tan E et al. 2002, Weitzner JM 1990, and De Weert J et al. 1984). Other potential promoters of the immune system, including drugs, toxins, infectious agents, and trauma may play roles in triggering the onset of AA (Geletko SM et al. 1996, Roselino AM et al. 1996, and Skinner RB Jr et al. 1995). Whatever the initiating factor, in a genetically susceptible individual, it could tip the balance of the immune system into autoimmunity, and facilitate initiation of AA.

Clinical presentation

Alopecia areata most commonly manifests as the sudden appearance of round or oval patches of hair loss. The scalp is the most common site affected by AA, but other parts of the body may also be involved (Safavi KH et al. 1995). The alopecic patches are well circumscribed and usually have a distinctive border where normal hair demarcates the periphery of the lesion. The underling skin appears normal with no epidermal scaling or any sign of follicular scarring (Wasserman D et al. 2007). The disease is usually relapsing-
remitting in nature, and may extend to involve the whole scalp or body and cause alopecia totalis or universalis (figure 1.2). AA tends to preferentially affect pigmented hairs, with relatively sparing white hairs. The affected hairs leave anagen prematurely, resulting in massive telogen hair shedding (Tobin DJ 1997). Characteristic "exclamation-mark hairs" may be seen within or around the alopecic patches (see below). Upon regrowth, hairs often initially lack pigment and are white in color (Cline DJ. 1998). Nail changes may be associated with AA. The most common nail dystrophy is nail pitting followed by other features such as longitudinal ridging and thickening (Safavi KH et al. 1995).

**Histopathology and pathodynamics**

The histological hallmark of AA is the peri- and intrabulbar infiltration of inflammatory cells, consisting mostly of CD4+ and CD8+ T lymphocytes, macrophages, and Langerhans’ cells (Sperling LC and Lupton GP 1995). The inflammatory infiltrate is more evident in areas of acute hair loss, and affects primarily anagen stage hair follicles. In contrast to the inflammatory scarring alopecias, little or none of the inflammatory infiltrate is seen around the isthmus of the hair follicle, the proposed site for hair follicle stem cells (Cotsarelis G et al. 1990). This may explain why hair follicles retain their ability to regrow hair even in long lasting lesions of AA, and are not permanently destroyed.

Hair follicle injury is a common feature of AA, although it is unclear which hair follicle cell sub-population(s) is the target of the disease process. The earliest histologic changes of AA are seen in the keratinocytes of the presumptive cortex and precortical matrix, suggesting this cell population as the primary target in AA (Messenger AG et al. 1984). Amorphous-staining regions seen at the junction of dermal papilla (DP) and matrix (MX) indicate disruption of pre-cortical or hair-shaft-forming keratinocytes. Moreover, activated macrophages are shown in association with degenerative keratinocytes and the DP/MX junction (Tobin DJ 1997).
The fact that AA preferentially affects pigmented hair and may spare white hair and that regrowing hair is often initially white (Gollnick et al. 1990) has led some to consider melanocytes as the potential pathogenic target in AA. Degeneration of hair-bulb melanocytes have been shown to occur in AA in a distinctly different pattern from catagen-associated pigmentation changes in normal hair follicles (Tobin DJ 1997). Moreover, Gilhar et al. (Gilhar A et al. 2001) identified five melanocyte derived peptides, which were able to activate AA lesional scalp T cells when cultured together in vitro. The authors concluded that multiple melanocyte epitopes can act as autoantigens in AA. Others have blamed dysfunction of the dermal papilla as the major cause of epithelial changes in AA, based on observation of the reduced volume and cell number of the DP in AA (van Scott EJ et al. 1958).

The damage to the follicular epithelium results in premature precipitation of anagen hair follicles into catagen and telogen. This is often manifested clinically as a sudden increase in telogen shedding, starts focally and spreads centrifugally as a wave, as more anagen hair follicles enter catagen and telogen prematurely (Eckert J et al. 1968). On horizontal sections of lesional scalp biopsies, a decreased anagen-to-telogen ratio of hair follicles is observed (Tobin, DJ et al. 1995). AA affected telogen HF is malformed and contains DP which is still metachromatic (a marker of anagen), suggesting that epithelial changes in AA differ from normal catagen induction (Tobin DJ et al. 1997). A subtype of telogen HF is called exclamation-mark hairs (EMH). These hair shafts fracture in their defective follicles at their distal end and taper proximally towards the scalp, giving them the appearance of an exclamation-mark. EMH, which appears to result from injury to pre-cortical keratinocytes, occurs in areas of acute hair loss (Tobin DJ et al. 1990).

After re-entry into anagen takes place, growth appears to be halted in anagen stage III (Messenger AG et al. 1986), resulting in a mixture of telogen and immature anagen hair follicles that is usually observed in the biopsies from established alopecic lesions. In long-standing lesions, marked miniaturization of the anagen follicles is observed. These miniature follicles produce a normal inner root sheath, but the hair cortex is incompletely
keratinized. It is uncertain whether dystrophic anagen follicles in AA return prematurely to telogen and continue to pass through truncated growth cycles, or remain suspended in early anagen. As regrowth occurs, follicles progress further into anagen (McDonagh AJ et al. 1996).

AA and collapse of immune privilege in hair follicles

The HF injury in AA is believed to be the consequence of breach in peripheral tolerance and failure of so-called HF immune privilege (IP). The proximal segment of anagen hair follicle exhibits all the features of an area of relative IP, including:

- Reduced or absent expression of MHC class I and II antigens on the sub-infandibular epithelium
- Limited presence and absent MHC class II antigen expression by antigen presenting cells
- Expression of immuno-suppressive molecules, such as TGF-β1, IGF-1, and α-MSH
- Expression of Fas ligand (FasL)
- Absence of lymphatics

The hair follicle IP is believed to be generated and maintained during each anagen phase (and then disassembled during catagen and telogen) in order to sequester potentially deleterious, anagen- and/or melanogenesis-associated autoantigens from immune recognition, especially via downregulation of MHC class I (Paus R et al. 2003). Such IP, accordingly, should be continuously maintained, and its efficacy might be subject to great variability in genetically distinct individuals. Its insufficient function or collapse could result in an autoimmune attack on the follicular epithelium and development of AA according to Paus et al.
The ‘immune privilege collapse model’ suggests the following scenario of AA pathogenesis: In a genetically susceptible individual, the aforementioned triggering factors, including psychoemotional stress, microtrauma, bacterial or viral infection, mast cell degranulation, or any other immuno-provocative insult lead to a peri- and/or intrafollicular rise in interferon (IFN)-γ secretion and upregulated expression of MHC Ia in the proximal hair follicle epithelium. This endangers maintenance of the hair follicle IP by potentially subjecting the follicular autoantigens to immune recognition. Consequently, once the hair follicle enters into anagen and active pigmentation is resumed, an as yet unknown autoantigen(s) is presented to the skin immune system. (Paus R et al. 2005).

**Animal models for AA**

In addition to ethical limitations of performing large-scale or therapeutic studies in men, the complexity of human AA and variations in clinical presentation, reflecting the mixed genetic background of the patients and the various environmental factors they are exposed to, entail employing appropriate animal models to explore specific aspects of the disease. Several animal species have been reported to develop hair loss resembling human AA, including dogs, cats, horses, rodents, and nonhuman primates (McElwee KJ et al. 2002). In the large species, however, AA is poorly characterized, the animals are outbred, and they are not readily available for study, which make them of little practical use as research models. Inbred rodents living in a controlled pathogen-free environment provide useful models due to their small size, and the extensive availability of rodent reagents for use in scientific research, and provide scientists with valuable tools to perform reproducible studies and a screening system for novel therapeutic approaches to the human disease. The C3H/HeJ mouse and the DEBR rat are the two extensively characterized and most frequently used models for AA and can be used for the study of genetic aspects, pathogenesis and therapy of the disease.
Severe combined immunodeficiency (SCID) mice

Another evidence implicating involvement of T cells in the pathophysiology of alopecia areata has been provided by studies on mice with severe combined immunodeficiency (SCID). In a set of experiments, Gilhar et al. (Gilhar A et al. 1998) demonstrated that lesional scalp skin grafts transplanted to SCID mice regrow hair, suggesting that AA affected hair follicles can resume hair growth once they are relieved from the deleterious effects of the immune system. When the recipient mice were injected with autologous T cells from lesional skin, AA was re-induced in the scalp explants. In particular, only T lymphocytes cultured with hair follicle homogenate and antigen presenting cells were capable of inducing AA in scalp explants. Both the clinical and histopathologic features of AA were reproduced in this model, including hair loss, perifollicular T lymphocyte infiltration, and expression of HLA-DR and ICAM-1 in the follicular epithelium. The induction of these changes was not a nonspecific effect of T cell activation, as lesions could not be produced by injection of IL-2 activated T cells from peripheral blood or scalp. Moreover, the fact that these changes could not be induced by lesional scalp T cells that were not cultured with follicular homogenate suggests that the T lymphocytes involved in AA are reacting to specific follicular antigens according to Gilhar et al (Gilhar A et al. 1998). As such, T cells targeted to follicular autoantigens appear to be key mediators in the pathogenesis of AA.

Dundee experimental bald rats (DEBR)

In DEBR rats, the initiation of overt onset of hair loss is most commonly observed in females aged 5–8 months and in males aged 7–10 months. Spontaneous expression of AA is apparent in 42% of individuals by 18 months of age in a colony; however, females are more likely to be affected than males by a ratio of approximately 3:1 (Lu W et al. 2006). Typically, alopecia first begins on the head, spreading backwards around the eyes, while separate patches of hair loss on the flanks gradually expand in size (McElwee KJ et al. 2002). These lesions may wax and wane and are frequently symmetrical. All types of hair
follicles are affected. The development of hair loss is correlated with the peri- and then intra-follicular mononuclear cell infiltration of hair follicles.

**C3H/HeJ mouse model for AA**

Aging C3H/HeJ mice develop a spontaneous, polygenic, inflammatory-based, AA-like hair loss. The first onset of AA develops from four months of age in females compared with six or more months in males, and affects up to 20% of a colony population with frequency equally distributed between males and females. Typically, alopecia initially develops on the ventral surface, expanding to cover the entire abdomen in a symmetrical pattern. Distinct foci of alopecia may develop elsewhere on the dorsal surface, bilaterally symmetrical or asymmetrical in appearance. Extensive hair loss, which may approach total body or universal alopecia, develops in 17% of AA-affected male and female mice. Complete, spontaneous remission is recognized in up to 3% of affected mice (Sundberg JP et al. 1994). Histologically, anagen hair follicles are affected by an intense peri-follicular infiltration of CD4+ and CD8+ T cells in a ratio of 1:2, respectively. Some dendritic cells and a variable number of granulocytes are also observed in the inflammation. Some T cells, primarily CD8+ cells, penetrate to intrafollicular positions in the bulb and infundibulum. Long term AA affected mice show the same infiltrate distribution with a reduced number of inflammatory cells.

The experimental use of the spontaneous C3H/HeJ mouse model is limited because affected mice are found in colonies in relatively low frequency, with unpredictable time of onset, and there is no easy way to determine what stage of disease they are in due to the waxing and waning nature of disease. McElwee et al. showed that AA can be serially transferred from AA affected C3H/HeJ mice to normal littermates (McElwee KJ et al. 1998). Skin grafts were removed from AA affected mice and transplanted onto normal haired recipient mice. Patchy hair loss was first observed on the ventral surface of recipient mice 49 to 63 days after surgery, later progressing to the dorsum similar to the progression of spontaneous AA observed in C3H/HeJ mice (figure 1.2). Histological studies showed a peri- and intra-follicular mononuclear cell infiltrate in both graft and
host skin typical of mouse AA. Sham grafting did not induce AA. The consistent induction of AA in C3H/HeJ mice provides a rapid, reliable model to study the initiation and progression of hair loss disease in a controlled and predictable manner.

AA in C3H/HeJ retains numerous similarities with human AA. Clinically, the disease is presented as patchy alopecia that wax and wane, migrate, and can become more extensive, as described above. Hair follicle dystrophy is associated with peri- and intra-follicular infiltration of CD4+ and CD8+ T cells as the dominant cell types, comparable with human AA. Only anagen hair follicles are affected with telogen and catagen hair follicles escaping from the attention of the immune system (Sundberg JP et al. 1994). AA is a non-scarring hair loss in both species with the possibility of spontaneous hair regrowth and a hair growth response to the same immunomodulatory therapies.

Hair follicle-specific IgG autoantibodies are present in significantly elevated concentrations in C3H/HeJ mice AA. These antibodies are also present, but in lower concentrations, in unaffected littermates without hair loss, but absent from strains of mice that have alopecia or inflammatory skin diseases other than AA (McElwee KJ et al. 1998). Antibodies in AA affected C3H/HeJ mice react with antigens in human hair follicles and vise versa.

The C3H/HeJ is an inbred strain; as such, the disease should be monomorphic between individuals and represent a specific type of AA if the disease is due to a single dominant or recessive gene. However, the unpredictable onset, variability in the phenotype presentation and expression frequency indicate that C3H/HeJ mouse AA is a complex, polygenic trait, like human AA (McElwee KJ et al. 2002). Genome-wide linkage analysis in C3H/HeJ mice has identified susceptibility intervals on mouse chromosomes 17, 9, 8, and 15 (Alaa1 – Alaa4) of which the chromosome 17 locus may be comparable to the human chromosome 6 interval found in humans (Sundberg JP et al. 2004).

Many associated symptoms and conditions have been reported in human AA that can also be found in mouse AA. C3H/HeJ mice occasionally exhibit nail deformities and retinal
dystrophy due to a genetic mutation *Pdeb* (McElwee KJ et al. 1998b). This strain of mouse also shows eczematous dermatitis in association with hair loss. Inflammatory bowel disease occurs in association with hair loss in C3H/HeJBir mouse substrain, which is developed from C3H/HeJ mice.

Comparable with humans, AA in C3H/HeJ mice is characterized with follicular antigen presentation and T cell activation. AA, induced by transferring skin grafts from AA affected to unaffected mice, is associated with increased expression of MHC class I and II antigens and ICAM-1 in the follicular epithelium, preceding the overt hair loss (McElwee KJ et al. 2002b, McElwee KJ et al. 2003). Further evidence has been provided by functional studies on C3H/HeJ mice. Transferring the combination of CD4+ CD25- cells and CD8+ cells from AA affected mice to their normal littermates results in significant hair loss (McElwee KJ et al. 2005). Inhibiting inflammatory cell migration using monoclonal antibodies (MAb) against CD44v10, commonly expressed on activated lymphocytes, blocks development of AA (Freyschmidt-Paul P et al. 2000). In addition, inhibition of AA onset has been demonstrated using MAb against CTLA-4 and B7 costimulatory antigens commonly expressed on antigen presenting cells (APCs) (McElwee KJ et al. 2002a).

Aberrant expression of several cytokines has been shown in mouse AA, including IL4, IL6, IL10, IL12, IFN-γ, and TNF-α (McElwee KJ et al. 2002b). In vitro and in vivo studies have shown that IFN-γ is able to induce expression of MHC-I and II antigens in the follicular epithelium and initiate the inflammatory response against the hair follicles in C3H/HeJ mice (except the injections were given systemically so the main action need not have been in the skin) (Paus R et al. 1999, Gilhar A et al. 2005). Furthermore, mice deficient for IFN-γ are completely resistant to the development of AA (Freyschmidt-Paul P et al. 2006). Mice deficient for IL10 or IL2 have also been shown to be partially resistant to the induction of AA (Freyschmidt-Paul P et al. 2006, Freyschmidt-Paul P et al. 2005). These and other data indicate important roles for cytokines in the development of hair loss in mouse AA.
Given the limited understanding of AA initiation and progression and limited success of therapeutic modalities, animal models appear vital in studying the pathogenesis of AA. C3H/HeJ offers the advantage of an inbred strain with a hair loss disease that exhibits numerous similarities to human AA, and can be reliably used in large-scale experiments that may be difficult or impossible to perform in humans.

**AA and the immune response**

The pathologic events that happen after initial onset of AA are not fully understood, but a significant body of evidence indicates that abnormal immune responses are involved in AA. As mentioned, affected hair follicles are heavily infiltrated by inflammatory cells, which consist mainly of T lymphocytes, macrophages, Langerhans' cells, and to lesser extent plasma cells (Tobin DJ 1997). While CD4+ T cells are preferentially located peri-bulgarly, skin biopsies from humans and rodents have demonstrated a predominance of CD8+ T cells in the intrafollicular infiltrate (Alexis AF et al. 2004). Taken together with increased expression of MHC class I and II antigens and endothelial cell adhesion molecule (ICAM)-1 in the proximal follicular epithelium (McElwee KJ et al. 2002), antigen presentation and T cell activation are suggested to play important roles in the development of AA.

In addition to the aforementioned HLA associations, increased antigen presentation, and autoreactive T cell activation, AA's associations with other autoimmune conditions suggest an autoimmune basis of the disease. The strongest associations have been shown with vitiligo and thyroid disorders (Shellow WV et al. 1992, Muller SAWR et al. 1963). The efficacy of immunomodulatory agents, including corticosteroids, photochemotherapy (PUVA), cyclosporin A, and topical sensitizers such as diphenylcyclopropenone in the treatment of AA further implicates immune responses in the pathogenesis of AA (Freyschmidt-Paul P et al. 2003).

The exact function of autoreactive T cells in AA, however, still remains an enigma. Antigen-specific activation of autoreactive T cells can initiate and/or propagate
autoimmune diseases by one of two distinct, yet often overlapping, pathways: humoral or cytotoxic (Chow S et al. 2005). Circulating antibodies to follicular structures have been found in both humans and animal models of AA, but their significance in the pathogenesis of AA is unclear (Hordinsky M et al. 2004). Antibodies to melanoma associated antigens as well as those bound to human anagen hair follicle extracts have been found in the sera of AA patients, but they were also found in the sera of some normal individuals (Alexis AF et al. 2004). The finding of AA in patients with impaired antibody production, such as in common variable immunodeficiency, has been further undermined the pathogenic role of autoantibodies in AA (McDonagh AJ et al. 1996).

A role for T cell mediated direct cytotoxicity against the follicular epithelium has been suggested based on observations of CD8+ T cell infiltration of intrabulbar region. Furthermore, it has been shown that the depletion of CD8+ cells by the intraperitoneal injection of monoclonal anti-CD8 antibodies partially restores hair growth in the Dundee experimental bald rat (DEBR) (McElwee KJ et al. 1996), suggesting a functional role for CD8+ cells in AA. In C3H/HeJ mouse model for AA, the transfer of CD8+ cells from AA affected mice into normal haired recipients resulted in localized hair loss exclusively at the site of injection (McElwee KJ et al. 2005). In contrast, some CD4+ and CD4+/CD25- cell-injected mice developed extensive, systemic AA, and a combination of CD8+ and CD4+/CD25- cells injected yielded the highest frequency of systemic AA induction. In another set of experiments, the activated CD8+ T cells derived from AA lesions were injected into the autologous human scalp grafts on SCID mice (Gilhar A et al. 2001), but failed to induce reproducible hair loss. However, when mice were injected with unseparated or the combination of CD8+ and CD4+ T cells, hair loss was reinduced. Accordingly, while CD8+ T cells appear to be functionally relevant in AA, their exact mechanism of function is presently unknown. Histological and electron microscopy studies have so far failed to show an association between the putative infiltrating cytotoxic cells and the potential target cells in AA (Tobin DJ et al. 1997). It is not currently known whether Fas- or perforin-mediated apoptosis of target cells occur in AA, and such studies are, therefore, of crucial importance in order to elucidate the role of T lymphocytes in the pathogenesis of AA.
Several clinical and experimental data point towards cytokines as potential inducers of hair loss in AA. Aberrant expression of cytokines such as IL-1β, IL-2, IL-6, IL-10, IL-12, TNF-α, IFN-γ, TGF-β have been widely reported in both human and mice with AA (Hoffmann R et al. 1994, McElwee KJ et al. 2002). Not only cytokines are involved in antigen presentation, initiation and propagation of immune responses, but they also play an important role in physiology and pathophysiology of the skin in general and hair follicles in particular. Several cytokines with hair growth inhibitory properties are reportedly upregulated in AA, including IL-1, IFN-γ, TNF-α, and IL-6 (Ito T et al. 2005, Yu M et al. 2008, Hoffmann R et al. 1996), suggesting that hair loss may occur because such cytokines interfere with the normal hair cycle, leading to premature precipitation of anagen hair follicles into catagen and telogen. This concept may explain typical clinical features of AA such as progression pattern in centrifugal waves and spontaneous hair regrowth in concentric rings, which suggests the presence of soluble mediators within affected areas of the scalp (Hoffmann R 1999).

Putting all together, the following mechanism of disease is suggested: after escaping the central tolerance and deletion in thymus, T cells with the potential to react with self antigens enter the peripheral blood and lymph nodes, where they encounter the self antigen(s) and become activated. Activated autoreactive T cells would then have to escape peripheral tolerance in order to proceed to the target organ, which is the hair follicle. The self antigen(s), which are normally sequestrated from the immune system, are now targeted as a result of the collapse of immune privilege in the hair bulb, leading to the disease induction. The exact factors that mediate the induction and/or progression of hair loss are not known at present, but possibilities include: (i) direct cytotoxicity against the follicular epithelium mediated by cytotoxic T cells or natural killer (NK) cells; (ii) antibody dependent cell-mediated cytotoxicity; (iii) cytokine-induced inhibition of the proliferation and/or differentiation of the follicular keratinocytes.

We, accordingly, sought to further study the nature of the immune response against HFs, using C3H/HeJ mouse model of AA. We hypothesized that if cell-mediated cytotoxicity
against HFs is a component of the hair loss, increased expression of typical cytotoxic products in the lesional skin, as well as elevated apoptosis activity within the follicular epithelium would be expected to occur in AA. In contrast, if cytokine-mediated hair loss is to be of any pathogenic significance, increased expression of cytokines is expected to be found in the skin and/or lymphoid organs of AA affected mice compared to normal haired controls. We, therefore, studied the mRNA expression levels of main Th1-, Th2, and Th17-associated cytokines, chemokines, and genes and products typical of cytotoxic cells in the skin, draining lymph nodes, spleen, and thymus of AA affected C3H/HeJ mice relative to their normal haired littermates, using reverse transcriptase PCR (RT-PCR) and immunohistochemistry.
Tables and figures

The figure has been removed because of copywrite restrictions.

Figure 1.1 Clinical findings in AA. AA is a relapsing-remitting, non-scaring, inflammatory disease of hair follicles manifesting as one or multiple patches of hair loss on the scalp or other parts of the body. In severe cases, all scalp or body hair may be lost. (Courtesy of the Department of Dermatology, University of Marburg).
Figure 1.2 The experimental induction of hair loss in C3H/HeJ mice. Donor AA affected mice were sacrificed, and full-thickness skin grafts (approximately 1-1.5 cm in diameter) were removed from the dorsal and ventral surfaces. Recipient mice from the same species were anesthetized, a circular piece of skin (approximately 1-1.5 cm in diameter) was removed from the dorsal surface and replaced with a donor skin graft. More than 95% of the recipient mice developed hair loss about 8 to 10 weeks after grafting, starting at the periphery of the grafts and extending to the other parts of the body.
References:


Chapter 2. Cell-mediated cytotoxicity

Introduction

Alopecia areata (AA) is a relapsing-remitting, non-scarring hair loss disease characterized by heavy inflammatory infiltration of anagen hair follicles (HF). Affected HFs leave anagen prematurely, resulting in massive telogen hair shedding and resultant alopecic patches that spread centrifugally. Histologically, there is a decreased ratio of anagen-telogen HFs with majority of HFs being in telogen or dystrophic anagen (Eckert J et al. 1968 and Tobin D.J. et al. 1995). AA is a polygenic trait with multiple recognized genes involved in disease susceptibility and various environmental factors in disease initiation and/or progression (Lu W et al. 2006).

Several mammalian species develop hair loss diseases that resemble AA in humans, of which C3H/HeJ mouse is the most available and extensively used. The genetics, histology, pathophysiology, and clinical presentation of the hair loss disease are comparable to human AA (McElwee KJ et al. 2002). Up to 20% of C3H/HeJ mice develop spontaneous hair loss by the age of 20 months (Sundberg JP et al. 1994). The disease can also be induced by grafting the lesional skin from AA affected mice to normal haired mice of the same strain. The recipient mice develop hair loss 8 to 12 weeks after grafting (McElwee KJ et al. 1998). This allows for the disease initiation and progression to occur in a predictable and controlled manner. As such, this inbred strain of mouse provides researchers with a rapid and reliable tool to study the genetic susceptibility, pathogenesis, and therapeutic approaches in AA.

The proximal segment of normal human and rodent’s HF is an area of relative immune privilege with reduced MHC expression. AA is believed to be the consequence of failure in maintaining such immune privilege (Paus R et al. 2003). The initiation of AA has been shown to be associated with increased expression of MHC antigens and intracellular

---

1 A version of this chapter will be submitted for publication. Barekatain A, Yu M, Sadeghi N, Lo B, Shapiro J, McElwee KJ
adhesion molecule (ICAM)-1 in the follicular epithelium and activated antigen presenting cells including macrophages and dendritic cells (Christoph T et al. 2000). It is postulated that exposure to certain environmental factors including psychoemotional stress, infection, microtrauma, or other immuno-provocative events leads to breakdown of HF immune privilege and presentation of as yet unknown autoantigen(s) to the immune system (Paus R et a. 2005).

Numerous experimental and clinical studies have demonstrated a pivotal role for T cells in the pathogenesis of AA. T cells constitute the primary cell population among the inflammatory infiltrate of HFs with the predominance of CD4+ T cells around, and CD8+ T cells inside, the affected HFs (Sperling LC and Lupton GP 1995). Antigen-specific T cell activation against the putative follicular target(s) in AA has been shown in a series of experiments on mice with severe combined immunodeficiency (SCID). The hair loss was re-induced in human lesional scalp skin explants on mice upon injection of activated autologous T cells. Intriguingly, only T cells cultured with hair follicle homogenate and antigen presenting cells were capable of inducing AA in scalp explants. Non-specific effect of T cells activation was ruled out as lesions could not be produced by injection of IL-2 activated T cells from peripheral blood or scalp (Gilhar A et al. 1998).

Although the functional relevance of T cells has been well documented in the literature, their precise mechanism of action in the development of AA still remains a mystery. Possibilities include direct cytotoxicity against the follicular epithelium mediated by cytotoxic T cells or natural killer (NK) cells, antibody dependent cell-mediated cytotoxicity, and T helper (Th)-1 or Th-2 cytokine-induced inhibition of hair growth (Bodemer C et al. 2000, Chow S et al. 2005, and Hoffmann R et al. 1994).

CD8+ T cell mediated cytolysis of the follicular epithelium has been offered as the possible mechanism of hair loss based on observation of their infiltrating the intrafollicular space (Sperling LC and Lupton GP 1995). Furthermore, the depletion of CD8+ cells by the intraperitoneal injection of monoclonal anti-CD8 antibodies has been shown to partially restore hair growth in the Dundee experimental bald rat (DEBR) (
McElwee KJ et al. 1996). The transfer of CD8+ cells from AA affected C3H/HeJ mice into the normal haired recipients also results in localized hair loss exclusively at the site of injection (McElwee KJ et al. 2005). In contrast, the activated CD8+ T cells derived from AA lesions and injected into the autologous human scalp grafts on SCID mice failed to induce reproducible hair loss (Gilhar A et al. 2002). Histological and electron microscopy studies of human AA did not exhibit any close association between T lymphocytes and the degenerative keratinocytes or melanocytes, the putative targets of the hair loss disease (Tobin DJ 1997). Such association appears crucial if cytotoxic cells are to be involved in direct cytotoxicity against the follicular epithelium.

Cytotoxic cells can induce apoptosis in target cells through two main mechanisms: (i) granule exocytosis pathway, involving secretion of the serine proteases granzyme A and B and the pore-forming molecule perforin; (ii) expression or release of tumor necrosis factor (TNF) family of ligands, including TNF-α, Fas ligand (FasL), TRAIL, whose interactions with corresponding death receptors result in cytolysis of target cells (Andersen MH et al. 2006).

If cell-mediated cytotoxicity is to be a component of hair loss mechanism in AA, it would be expected to see an increased apoptosis activity in the follicular epithelium of AA affected individuals, mediated by either one or both cytolytic pathways mentioned above. Surprisingly, such a fundamental concept had not been addressed thoroughly in the previous studies of the pathogenesis of AA. Accordingly, we set up a series of experiments to further elucidate the role of infiltrating inflammatory cells in the development of AA, and to examine the relevance of cell-mediated cytotoxicity to the hair loss mechanism, using C3H/HeJ mouse model for AA. Two principle questions that were addressed in this study are whether expression profiles and patterns of genes and products typical of cytotoxic cells are consistent with direct cytotoxicity against the follicular epithelium, and whether an increased apoptosis activity of HFs is a component of the hair loss disease development.
Material and methods

Animals

We used C3H/HeJ mice as our model for alopecia areata (AA). Mice without hair loss were purchased from The Jackson Laboratories (Bar Harbor, ME). Induction of hair loss was achieved by grafting full-thickness alopecic skin from AA-affected mice onto the back of normal mice according to the techniques as described before (McElwee et al. 1998). Noticeable hair loss occurred in more than 95% of recipient mice by the twelfth week after grafting. Three to six months after grafting, AA-affected mice were sacrificed and skin samples were collected for reverse transcriptase-PCR or immunohistochemical studies. All normal control mice were age and sex matched. All animal studies were conducted according to the institutional guidelines with ethics committee approval.

Immunohistochemistry

Longitudinal skin sections from 6 AA-affected and 6 normal mice (18- to 20-month-old females were embedded in the O.C.T compound (Sakura, Torrance, CA), snap frozen and stored overnight at -80°C before being cut onto aminoalkylsilane coated slides (Sigma-Aldrich, Oakville, Canada). Sections were fixed in 4% paraformaldehyde for 10 minutes and air dried. Non-specific antibody binding was prevented by incubating sections with 10% normal donkey serum (Santa Cruz biotechnology, Santa Cruz, CA) or 10% normal rabbit serum (Vector Laboratories, Burlingame, CA) for 90 minutes as appropriate. Endogenous biotin activity was blocked by application of an avidin-biotin blocking kit solution (Vector Laboratories, Burlingame, CA). Sections were subsequently incubated with the following primary antibodies for 2 hours at room temperature: Goat anti mouse granzyme B (clone N-19), rabbit anti mouse Fas (clone FL-7886), and rabbit anti mouse FasL (clone N-20, all from Santa Cruz), rat anti perforin (clone CB5.4, Abcam Inc, Cambridge, MA), or rat anti CD8 (clone 53-6.7, BD Bioscience, Mississauga, Canada) all diluted in the antibody diluent with background reducing components (Dako, Mississauga, Canada).
Sections were then incubated with the following biotinylated secondary antibodies, diluted 1:100 in PBS buffer for 30 minutes at room temperature: donkey anti-goat IgG-B (Cat. No. sc-2042), donkey anti-rabbit IgG-B (Cat. No. sc-2089, both from Santa Cruz), and rabbit anti-rat IgG (Cat. No. BA-4001, Vector Laboratories). Avidin DH and biotinylated alkaline phophatase H (ABC-AP, Vector Laboratories) was used as the enzyme. To further amplify the signals, sections were additionally incubated with the primary antibodies overnight at 4°C, corresponding secondary antibodies and ABC-AP. The signal was developed using Vector Red (Vector Laboratories, Burlingame, CA). Endogenous alkaline phosphatase activity was inhibited by the addition of levamisole (Vector Laboratories, Burlingame, CA) to the buffer used to prepare the substrate solution. Finally, sections were counterstained with Mayer's hematoxylin, dehydrated, cleared and permanently mounted in non-aqueous media (Fisher Scientific, Ottawa, Canada). Negative controls were included on all slides by replacing primary antibody with the diluent.

**Real-time reverse transcriptase-PCR analysis for gene expression**

Six AA affected mice and 6 normal haired littermates were sacrificed. Tissue samples from the skin, draining lymph nodes, spleen and thymus were excised, placed in RNA stabilizing reagent (RNAlater, Qiagen, Mississauga, Canada) and stored at -80 until used. Total RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen, Mississauga, Canada). Eleven apoptosis-associated molecules were chosen for RT-PCR analysis. The sequences of the complete genome were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). A pair of oligonucleotide primers was designed for each sequence using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with the following criteria: 50-65°C melting temperature, 40-60% G+C content, 18-25bp primer length, and 75-250bp amplicon size. Primers were tested for their specificity for the target genes and redesigned when necessary. The primers’ sequences are listed in table 2.1.
Two micrograms of total RNA from each sample were subjected to reverse transcription using the Superscript first-strand cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The RT-PCR reactions were conducted using a total of 20μL of reaction mixture (5 μL of cDNA, 10 μL of SYBR Green PCR Master Mix (Finnzymes, Burlington, Canada), 5 μL of each 5 μmol/L forward and reverse primers (Invitrogen Life Technologies, Carlsbad, CA) in an Opticon™ DNA Engine (MJ Research, MA, USA). The PCR program was initiated for 10 minutes at 95°C before 41 thermal cycles, each of 15 seconds at 94°C, 30 seconds at 60°C and 30 seconds at 72°C. β-actin was used as the reference gene for all analyses described.

For data analysis, relative quantification was used to determine the ratio between the quantity of a target molecule in a sample (AA-affected) and in the calibrator (normal haired controls). Both the sample and the calibrator data were first normalized against variation in sample quality and quantity and normalized values, ΔC(t)s, were calculated. The ΔΔC(t) was then determined using the following formula: ΔΔC(t) = ΔC(t)sample-ΔC(t)calibrator. At the end, the expression level of the target gene normalized to the reference gene and relative to the calibrator was determined as 2^-ΔΔC(t). Melting curves for each PCR reaction were generated to ensure the purity of the amplification product.

For statistical significance, the ΔCt values for AA affected mice and normal haired controls were subjected to student-T-test, which yielded the estimation of ΔΔCt. P-values were derived from testing the null hypothesis that ΔΔCt are equal to 0. Therefore, a small P-value (<0.05) indicates that the ΔΔCt is significantly different from 0, which demonstrates a significant effect in the mRNA expression level of target molecule in AA affected mice relative to their normal haired littermates (appendix 1).

Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Snap-frozen, longitudinal skin sections from six AA affected and six normal haired mice were fixed in 4% paraformaldehyde solution in 0.1 M phosphate buffer at room temperature for 20 minutes and subjected to the TUNEL assay by using the In Situ Cell
Death Detection Kit, alkaline phosphatase (Roche Applied Science, Mannheim, Germany) following the protocol of the manufacturer. Briefly, 10\(\mu\)m thick skin sections were washed in PBS, PH 7.4, for 30 minutes, incubated in the freshly prepared 0.1% Triton X-100, 0.1% sodium citrate permeabilizing solution for 2 minutes on ice, and rinsed twice in PBS. The sections were then incubated with terminal deoxynucleotidyl transferase (TdT) for 60 minutes at 37°C in a humidified chamber in the dark, in order to label DNA strand breaks. One negative control per slide and at least two positive controls were included in each experimental set up. For negative controls, TdT was replaced with the label solution provided by the manufacturer, while positive controls were incubated with 300 U/ml DNase I recombinant (Roche Applied Science, Mannheim, Germany) diluted in 50mM Tris-HCL, PH 7.5, 10 mM MgCl₂ and 1 mg/ml BSA solution for 10 minutes at room temperature to induce DNA strand breaks, prior to labeling procedures. Labeling of DNA strand breaks was followed by applying the alkaline phosphatase anti-fluorescein antibody (Converter-AP) in order to detect the incorporated fluorescein. Finally, the sections were developed with Vector Red (Vector Laboratories, Burlingame, CA) in TBS for 30 minutes at room temperature, counterstained with hematoxylin, and mounted in non-aqueous media.

**Alcian Blue-Safranin staining of mast cells**

Snap-frozen skin sections were stained with alcian blue 8GX (product No. A3157, Sigma-Aldrich Canada Ltd, Oakville, Canada) for 20 minutes followed by washing for 5 minutes in 0.7 N HCL. The alcian blue solution was prepared in the following way: 1 gram of alcian blue powder was dissolved in 100 ml of 0.7 N HCL. Concentrate HCL was added to lower the solution to PH 0.2, in order to allow stained mast cells to stand out clearly against the underlying tissue. Some slides were then used in immunohistochemical studies (above). Other sections were then counter-stained in 0.5 % Safranin O (product No. S8884, Sigma-Aldrich Canada Ltd, Oakville, Canada) in 0.125 N HCL for 5 minutes, rinsed in tap water, and were dehydrated, cleared, and mounted for mast cell number analysis.
Cell counting and data analysis

Mast cells and granzyme B+ cells were counted separately under the magnification of 10X, in at least 10 visual fields per section, three to five sections per specimen. The average number of cells was then calculated per square millimeter of the skin in AA affected mice and their normal haired littermates (n=6 each).

Combined histological and immunohistological staining was achieved by first incubating skin sections with the dye alcian blue, as discussed above. Sections were then washed in tap water, and were subjected to the aforementioned immunohistochemical staining protocol, using antibodies against mouse granzyme B, Fas, and FasL. The total number of mast cells, the number of mast cells stained positively for granzyme B, the number of mast cells expressing FasL, the total number of granzyme B+ and FasL+ cells were counted under the magnification of 40X, in at least 10 visual fields per section, three sections per specimen. The proportion of mast cells expressing granzyme B or FasL, as well as the proportion of granzyme B+ or FasL+ cells which were also stained for mast cells, were calculated in the AA affected and normal haired mice (n=6 each).

For statistical significance, data were analyzed using student-T-test. A P-value of less than 0.05 indicates that there is a statistically significant difference in the number and/or proportion of cells between AA affected mice and their normal haired littermates.
Results

**Granzyme expression.** RT-PCR revealed a marked increase in the mRNA expression levels of granzymes A and B in the skin of AA affected mice compared with normal haired controls (figure 2.1). They were also upregulated in the skin draining lymph nodes, spleen and thymus of mice with AA (except for granzyme A in the spleen, all changes were statistically significant). Immunohistochemical studies on the frozen skin sections confirmed the results of RT-PCR; granzyme B producing cells were scattered throughout the dermis, mainly around the distal portion of hair follicles, at the junction of the dermis and subcutaneous tissue and throughout the subcutaneous tissue. Moreover, granzyme B was expressed by the epidermal and follicular epithelium in both the AA and normal skin sections (figure 2.2). While infiltrating granzyme B+ cells were abundant in the AA affected skin, they were considerably less populated in the normal skin sections. Averagely, more than 20 cells were counted per square millimeter of the skin in AA affected mice; whereas only less than 2 cells per square millimeter were identified in the normal skin sections (figure 2.3).

**Perforin expression.** The mRNA expression of perforin was only modestly upregulated in the skin of AA affected mice, in contrast to the increase in the mRNA levels of granzymes A and B (figure 2.1). Furthermore, immunohistological studies revealed a limited number of perforin expressing cells in the skin of AA affected mice, distributed diffusely rather than located exclusively in the anticipated peri-follicular infiltrate location. Perforin expressing cells were not identified in the skin sections from the normal haired mice (figure 2.2).

**Fas and Fas ligand (FasL) expression.** mRNA expression levels of FasL were dramatically higher in the skin of AA affected mice compared with normal haired controls, as revealed by RT-PCR (figure 2.4). Using immunohistochemistry, FasL expression was further studied in the skin sections from the normal and AA affected mice. Infiltrating FasL+ cells were plentiful in AA affected mice, spread throughout the dermis and not necessarily in the focal infiltrate of hair follicles. FasL was expressed strongly by the epidermal and follicular keratinocytes as well, in both the normal and AA...
affected skin sections. However, inflammatory FasL expressing cells were identified in substantially fewer numbers in the normal skin (figure 2.5).

Fas mRNA levels were slightly, but significantly, upregulated in the skin of AA affected mice (figure 2.4). Infiltrating Fas+ cells were found in a similar expression pattern as FasL expressing cells in AA affected mice, diffused across the skin sections. Infiltrating Fas expressing cells were also present in the normal skin sections, but in a significantly reduced numbers. Like FasL, Fas was expressed by the inner and outer root sheaths of hair follicles as well as the epidermal epithelium (figure 2.5).

Other members of TNF family of ligands and receptors. mRNA expression levels of TNF-α, along with only one of its receptors TNF-R2, were significantly upregulated in the skin of AA affected mice compared with normal haired littermates. TRAIL-R mRNA expression was down-regulated (p-value<0.05) in the skin of AA affected mice; other members did not show significant changes between the normal and AA affected skin (figure 2.6).

CD8+ cells. Immunohistochemical studies on the frozen skin sections exhibited a very dense infiltration of CD8+ cells, mainly intra-follicular, in AA affected mice. They were also detected, in fewer numbers, in the upper dermis and epidermis. CD8+ infiltrating cells were not identified in the normal skin sections (figure 2.5).

Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). TUNEL technology was employed in order to detect and quantify apoptosis activity, at the single cell level, in the skin. Significant apoptosis activity was identified associated with the inflammatory infiltrate of hair follicles in all the AA affected skin sections studied. Apoptosis was also detected in the AA affected hair follicles; however, the extent of apoptosis activity was largely variable between different hair follicles in the same tissue section and between the sections from different AA affected mice. Overall, a limited number of apoptotic cells were identified in the AA affected follicular epithelium, mainly in the inner and outer root sheaths (figure 2.7).
Apoptosis activity was confined to the follicular and epidermal epithelium in the skin of normal haired mice. Apoptotic cells were mostly in the inner and outer root sheaths of the hair follicles and, in lesser numbers, in the matrix and hair cortex. The proportion of hair follicles with any extent of apoptosis activity was not significantly different between the normal and AA affected skin (figure 2.7).

**Mast cells.** The majority of granzyme B+ and FasL+ cells were morphologically consistent with mast cells: large cells with round to oval nuclei. Accordingly, in order to further characterize these cells, immunohistologically developed skin sections were incubated with Alcian blue; a phthalocyanine dye that stains the acid mucopolysaccharide content of mast cells' granules. Mast cells were identified in both the normal and AA affected skin sections, mostly located in the superficial papillary dermis and at the junction of the dermis and subcutaneous tissue (figure 2.8). Mast cells were significantly more populated in the AA skin sections (p-value<10^-30) than in the normal sections (figure 2.9).

Granzyme B was identified in the granules of mast cells (figure 2.8). Immunohistochemical and histological staining also revealed that skin mast cells from AA affected mice were more active in terms of producing granzyme B than those from normal mice. A greater proportion of mast cells were stained positively with anti-granzyme B antibody in AA affected skin than in normal skin sections (figure 2.9). Mast cells were also shown to express Fas and FasL, with FasL expressing cells occurring more abundantly in the AA affected skin (figures 2.8 and 2.9). Also of note, the vast majority of granzyme B producing and FasL expressing cells, in both the normal and AA affected mouse skin, were revealed to be mast cells (figure 2.9).
Discussion

Granzyme B may play an important, perforin-independent, role in the inflammatory hair loss disease mechanism

The marked increase in the mRNA expression of granzyme A and granzyme B in the skin of AA affected mice, revealed by RT-PCR, suggests they may play an important role in AA pathogenesis. This is further supported by our observations from immunohistochemistry where granzyme B expressing cells were found to be substantially more abundant in the skin sections from AA affected mice than from normal haired controls.

Granzyme B is the most studied member of the family of granzymes, involved in immune-mediated killing of virally infected and tumor cells, as well as autoimmune diseases and transplant rejection. granzyme B is expressed in cytotoxic T cells, natural killer (NK) cells, mast cells, blood basophils, CD4+/CD25+ regulatory T cells, and keratinocytes (Chamberlain CM and Granville DJ. 2007). Translated as a zymogen, granzyme B is proteolytically activated by cathepsin C in the cytoplasmic granules, thus protecting the effector cell from autolysis. Upon activation, cytotoxic cells exocytose the constituents of their granules, including granzyme B and perforin, into the immunological synapse formed between the effector and target cells. Granzyme B is then endocytosed independently through its receptor mannose 6 phosphate (M6P) into the target cell and the endoplasmic reticulum (Veugelers K et al. 2006).

Granzyme B is absolutely dependant on perforin in order to enter the cytoplasm and initiate the apoptotic pathways (Pinkoski MJ et al. 1998). Therefore, in the absence of perforin, granzymes are regarded as incapable of inducing rapid target cell apoptosis during granule-mediated killing (Froelich CJ et al. 1996). However, granzyme B has also a potent extracellular matrix remodeling activity. In the absence of perforin, granzyme B can induce cell detachment and anoikis in certain cells, including endothelial and vascular smooth muscle cells, by cleaving extracellular proteins such as fibronectin, vitronectin, and laminin (Buzza MS et al. 2005 and Choy JC et al. 2004).
RT-PCR showed only a modest increase in the mRNA expression of perforin and immunohistochemistry revealed a very limited number of perforin expressing cells in the skin of AA affected mice compared with normal haired controls. These observations suggest that, if granzyme B plays any role in mouse AA, it could have some perforin-independent effects on the follicular epithelium.

Granzyme B is one of the key mechanisms through which CD4+/CD25+ T regulatory (Treg) cells execute their immunosuppressive functions. Proper suppression of CD4+ T cell proliferation by Treg cells is shown to be dependent on granzyme B, and this occurs in a perforin-independent fashion (Gondek DC et al. 2005). Treg cells, on the other hand, are thought to play an important part in peripheral self-tolerance and in preventing and confining autoreactivity (Loser K and Beissert S. 2007). Studies on C3H/HeJ mice have demonstrated a low expression level for Treg cells in the skin of AA affected mice relative to their normal haired littermates (Zöller M et al. 2002). Taken together with our observations where mast cells proved to be the major cellular source of granzyme B, as discussed below, Treg cells do not appear as a potential source of granzyme B in the skin of AA affected mice. Therefore, quantitative and/or functional defects of Treg cells may be a contributing factor in the failure of immune homeostasis in the inflammatory hair loss disease AA.

In agreement with our data, Pardo et al. have recently shown that mast cells from mouse skin can express granzyme B (Pardo J et al. 2007). They also suggested that granzyme B secreted by mast cells can facilitate trans-endothelial migration of leukocytes and their recruitment to the skin by inducing the disorganization of endothelial cell-cell contacts. Mast cells’ involvement in leukocyte migration has also been shown by others (Cavill I et al. 1977 and Huang C et al. 1998). Furthermore, it has been demonstrated that mouse skin mast cells can promote the migration of dendritic cells into the skin and lymph nodes (Heib V et al. 2007, Jawdat DM et al. 2004, and Suto H et al. 2006). Given the fact that the majority of granzyme B producing cells in the mouse skin proved to be mast cells, as revealed by our observations from histological and immunohistological studies on the
mouse skin sections, it is suggested that granzyme B-producing mast cells might in principle play an important part in recruiting antigen presenting cells and other leukocytes to the skin of AA affected mice.

**Mast cells may be involved in the pathogenesis of AA through a number of potential mechanisms of action**

To our best knowledge, we are the first to report a substantially increased number of mast cells in the alopecic lesions of C3H/HeJ mouse model of AA, compared with normal haired controls. Mast cells are highly granulated tissue dwelling cells, widely distributed throughout the body in connective tissues and on mucosal surfaces (Puxeddu I et al. 2003). Mast cells have been mostly known for their pivotal role in allergy; upon interaction between allergens and surface IgE receptors, mast cells become activated, resulting in the release of a series of preformed and rapidly synthesized substances that mediate allergic reactions (Borish L and Joseph BZ 1992). AA, on the other hand, has been shown to occur in association with atopic disease; up to 60 percent of AA patients have been reported to have atopic history (Tan E et al. 2002, Sharma VK et al. 1996, and Sukhjot S et al. 2001). Furthermore, AA tends to manifest more severely in the presence of atopy (Sukhjot S et al. 2001, Goh C et al. 2006, and De Weert J et al. 1984). Therefore, it is not unreasonable to postulate that mast cells may be important determinants of the course and severity of AA.

Recently mast cells have been proven to play an important role in stress-induced hair loss. It was shown that mice exposed to sonic stress exhibit premature hair growth regression (catagen) associated with skin mast cell activation (Arck PC et al. 2003); furthermore, mast cell deficient W/W<sup>v</sup> mice were resistant to stress-triggered premature catagen induction (Arck PC et al. 2005). It is believed that mast cells’ hair growth inhibitory properties are mediated, at least in part, by the neuropeptide substance P (SP) (Arck PC et al. 2001). In fact, SP has been shown to be substantially involved in the pathogenesis of AA; during the development of mouse AA, the number of SP-immunoreactive nerve fibers is increased in the skin, and that the additional supply of SP
to the skin of AA affected C3H/HeJ mice resulted in a significant increase of mast cell degranulation and hair follicle regression (catagen) (Siebenhaar F et al. 2007). While SP-induced inhibition of hair growth has been demonstrated, as mentioned above, other products of mast cells, including granzyme B, may also play a significant part, and further studies are warranted in order to elucidate their role in hair loss mechanisms. These findings, nonetheless, provide invaluable evidence to support a linkage between AA and the brain, with mast cells possibly being important players involved in neuroimmune interactions modulating the development of AA.

Hair growth inhibitory properties of mast cells have been illustrated in a number of studies; Maurer et al. have shown that mast cell activation results in induction of premature and dystrophic catagen hair follicles, and inhibitors of mast cell degranulation retard normal catagen development (Maurer M et al. 1997). Given their ability to generate a battery of potent growth-modulatory agents, including biogenic amines, proteoglycans, proteases, leukotrienes, prostaglandins, and cytokines, mast cells have been proposed as important modulators of hair follicle growth cycle (Maurer M et al. 1995).

Recently, however, some research work has shown a potential immuno-protective role for mast cells. First, it was observed that cultures of Treg cells tend to be contaminated with 'unwanted' mast cells (Cobbold SP et al. 2003). Furthermore, when mice were transplanted with skin grafts, it was found that not only the grafts were infiltrated with Treg cells but also with mast cells (Zelenika D et al. 2001). Lu et al. extended these findings by using mice deficient in mast cells as transplant recipients where their efforts to induce therapeutic tolerance failed, as all the grafts were rejected. Intriguingly, however, when they injected the recipient mice with mast cells, the host’s ability to accept the grafts for prolonged time was restored (Galli SJ et al. 2005). Based on these observations, and given the critical positioning of mast cells around the blood vessels, nerve fibers, and hair follicles, mast cells were proposed as potential inducers of the peripheral tolerance in immune privileged sites (Waldmann H 2006); therefore, their
malfunction might be involved in the collapse of the hair follicle immune privilege, which is believed to be responsible for the development of AA (Paus R et al. 2005).

Alopecia areata is not the only hair loss disease with aberrant mast cell features. Some forms of scarring alopecias have been reportedly associated with mastocytosis and increased number of skin mast cells (Xu X et al. 2003, and Reed RJ et al. 1973). Actively degranulating mast cells have also been shown in close association with infiltrating mononuclear cells within follicular sheaths in male pattern alopecia (Jaworsky C et al. 1992). Mast cells, therefore, may play important roles in hair growth disorders.

RT-PCR and immunohistological studies of mouse skin revealed a substantially elevated mRNA and protein expression of FasL in AA affected mice. This is consistent with studies on Fas and FasL deficient mice where it was shown that in the absence of FasL, mouse is relatively resistant to the development of AA (Freyschmidt-Paul P et al. 2003). Here we show for the first time that mast cells are the primary cellular source of FasL in the skin of AA affected mice; more that 95 percent of FasL expressing cells turned out to be mast cells. This further supports our putative hypothesis that mast cells could play an important pathogenetic role in AA.

In spite of increased expression of FasL in the mast cells of AA affected skin compared to normal skin, no significant difference in apoptosis activity was found between hair follicles of normal and AA affected mice, suggesting that mast cells may not be involved in FasL-mediated cytotoxicity in mouse AA. This is consistent with a previous study where murine mast cells were revealed to express FasL but unable to induce lysis in target cells (Wagelie-Steffen AL et al. 1998). FasL protein was shown to be exclusively located within the cell; and, therefore, it was suggested that the inability of mast cells to execute FasL-mediated cytolysis is due to their failure to express FasL on the cell surface. We have also shown that mast cells express Fas; however, the functional significance of the Fas-FasL pathway in mast cells is not exactly known.
Cell-mediated cytotoxicity appears as an unlikely mechanism of hair loss in chronic mouse AA

We identified a dense follicular infiltration of CD8+ cells in the AA affected mice. This has also been shown in a number of other studies in both humans and mice (McElwee KJ et al. 2003, Todes-Taylor N et al. 1984, Hull SM et al. 1991, and McElwee KJ et al. 2002). To examine whether infiltrating CD8+ cells are involved in cellular cytotoxicity against the follicular epithelium, we studied expression levels and patterns of genes and products typical of cytotoxic cells. It was revealed that the distribution patterns of granzyme B and FasL expressing cells are not consistent with that of CD8+ cells. While CD8+ cells were identified mostly in the focal infiltrate of hair follicles in the AA affected mice, granzyme B and FasL positive cells were scattered throughout the dermis and not exclusively in the peri-follicular region. In addition, more than 90 percent of granzyme B and FasL expressing cells proved to be mast cells. The few identified perforin expressing cells in the skin of AA affected mice demonstrated no association with the peri-follicular infiltrate. These observations may be interpreted as failure of peri- and intra-follicular infiltrating CD8+ cells to express either FasL, granzyme B or perforin.

Our data is in agreement with what others have found in studies of AA in humans; in a study of chronic AA patients, Sato-Kawamura et al. also failed to demonstrate any association between the inflammatory infiltrate of hair follicles and FasL or perforin expressing cells (Sato-Kawamura M et al. 2003). Kehren and colleagues showed that in order for CD8+ T cells to mediate the skin inflammation through their cytotoxic activity, they have to use either the granzymes-perforin or the Fas-FasL pathways. Furthermore, they demonstrated that CD8+ T cells from mice double deficient for perforin and FasL are not able to execute their lytic activity (Kehren J et al. 1999). Taken together, a role for CD8+ T cells in direct cytotoxicity against the follicular epithelium appears unlikely in AA.
Given the unlikely involvement of CD8+ T cells in cell-mediated cytotoxicity against the follicular epithelium in mouse AA, a question is raised about the functional role of infiltrating CD8+ T cells. Recently a subset of CD8+ T cells, known as CD8+ regulatory T (CD8+ Treg) cells, has been shown to play an important role in regulation of a number of autoimmune diseases (Najafian N et al. 2003). CD8+ Treg cells, characterized as CD8αα+ TCRαβ+ cells, are believed to be involved in a negative feedback mechanism for the regulation of pathogenic CD4+ T cells reactive to a self-Antigen (Tang X et al. 2006). Little is known about their role in AA.

CD8+ T cells can also produce a battery of cytokines and chemokines. Upon activation, CD8+ T cells secrete TNF-α and IFN-γ, among other cytokines (Walter U and Santamaria P 2005). Not only can these cytokines boost antigen presentation by the follicular epithelium and promote maturation and antigen presentation of infiltrating dendritic cells, but also they exhibit strong hair growth inhibitory properties (McDonagh AJ et al. 1993, Ito T. et al, 2005). Chemokines are the main regulators of lymphocyte trafficking and recruitment, and CD8+ T cells have been demonstrated as potential producers of such chemokines as CXCL10 and RANTES (Ejrnæs M et al. 2005). Accordingly, CD8+ T cells may play a role in autoimmune processes by recruiting other immune cell types to the site of inflammation.

We used the mAb 53-6.7 which reacts to the α chain of CD8 (CD8a). In addition to cytotoxic T (Tc) cells and CD8+ Treg cells, CD8a is also expressed on subsets of γδ TCR-bearing T cells, and dendritic cells (MacDonald HR et al. 1990, and Vremec D et al., 1992). Accordingly, Immunofluorescent studies with a panel of antibodies against the α and β chains of CD8 and different subunits of TCR receptor appear appropriate in order to further characterize the population of infiltrating CD8+ cells in the skin of AA affected mice.

If cell-mediated cytotoxicity was to be a component of hair loss in mouse AA, an increased apoptosis activity in HFs of AA affected mice would be expected. However, our findings from TUNEL studies showed no significant difference in apoptosis activity
between hair follicles from AA affected and normal haired mice. Taken together, our data is not consistent with cell-mediated apoptosis of hair follicles as a mechanism of hair loss in mouse AA.

Conclusion

Given the relatively limited apoptosis activity in the follicular epithelium of AA affected mice and lack of conclusive evidence for the lytic activity of CD8+ cells, cellular cytotoxicity against the follicular epithelium seems to be an unlikely mechanism of the hair loss in chronic mouse AA. Accordingly, the aberrant hair growth cycle and the formation of dystrophic anagen hair follicles may not be the result of altered apoptosis activity in AA affected hair follicles. On the other hand, mast cells, with their potential hair growth inhibitory properties and their involvement in allergic reactions and stress-induced alopecia, may play more important roles in the pathogenesis of AA than previously thought.
## Tables and figures

**Table 2.1: sequences of primers used in RT-PCR (from 5’ to 3’)**

<table>
<thead>
<tr>
<th>target gene</th>
<th>forward primer sequence</th>
<th>reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granzyme A</td>
<td>ATGGGAGGAGACACGGTTGT</td>
<td>GCCTCGCAAAATACCATCACA</td>
</tr>
<tr>
<td>Granzyme B</td>
<td>TCGACCCTACATGGCCTTAC</td>
<td>TGGGGAATGCAATTACACC</td>
</tr>
<tr>
<td>Perforin</td>
<td>TGAGGTAGGAGACTGCTGAA</td>
<td>ATAGCCTGTCTCAGAGGCTCC</td>
</tr>
<tr>
<td>Fas</td>
<td>ATGCACACTCTGCGATGAAG</td>
<td>CAGTTTCACAGCCAGGAGA</td>
</tr>
<tr>
<td>FasL</td>
<td>GCCCATGAATTACCCATGTCC</td>
<td>CGGTGGTAGGTGTGACACAG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CTTCAATGGGCTGGGACTAA</td>
<td>GGCTCAGTAAAGGTGCAAAGC</td>
</tr>
<tr>
<td>TNFR1</td>
<td>GAACCCAGTTCACAACGCTACC</td>
<td>CCTACAGGGGATTCACGC</td>
</tr>
<tr>
<td>TNFR2</td>
<td>TACCAAGGTGGCATCTCTC</td>
<td>AGGGCTTTTCTTCTCCTGC</td>
</tr>
<tr>
<td>TRAIL</td>
<td>CCCTGCTTGAGGTAAAGAG</td>
<td>GGCTAAGGTCTTCCATCC</td>
</tr>
<tr>
<td>TRAILR</td>
<td>CCGACAGACATCTAGCAGCA</td>
<td>TTACCGAGACAGCAACCTTC</td>
</tr>
<tr>
<td>TRAMP</td>
<td>CCAGGTGCTTTCTAGGAGTC</td>
<td>TGAGAGATGGGAGGTCTGTG</td>
</tr>
<tr>
<td>Annexin V</td>
<td>AACCTGTGACATCCGAAG</td>
<td>AGAGTCGTGAGGGTCATC</td>
</tr>
<tr>
<td>Caspase3</td>
<td>ATGGGAGCAAGTGCTGAGC</td>
<td>CAGAGCGAGATGACATTTCCA</td>
</tr>
</tbody>
</table>
Figure 2.1 Granule exocytosis pathway gene expression in murine alopecia areata.

Y-axis shows fold change in the mRNA levels of granzyme A, granzyme B, and perforin in AA affected mice compared with normal haired controls (n=6 each). The expression levels of target genes were studied in the skin and lymphoid organs of C3H/HeJ mouse model for AA, using quantitative RT-PCR. For statistical significance, the ΔCt values for AA affected and normal haired mice were subjected to student-T-test, which yielded the estimation of ΔΔCt. A small P-value (<0.05), marked with an asterisk, indicates that the ΔΔCt is significantly different from 0. AA, alopecia areata; Ct, threshold cycle; ΔCt, Ct(target) – Ct(reference); ΔΔCt, ΔCt(sample) - ΔCt(calibrator).
Figure 2.2 Immunohistochemical studies of granzyme B and perforin. Granzyme B expressing cells (marked with arrows) are shown in the skin of (a and b) AA affected and (c) normal haired mice. While granzyme B+ cells are frequent in the skin of AA affected mice, they are virtually absent from the skin of normal haired controls. Infiltrating granzyme B+ cells are scattered throughout the dermis and subcutaneous tissue, and not exclusively located in the focal infiltrate of hair follicles. (d and e) Perforin+ cells in the skin of AA affected mice are marked with arrows. The few identified perforin expressing cells do not show any association with the perifollicular infiltrate. (f) Perforin+ cells are absent from the normal skin (n=6 each). AA, alopecia areata; GrB+, granzyme B+ cells; NL, normal haired; Per+, perforin+ cells.
Figure 2.3 The average number of granzyme B expressing cells per square millimeter of the skin. Cells were counted under the magnification of 10X, in at least 10 visual fields, three sections per specimen (n=6 each). The average number of granzyme B+ cells per square millimeter of the skin differ significantly between AA affected mice and their normal haired littermates (P-value<0.001), as revealed by student-T-test.
Figure 2.4 The Fas-FasL pathway gene expression in murine alopecia areata. There is a marked increase in the mRNA expression level of FasL in the skin of AA affected mice compared with normal haired controls, as revealed by quantitative RT-PCR (n=6 each). Expression of the target gene normalized to the reference gene and relative to calibrator (control) is given by $2^{\Delta\Delta Ct}$. For statistical significance, the $\Delta Ct$ values for AA affected and normal haired mice were subjected to student-T-test, which yielded the estimation of $\Delta\Delta Ct$. A small P-value (<0.05), marked with an asterisk, indicates that the $\Delta\Delta Ct$ is significantly different from 0. AA, alopecia areata; Ct, threshold cycle; $\Delta Ct$, Ct(target) – Ct(reference); $\Delta\Delta Ct$, $\Delta Ct$(AA) - $\Delta Ct$(normal); FasL, Fas ligand.
Figure 2.5 The expression patterns of FasL+, Fas+, and CD8+ cells in the skin of C3H/HeJ mice. The distributional patterns of FasL+ and Fas+ cells are significantly different from that of CD8+ cells, as revealed by the immunohistological studies of the mouse skin. While CD8+ cells are mostly seen in the intra- and peri-follicular regions, Fas+ and FasL+ cells are scattered throughout the dermis and subcutaneous tissue, showing poor association with the focal infiltrate of hair follicles. (a and b) FasL+ cells are abundant in the skin of AA affected mice, while (c) they are relatively absent from the skin of normal haired littermates; (d, e, and f) exhibit Fas expressing cells in the skin of AA affected and normal haired mice; (g and h) there is a large peri- and intrafollicular population of CD8+ cells in the AA affected skin; (i) CD8+ cells are absent from the normal skin (n=6). Positively stained cells are marked with arrows. AA, alopecia areata; FasL, Fas ligand; NL, normal haired.
Figure 2.6 Genes expression of the TNF family of receptors and ligands in murine alopecia areata. A total number of 6 AA affected and 6 normal haired mice were sacrificed, and tissue samples from the skin, draining lymph nodes, spleen, and thymus were subjected to RT-PCR. TNF-α, along with one of its receptors, is upregulated in the skin of AA affected mice relative to their normal haired littermates. Expression of the target gene normalized to the reference gene and relative to calibrator (control) is given by $2^{-\Delta\Delta Ct}$. For statistical significance, the ΔCt values for AA affected and normal haired mice were subjected to student-T-test, which yielded the estimation of $\Delta\Delta Ct$. A small P-value (<0.05), marked with an asterisk, indicates that $\Delta\Delta Ct$ is significantly different from 0. AA, alopecia areata; Ct, threshold cycle; ΔCt, Ct(target) – Ct(reference); $\Delta\Delta Ct$, ΔCt(AA) - ΔCt(normal); TNF-α, tumor necrosis factor α; TNF-R1, TNF receptor 1; TNF-R2, TNF receptor 2; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor; TRAMP, TNF receptor-related apoptosis-mediating protein.
Figure 2.7 Apoptosis activity in the skin of C3H/HeJ mice.
TUNEL staining was performed on skin sections from 6 AA affected and 6 normal haired mice in order to detect the extent of apoptosis activity among and within hair follicles. (a and b) Limited apoptosis activity occurs in the inner and outer root sheaths of AA affected hair follicles. (c and d) Normal anagen stage hair follicles also show some extent of apoptosis activity. (e) The proportion of hair follicles with any extent of apoptosis activity per square millimeter of the skin is compared between AA affected and normal haired mice. Hair follicles with or without apoptosis activity were counted under the magnification of 10X, in at least 10 visual fields per section, three sections per specimen. A total number of 6 AA affected and 6 normal haired mice were studied. There is no statistically significant difference between the two groups (P-value= 0.16). Positively stained cells are marked with arrows. AA, alopecia areata; NL, normal haired.
Figure 2.8 Mast cells in the skin of C3H/HeJ mice. (a) Mast cells are abundant in the skin of AA affected mice, mostly populating the superficial papillary dermis and the junction of dermis and subcutaneous tissue. (b) Mast cells are also present in the normal skin, albeit in much fewer numbers (n=6 each). (c) Mast cells in the AA affected mouse skin express granzyme B (marked with arrows). (d) and (e) skin mast cells also express Fas and FasL in AA affected mice (n=6). Mast cells are marked with arrows. Mast cells were identified by incubating skin sections with the dye Alcian blue. Conventional immunohistochemical protocols were then employed to detect granzyme B, Fas, or FasL expressing cells, using antibodies against mouse granzyme B, Fas, and FasL. AA, alopecia areata; FasL, Fas ligand. MC, mast cell; NL, normal haired.
Figure 2.9 Mast cells in AA.
Comparisons of (a) the total number of mast cells, (b) proportion of mast cells expressing granzyme B, (c) proportion of mast cells expressing FasL, per square millimeter of the skin, were made between AA affected mice and normal haired controls. Mast cells were counted under the magnification of 10X, in at least 10 visual fields, three sections per specimen (n=6 AA and 6 normal mice). Granzyme B+, or FasL+ mast cells were then counted, and the proportion of mast cells expressing granzyme B, or FasL was calculated by dividing the number of granzyme B+, or FasL+ mast cells by the total number of mast cells. (d) and (e) The majority of FasL+ and granzyme B+ cells in the skin of both AA affected and normal haired mice are mast cells. The proportion of granzyme B or FasL expressing cells which were proved to be mast cells was calculated by dividing the number of granzyme B+ or FasL+ mast cells per square millimeter of the skin by the total number of granzyme B+ or FasL+ cells. Comparisons between AA affected mice and their normal haired controls were made, using student-T-test. P-values< 0.05 demonstrate statistical significance. AA, alopecia areata.
References:


Chapter 3. NK cells in alopecia areata

Introduction

Natural killer (NK) cells are specialized leukocytes of the innate immune system, involved in cytotoxicity against virally-infected and transformed cells, as well as regulation of autoimmune diseases (Baxter AG et al. 2002). NK cells execute their effector function by one of the two major mechanisms: the granule exocytosis pathway, which involves exocytosis of cytotoxic granules' constituents, including granzymes and perforin, and signaling through the tumor necrosis factor (TNF) family of death receptors and ligands (Smyth et al. 2002). In addition, NK cells can release a variety of cytokines (e.g. interferon (IFN)-γ, TNF-α) and chemokines (e.g. CCL3, CCL4, and CCL5), and therefore are able to mediate inflammatory responses (Biron CA et al. 1999).

An array of inhibitory and stimulatory receptors regulates the effector functions of NK cells. Inhibitory receptors include the Ly49 receptors in rodents, killer cell immunoglobulin-like receptors (KIRs) in primates, and the CD94/NKG2A complex in both rodents and primates, whose interactions with MHC class I antigens on target cells block cytotoxicity and cytokine production by NK cells (Long EO et al. 1997).

Several different classes of activating NK cell receptors have been described, including NKG2D, natural cytotoxicity receptors (NCRs) such as NK1.1, and NK cell antibody receptors such as CD16. Stimulatory receptors are considered essential for the initial activation of NK cell effector functions, and in some circumstances, signals from activating receptors are sufficient to stimulate NK cells even in the presence of inhibitory signals provided by MHC class I engagement (Smyth MJ et al. 2005). NK cell responsiveness, therefore, appears to be determined by an integration of inhibitory and stimulatory signals.

1 A version of this chapter will be submitted for publication. Barekatain A, Yu M, Sadeghi N, Lo B, Shapiro J, McElwee KJ
Alopecia areata (AA) is considered as an organ-specific autoimmune disease of anagen stage hair follicles, characterized by an inflammatory infiltrate comprised primarily of CD4+ and CD8+ T cells, marked response to immunomodulatory treatments, and associations with other autoimmune disorders such as autoimmune thyroiditis and vitiligo (Sperling LC et al. 1995, Shellow WV et al 1992, Muller SA WR et al 1963, Freyschmidt-Paul P et al. 2003). Anagen stage hair follicles are sites of relative immune privilege with reduced expression of MHC class I, and decreased presence and function of antigen presenting cells (Harrist TJ et al. 1983, Paus R et al. 2003). AA is believed by some to occur as a result of failure in maintaining the immune privilege in hair follicles, and the consequent presentation of as yet unknown autoantigen(s) to the immune system (Paus R et al. 2005).

C3H/HeJ mice spontaneously develop a hair loss disease which is highly comparable to AA in humans (Sundberg JP et al. 1994). Hair loss can also be induced by grafting the lesional skin from AA affected mice to normal haired littermates. The recipient mice develop a generalized hair loss 10 to 12 weeks after grafting (McElwee KJ et al. 1998). This allows the disease initiation to be predictable and controlled, and offers this inbred strain of mouse as a useful model to study the pathogenesis of AA.

The fact that anagen stage hair follicles express low MHC class I antigens, and very few NK cells are present around normal human hair follicles (Christoph T et al. 2000) raises the question as how hair follicles manage to escape from recognition and cytolysis by NK cells. Recently, it has been shown that the hair follicle immune privilege may be preserved in part by increased expression of the NK cell suppressor macrophage migration inhibitory factor (MIF), which is downregulated in AA affected hair follicles (Ito T et al. 2007). Along with increased presence of NK cells around AA affected hair follicles, and NK cells' elevated expression of the activating receptor NKG2D, a potential role was suggested for NK cells in the pathogenesis of AA (Ito T et al. 2007).

However, the role of NK cells in autoimmune diseases can be both enhancing and preventing based on which cell types become the targets of NK cells. If the target cells
are in organized tissues, NK cells can initiate the autoreactive responses, and therefore are involved in the induction of autoimmune diseases. In contrast, if immune cells become the targets, NK cells respond by recognizing and killing the autoreactive immune cells, and acting as regulators to prevent and/or confine the autoimmune response (Jie HB et al. 2004).

The ability of NK cells to secrete an array of cytokines such as IFN-γ, TNF-α, IL-10, IL-13 and chemokines such as MIP-1α, MIP-1β, and RANTES further contributes to their regulatory effects in autoimmune diseases. Activated NK cells boost antigen presentation of dendritic cells and promote the differentiation of Th1 cells by producing IFN-γ (Zhang C et al. 2006). On the other hand, NK cells can inhibit the function of dendritic cells by directly killing immature dendritic cells (Cooper MA et al. 2004).

As discussed in detail in the previous chapter, CD8+ T cells appear unlikely to be involved in direct cytotoxicity against the follicular epithelium in chronic mouse AA. Accordingly, we sought to study the role of NK cells, the cytotoxic cells of the innate immune system, to further elucidate the role of cell-mediated cytotoxicity in the pathogenesis of AA. Moreover, to examine whether positive or negative regulatory roles of NK cells are predominant, the mRNA expression levels of major inhibitory and stimulatory NK cell receptors were studied in the skin and lymphoid organs of AA affected mice compared to normal haired controls, using quantitative reverse transcriptase PCR (RT-PCR). In a series of immunohistological studies, we also attempted to examine the expression pattern of NK cells in the skin of AA affected and normal haired C3H/HeJ mice, using antibodies against various NK cell’s characteristic markers.
Material and methods

Animals

We used C3H/HeJ mice as our model for alopecia areata (AA). Mice without hair loss were purchased from The Jackson Laboratories (Bar Harbor, ME). Induction of hair loss was achieved by grafting full-thickness alopecic skin from AA-affected mice onto the back of normal mice according to the techniques as described before (McElwee et al., 1998). Noticeable hair loss occurred in more than 95% of recipient mice by the twelfth week after grafting. Three to six months after grafting, AA-affected mice were sacrificed and skin samples were collected for reverse transcriptase-PCR. All normal control mice were age and sex matched. All animal studies were conducted according to the institutional guidelines with ethics committee approval.

Real-time reverse transcriptase-PCR analysis for gene expression

Six AA affected mice and 6 normal haired littermates were sacrificed. Tissue samples from the skin, draining lymph nodes, spleen and thymus were excised, placed in RNA stabilizing reagent (RNAlater, Qiagen, Mississauga, Canada) and stored at -80 until used. Total RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen, Mississauga, Canada). Nine major NK cell markers were chosen for RT-PCR analysis. The sequences of the complete genome were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). A pair of oligonucleotide primers was designed for each sequence using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi) with the following criteria: 50-65°C melting temperature, 40-60% G+C content, 18-25bp primer length, and 75-250bp amplicon size. Primers were tested for their specificity for the target genes and redesigned when necessary. The primers’ sequences are listed in table 3.1.
Two micrograms of total RNA from each sample were subjected to reverse transcription using the Superscript first-strand cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The RT-PCR reactions were conducted using a total of 20μL of reaction mixture (5 μL of cDNA, 10 μL of SYBR Green PCR Master Mix (Finnzymes, Burlington, Canada), 5 μL of each 5 μmol/L forward and reverse primers (Invitrogen Life Technologies, Carlsbad, CA) in an Opticon™ DNA Engine (MJ Research, MA, USA). The PCR program was initiated for 10 minutes at 95°C before 41 thermal cycles, each of 15 seconds at 94°C, 30 seconds at 60°C and 30 seconds at 72°C. β-actin was used as the reference gene for all analyses described. For data analysis, relative quantification was used to determine the ratio between the quantity of a target molecule in a sample (AA-affected) and in the calibrator (normal haired controls). Both the sample and the calibrator data were first normalized against variation in sample quality and quantity and normalized values, ΔC(t)s, were calculated. The ΔΔC(t) was then determined using the following formula: ΔΔC(t) = ΔC(t)sample-ΔC(t)calibrator. At the end, the expression level of the target gene normalized to the reference gene and relative to the calibrator was determined as $2^{\Delta \Delta C(t)}$ (appendix 1). Melting curves for each PCR reaction were generated to ensure the purity of the amplification product. For statistical significance, the ΔCt values for AA affected mice and normal haired controls were subjected to student-T-test, which yielded the estimation of ΔΔCt. P-values were derived from testing the null hypothesis that ΔΔCt are equal to 0. Therefore, a small P-value (<0.05) indicates that the ΔΔCt is significantly different from 0, which demonstrates a significant effect in the mRNA expression level of target molecule in AA affected mice relative to their normal haired littermates (appendix 1).

**Immunohistochemistry**

Longitudinal skin sections from 6 AA-affected and 6 normal mice (18- to 20-month-old females) were embedded in O.C.T compound (Sakura, Torrance, CA), snap frozen and stored overnight at -80°C before being cut onto aminoalkysilane coated slides (Sigma-
Aldrich, Oakville, Canada). The following fixation protocols were tried, in an attempt to determine which fixative yields the best immunohistochemistry results: 4% paraformaldehyde for 15 minutes at room temperature (RT), or 100% Acetone for 10 minutes at -20°C. Tissue sections were also subjected to antigen retrieval, using antigen unmasking solution (Cat. No. H-3300, Vector Laboratories, Burlingame, CA), according to the manufacturer protocol.

Non-specific antibody binding was prevented by incubating sections with normal serum from the species from which the secondary antibodies had been obtained. Endogenous biotin activity was blocked by application of an avidin-biotin blocking kit solution (Vector Laboratories). Subsequently, sections were incubated with the following primary antibodies, for two hours at room temperature: rat monoclonal antibody against mouse Ly49G (clone 4D11, BD Bioscience, Mississauga, Canada), goat polyclonal antibody against mouse Ly49 (clones V-17, P-17, and D-20, Santa Cruz biotechnology, Santa Cruz, CA), rat monoclonal antibody against mouse NKG2D (clone 191004, R&D systems, Burlington, Canada). Sections were then incubated with the following biotinylated secondary antibodies for 30 to 90 minutes at RT: rabbit anti-rat IgG (Cat. No. BA-4001, Vector Laboratories), or donkey anti-goat IgG-B (Cat. No. sc-2042, Santa Cruz), as appropriate.

In order to determine the optimal working concentrations of the antibodies used, and to examine whether there are any benefits to using a particular diluent, sections were incubated with 1:1000 to 1:10 concentrations of primary and secondary antibodies, diluted in various diluents, including phosphate buffered saline (PBS), Tris buffered saline (TBS), TBS with 10% Tween-20 (TBST), or the antibody diluent with background reducing components (Dako, Mississauga, Canada). Avidin DH and biotinylated alkaline phosphatase H (ABC-AP, Vector Laboratories) was used as the enzyme. To further amplify the signals, sections were additionally incubated with the primary antibodies overnight at 4°C, corresponding secondary antibodies and ABC-AP. The signal was developed using Vector Red (Vector Laboratories, Burlingame, CA). Endogenous alkaline phosphatase activity was inhibited by the addition of levamisole (Vector
Laboratories, Burlingame, CA) to the buffer used to prepare the substrate solution. Finally, sections were counterstained with Mayer's hematoxylin, dehydrated, cleared and permanently mounted in non-aqueous media (Fisher Scientific, Ottawa, Canada). Negative controls were included on all slides by replacing primary antibody with the diluent.
Results

RT-PCR studies of the skin and lymphoid organs of C3H/HeJ mice

mRNA expression levels of the major NK cells markers were studied in the skin, draining lymph nodes, spleen, and thymus of mice with AA relative to normal haired controls, using quantitative RT-PCR. The gene expression of the activating receptors CD16, and NKG2D were found upregulated in the skin of AA affected mice, whereas the expression of CD161 was downregulated. In contrast, RT-PCR revealed increased mRNA levels for the NK cells’ inhibitory receptors, including CD94, Ly49g, and Gp49B1 in the AA affected skin compared with normal skin (all statistically significant with p-values<0.05) (figure 3.1).

The study of the gene expression profiles of the NK cell markers also demonstrated significant changes in the lymphoid organs of mice with AA relative to normal haired littermates. In the skin draining lymph nodes, the expression levels of CD94, and NKG2D were found increased, while the inhibitory receptors CD94, Ly49g, and Gp49B1 were identified downregulated in the spleen, and upregulated in the thymus of AA affected mice (except for Ly49g in the spleen, all values were statistically significant) (table 3.2).

Immunohistochemistry

In order to further characterize the expression profile and pattern of NK cells, immunohistological staining of the skin was performed in AA affected and normal haired mice, using antibodies against the Ly49 family of NK cells’ inhibitory receptors and the stimulatory receptor NKG2D. Since RT-PCR had revealed the highest change in the skin for Ly49g, with more than eight-fold increase in AA affected mice relative to normal haired controls, we attempted to use the anti-Ly49g monoclonal antibody (mAb) 4D11 to identify NK cells in the skin.
Immunohistological detection of NK cells in the skin of C3H/HeJ mice, however, was elusive in our hands. The 4D11 mAb failed to identify Ly49g+ cells in the frozen skin sections of mice (figure 3.2, a and b). To determine whether inability to detect Ly49g was due to applying inappropriate concentrations of primary or secondary antibodies, a range of concentrations from 1:1000 to 1:10 were titrated, but did not result in specific staining. Other measures such as employing different fixation protocols, blocking reagents, or antibody diluents also failed to return any results.

In another trial, the polyclonal antibody P-17, reactive to the internal epitope of murine Ly49 antigen, was applied to the skin sections, and resulted in strong labeling of the epidermal as well as follicular keratinocytes, but failed to identify any infiltrating cells (figure 3.2, e and f). Two other clones, namely V-17 and D-20 with the ability to recognize different epitopes of the C-terminus of murine Ly49 antigen, were employed. Both antibodies strongly stained keratinocytes; D-20, however, also detected a population of infiltrating cells in the dermis of both AA affected and normal haired mice, more frequently inhabiting the former (figure 3.2 c and d). Nevertheless, these results were not reproducible, and the identification of Ly49+ inflammatory cells could not be replicated, despite many attempts under similar conditions. The very strong labeling of keratinocytes by the aforementioned antibodies, resulting in an intensely stained background, may not have allowed putative Ly49+ infiltrating cells to stand out clearly.

We also attempted to detect NK cells in the skin of C3H/HeJ mice, using an anti-NKG2D mAb, but failed to identify any NKG2D+ cells in the skin of AA affected mice and their normal haired littermates, despite of employing different immunohistochemical staining protocols.
**Discussion**

Natural killer (NK) cells distinguish between normal healthy cells and abnormal cells by using a sophisticated repertoire of cell surface receptors that control their activation, proliferation, and effector functions (Spits H et al. 1998). NK cell recognition entails binding to potential target cells, interactions between activating and inhibitory receptors with ligands available on the target, and the integration of signals transmitted by these receptors, which determines whether the NK cell detaches and moves on or stays and responds (Davis DM et al. 1999).

**NK cell inhibitory receptors**

**Ly49 Receptors**

Comprising at least 23 members (Ly49A through W), the mouse Ly49 family of receptors are type II transmembrane glycoproteins homologous to C-type lectins, characterized by both inhibitory and activating receptors (Dimasi N et al. 2004). While the killer cell immunoglobulin-like (KIR) receptors serve as the major functional NK receptor family recognizing MHC-I molecules in humans, members of the Ly49 family carry out the analogous function in mice (Natarajan K et al. 2002). The inhibitory receptors, including Ly49A, Ly49C/I, and Ly49G (Dokun AO et al, 2001), contain immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domains, which recruit protein tyrosine phosphatases such as SHP-1 and SHP-2. The recruited phosphatases mediate the inhibition of NK cell activation pathways.

We found more than an eight-fold increase in the mRNA levels of Ly49g in the skin of mice with AA relative to normal controls, as revealed by RT-PCR. Ly49g is expressed on about 50% of mouse NK cells, as well as subsets of NKT and T cells (Mason LH et al. 1995). This finding suggests that NK cells or NK-like cells may have a regulatory role in the inflammatory hair loss disease.
Gp49B1

Originally characterized as a mast cell inhibitory receptor, Gp49B1 has been reported to be expressed on all NK cells derived from C57BL/6 or BALB/c mice (Wang LL et al. 1997). Gp49B1 is an ITIM-containing type I transmembrane protein belonging to the Ig superfamily, and therefore is regarded as the structural homologue of human KIR. It has also been demonstrated that Gp49B1 is expressed on activated CD4+ and CD8+ T, and inhibits IFN-γ production by both subsets of T cells as well as NK cells following acute infection, without affecting their cytotoxic function (Gu X et al. 2003).

We detected a significant upregulation for this NK cell inhibitory receptor in the skin and thymus of mice with AA relative to their normal haired littermates, as revealed by RT-PCR. This further suggests a potential immuno-protective effect for NK cells in mouse AA.

NK cell activating receptors

NKG2D receptor

The NKG2D receptor is expressed on all human and mouse NK cells as well as γδTCR+ T cells activated CD8+ T cells, and recognizes cell surface glycoproteins structurally related to MHC class I (Bauer S et al. 1999). Stimulation of NK cells through NKG2D triggers cell-mediated cytotoxicity and in some cases induces secretion of cytokines and chemokines (Raulet DH. 2003). NKG2D has been reportedly implicated in immunity against viruses and tumors; in contrast, the deleterious effects of NKG2D-mediated responses have been shown in a number of autoimmune conditions such as rheumatoid arthritis and autoimmune diabetes in NOD mice (Groh V et al. 2003, and Ogasawara K et al. 2004). Recently, the expression of the MHC class I chain-related A (MICA) molecule, the ligand to which NKG2D receptor binds, has been reported highly upregulated in the lesional skin from AA patients compared to the normal human skin (Ito T et al. 2007),
suggesting a potential mechanism by which NKG2D-mediated responses and NK cells could be involved in the pathogenesis of AA.

We identified, by RT-PCR, significantly elevated mRNA levels of NKG2D in the skin, draining lymph nodes, and thymus of AA affected mice compared to normal haired controls. This is consistent with the immunohistological studies of AA in humans where increased presence of CD56+/NKG2D+ cells were observed around the hair follicles in the lesional scalp skin sections compared with normal skin (Ito T et al. 2007). These findings point toward a potential role for NK cells as instigators of the disease mechanism in AA.

**CD16**

CD16 (FcγRIII) is the low-affinity Fc receptor for IgG that is responsible for antibody-dependent cellular cytotoxicity (ADCC), containing an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. CD16 expressed on the majority of, but not all, human and mouse NK cells, as well as on activated monocytes, and a subset of T cells (Daeron M 1997). Upon CD16-mediated activation, NK cells secrete cytokines, mediate antibody-dependent cellular cytotoxicity (ADCC), and may undergo apoptosis (Lanier LL 1998).

RT-PCR revealed an increased expression level for CD16 in the skin of AA affected mice compared with normal haired controls. Consistent with our data, Zoller et al. have shown that CD16 expressing cells are upregulated in the peripheral blood mononuclear cells (PBMC) of patients with both the progressive and the regressive stages of AA, as compared with normal healthy individuals (Zöller M et al. 2004). The increase in the number of CD16+ cells in the PBMC of patients with AA has also been reported in the Japanese literature (Imai R et al. 1988). Based on these observations, a potential role for ADCC, mediated by NK cells or monocytes, appears possible in the pathogenesis of AA.
Taken together, the alterations in the expression levels of an array of NK cell receptors, as revealed by RT-PCR, suggest a potential role for these leukocytes in the pathogenesis of AA. However, none of these receptors are exclusively specific for NK cells, and they are also expressed by other immune cells, including T cells, mast cells, and monocytes. RT-PCR studies of C3H/HeJ mouse model for AA also returned confusing results about the activation status of putative NK cells, as both the inhibitory and stimulatory receptors were found significantly upregulated in the skin of mice with AA relative to their normal haired littermates. We, therefore, attempted to further examine the role of NK cells, using immunohistological staining of the mouse skin.

**Immunohistochemistry**

The in situ visualization of NK cells is hampered by the lack of specific reagents, especially in mice. In humans, NK cells are identified as leukocytes with a combination CD3-CD16+CD56+ marker profile (Sigal LH 2003). In mice however, CD56 is not expressed on NK cells, and staining with anti-NK1.1, anti-Ly49G2, anti-CD49b, or anti-NKG2D antibodies is often used to label NK cells. However, as mentioned above, none of these antibodies are entirely NK cell specific, or expressed on all NK cells (Grégoire C et al. 2007).

The identification of murine NK cells in situ first became possible by using the NK1.1 marker recognized by the monoclonal antibody (mAb) PK136 (Daniel M. et al. 2001). NK1.1 is expressed on all NK cells, and is rarely found on T cells, except for NKT cells (Ballas ZK et al. 1990). However, its expression on NK cells is limited to only a few mouse strains (e.g., C57BL/6 or C57BL/10), while most other inbred mouse strains, including C3H/HeJ mice, do not express NK1.1 (Koo JC et al., 1984).

Alternatively, the mAb DX5 has been introduced that stains NK cells and a small subset of T cells in all strains of mice that have been analyzed. DX5, therefore, has been offered as a useful reagent to identify and isolate NK cells, by flow cytometry, from mice lacking expression of NK1.1. DX5 recognizes CD49b, an α2 integrin which is known to bind to
collagen or laminin and is involved in cellular adhesion. However, its function on NK cells is currently unclear (Plow EF et al. 2000, and Arase H et al. 2001). We did not find a significant difference in the mRNA levels of this antigen between the skin or lymphoid organs of mice with AA and normal haired controls (Figure 3.1). Moreover, given the limited reactivity of this mAb by immunohistochemistry (Dokun AO et al. 2001), DX5 appeared to be an unsuitable candidate to identify NK cells in the immunohistological studies of murine tissues.

In a series of experiments, Dokun et al. examined the reactivity of variety of NK cell receptor-specific mAbs by immunohistochemistry, and found the mAb 4D11, specific for Ly49g, to be reactive with C57BL/6 tissue sections (Dokun AO et al. 2001). Since we had also detected an increased mRNA expression level for Ly49g, as revealed by RT-PCR, the mAb 4D11 was subsequently used in an attempt to identify Ly49+ cells in the skin of C3H/HeJ mice.

Nevertheless, we were unable to detect any positive signals in the skin of AA affected mice or their normal haired littermates, using the mAb 4D11. A number of other mouse anti-Ly49 antibodies were also tried, and except for only one staining session which was not replicable, no Ly49+ cells were identified, despite employing several different immunohistological staining protocols. Our efforts to identify putative NK cells in the skin of C3H/HeJ mice using the mAb against the mouse NKG2D receptor also failed to yield specific staining results.

**Conclusion**

RT-PCR and immunohistological studies of the skin in C3H/HeJ mice did not return conclusive results about the gene and protein expression profile of major NK cells’ markers. This may be in part due to unavailability of appropriate specific reagents to identify murine NK cells by immunohistochemistry, or simply because of the absence of NK cells from the skin of AA affected and normal haired mice. NK cells are circulating,
and not tissue-residing, lymphocytes (Yokoyama WM 1999), and one would not expect to find them in the skin if they are not involved in the hair loss disease.

One possible weakness of our study was the lack of appropriate positive control, which could be obtained by infecting mice with virulent viruses such as murine cytomegalovirus (CMV). NK cells are important mediators of host defense against viral infections, and their response is expected in the visceral organs of affected mice in the acute phase of infection with CMV (Farrell HE et al. 1999).

Alternatively, the role of NK cells in AA could be further examined, using flow cytometric studies of the spleen, draining lymph nodes, or the inflammatory cell population of the skin. This might allow a more reliable labeling, given the availability of a larger number of mAbs compatible with flow cytometric applications, to study the frequency and activation status of NK cells. *In situ* hybridization studies of the frozen skin sections could also be performed using radioactive probes specific for NK cell’s surface markers, activating as well as inhibitory receptors. This would allow visualization of the distribution of putative NK cells. Finally, putative NK cells could be enriched from the skin, cultured *in vitro*, and the cytokine profile of NK cells could be studied using ELISA.
Tables and figures

Table 3.1 The sequences of primers used in RT-PCR.

<table>
<thead>
<tr>
<th>target gene</th>
<th>forward primer (5’ to 3’)</th>
<th>reverse primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16</td>
<td>TGTTTGGCTTTTCGACAGACGG</td>
<td>CGTGACTTGCTTGGACCT</td>
</tr>
<tr>
<td>CD56</td>
<td>TGCTTTTCTGGGAACTGC</td>
<td>TCTCTGCCACTTGGCAACAG</td>
</tr>
<tr>
<td>CD49b</td>
<td>CGGAGAAAGCAAGTAGTACCG</td>
<td>GCAGTCATAGCCACAGCAA</td>
</tr>
<tr>
<td>CD94</td>
<td>TCACCTCCTGGGAGACTGATG</td>
<td>AGTGGTGTTGGGAAGGGTG</td>
</tr>
<tr>
<td>CD161</td>
<td>GAAAAGGAGCCACTTTGCTG</td>
<td>CATGTCTGGCAATGTAACC</td>
</tr>
<tr>
<td>CD244</td>
<td>AGCCCTGGACTAATGGGACT</td>
<td>AAGTCAGGGTGTGGTTG</td>
</tr>
<tr>
<td>Gp49B1</td>
<td>TTACCTGCTGTCAGGGTCC</td>
<td>GGACTTGAGTGTTCCAAGGA</td>
</tr>
<tr>
<td>Ly49G</td>
<td>GTTGCAGAAACTAGTGAGGAC</td>
<td>GATGTATGATTACCACAGTCC</td>
</tr>
<tr>
<td>NKG2D</td>
<td>GGCAATTCGATTCCACCTTA</td>
<td>ATCCAGTTGTAGGGCCTGG</td>
</tr>
</tbody>
</table>
Figure 3.1 NK cell surface marker gene expression in murine alopecia areata.

mRNA expression levels of major NK cell surface markers in AA affected mice relative to normal haired controls are shown, as revealed by RT-PCR. (n=6 each). Expression of the target gene normalized to the reference gene and relative to calibrator (control) is given by $2^{-\Delta\Delta Ct}$. For statistical significance, the $\Delta Ct$ values for AA affected and normal haired mice were subjected to student-T-test, which yielded the estimation of $\Delta\Delta Ct$. A small P-value ($<0.05$), marked with an asterisk, indicates that the $\Delta\Delta Ct$ is significantly different from 0. All plates were run in duplicates. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product. AA, alopecia areata; Ct, threshold cycle; $\Delta Ct$, Ct(target) – Ct(reference); $\Delta\Delta Ct$, $\Delta Ct$(AA) - $\Delta Ct$(normal).
Figure 3.2 Immunohistological staining of NK cells in the skin of C3H/HeJ mice. Antibodies against several mouse NK cell surface markers were used in an attempt to identify putative NK cells. While the anti-Ly49g mAb 4D11 did not return any specific staining, the polyclonal antibody against mouse Ly49 Ag, D-20, identified a population of infiltrating cells in the skin of AA affected mice, although it was not reproducible. Two other antibodies against Ly49 Ag, namely V-17 and P-17, were tried, but resulted in strong staining of epidermal and follicular keratinocytes without identifying any infiltrating cells (n=6 AA affected and 6 normal haired mice).
References:


Chapter 4. Cytokines in alopecia areata

Introduction

Alopecia areata (AA) is manifested by sudden appearance of alopecic patches on the scalp and other body parts. In the majority of cases, the disease is self-limiting, but it may extend to involve the whole scalp or body (Dawber R and Neste DV 2004). A hair loss disease with close genetic, histological and pathophysiologic similarities has been recognized in rodents (Sundberg JP et al. 1994). C3H/HeJ mice develop AA-like hair loss spontaneously. The disease can also be induced by grafting lesional skin from AA affected mice to normal haired mice. The recipient mice develop hair loss 10 to 12 weeks later (McElwee KJ et al. 1998). The C3H/HeJ mouse, therefore, provides a useful model to perform large-scale experiments in a controlled and predictable setting.

AA in both humans and rodents is characterized under the common denominator of antigen presentation and T cell activation. Hair loss is associated with a peri- and intra-follicular infiltration composed primarily of CD4+ and CD8+ T cells (Sperling LC and Lupton GP 1995, Sundberg JP et al. 1994). In addition, there is increased presence of antigen presenting cells around and within affected hair follicles, in close association with follicular keratinocytes, and characterized by increased expression of MHC II (Messenger AG et al. 1984, Paus R et al 1998). There is also increased expression of MHC class I and II antigens and ICAM-1 on hair follicle epithelial structures (Paus R et al. 2003).

Cytokines are small proteins that act in an autocrine or paracrine manner. Cytokines fall into different families, including interleukins, interferons, colony-stimulating factors, tumor necrosis factors, growth factors, and chemokines, and are involved in cell proliferation, differentiation, growth inhibition, apoptosis, and chemotaxis (Roitt et al. 2001).

1 A version of this chapter will be submitted for publication. Barekatain A, Yu M, Sadeghi N, Lo B, Shapiro J, McElwee KJ
One of the major effects of cytokines is to help to select the effector function of T lymphocytes by determining their differentiation into T helper (Th) 1, Th2, or Th17 subsets. In this context for example, IL-12 induces the generation of Th1 subsets, IL-4 promotes Th2 type cells, and IL-21, IL-6 and TGF-β are involved in the generation of Th17 cells from naïve T cells (Mosmann TR et al. 1989; Deenick EK et al. 2007). Each of the Th1, Th2, and Th17 subsets are characterized by the production of distinct cytokines, which help determine their effector functions. IFN-γ, TNF-α, and IL-18, are expressed by, and involved in, Th1 immune responses, including cell-mediated and delayed-type hypersensitivity responses. In contrast, Th2 type cell subsets produce cytokines such as IL-4, IL-5, IL-6, and IL-13 and promote humeral immunity and allergic responses (Mosmann TR et al. 1996, and Belardelli F et al. 2002). The newly discovered Th17 lineage, typified by the production of the pro-inflammatory cytokine IL-17, has been shown to be associated with inflammation and tissue damage in several animal models of human autoimmune conditions (Afzali B et al. 2007).

CD8+ cytotoxic T (Tc) cells can also be subdivided into Tc1 and Tc2 based on their cytokine profile. Tc1 cells produce large amounts of IFN-γ while Tc2 cells secrete IL-4 but not IFN-γ (Sad S et al. 1995). However, CD8+ T cell subsets respond differently to cytokines. IL-12 enhances Tc1 but not Tc2 cell growth while IL-4 inhibits Tc1 cell proliferation but has no effect on Tc2 cells (Kemeny DM et al. 1999).

The association of AA with autoimmune conditions particularly vitiligo and thyroid disorders, and its response to a range immunomodulatory treatments, have proposed AA as an organ-specific autoimmune disease, typified by Th1 responses (Shellow WV et al. 1992, and Freyschmidt-Paul P et al. 2003). In agreement with this concept, a number of Th1 cytokines, including IFN-γ, have been reportedly associated with AA, although the aberrant expression of type 2 cytokines, such as IL-4, has also been demonstrated in AA. Little is known about the role of Th17 cells in the pathogenesis of AA (McElwee KJ et al. 2002, and Hoffmann R et al. 1994)
Chemokines belong to a family of more than 40 relatively small peptides, which are involved in chemoattraction and activation of leukocytes to the site of inflammation and in the induction of cytokine production, and are thus key determinants of inflammatory reactions and immunity (Zlotnik A et al. 2000). These peptides are secreted by tissue cells, leucocytes and activated epithelial cells and act through a family of chemokine receptors, which are present on cell types including leukocytes, dendritic cells and endothelial cells (Baggiolini M et al. 1997).

Chemokines fall into four different subfamilies based on the highly conserved presence of the first two cysteine residues, which are either separated or not by other amino acids; the CC chemokines, the CXC chemokines, the CX3C chemokines and the C chemokines (Rossi D and Zlotnik A 2000). CC chemokines form the largest group and include monocyte chemotactic protein-1 (MCP-1), RANTES, and macrophage inflammatory protein (MIP)-1α and MIP-1β, which mostly attract monocytes, activated T cells, and natural killer (NK) cells. Most CXC chemokines (CXCL 1,2,3,5,6,7, and 8) recruit neutrophils. Others including CXCL 9,10, and 11 recruit a unique subpopulation of T cells and NK cells that produce IFN-γ. The last two groups are small, representing by CX3CL1 (fractalkine) and XCL1 (lymphotactin), respectively, and are involved in chemoattraction of T cells, NK cells and monocytes (Kimura H et al. 2007).

Given their crucial role in leukocyte chemoattraction, chemokines may be enormously relevant in the pathogenesis of AA. It has been demonstrated that extravasation of leukocytes is a crucial step in the development of AA, and AA can not be induced in C3H/HeJ mice if the lymphocytic and/or monocytic migration to the skin is impaired (Freyschmidt-Paul P et al. 2000). Furthermore, it has been shown that lesional AA scalp skin explants regrow hair when they are transplanted onto severe combined immunodeficient (SCID) mice, suggesting that hair follicles resume hair growth once they are relieved from the malicious effects of the immune system (Gilhar A et al. 1998). Leukocyte homing and activation, therefore, appears crucially important in initiation and persistence of hair loss in AA.
Different chemokines act upon different sets of immune cells, including Th1 and Th2 cells, according to their expression of specific receptors. For example CCR3, a receptor for RANTES, is selectively expressed on human Th2 cells, while Th1 cells express CCR5, which is the receptor for MIP-1α and MIP-1β (Sallusto F et al. 1998). Accordingly, studying the expression profiles of chemokines may help determine which effector T cell responses are of more pathologic significance in AA.

There are three main hypotheses about the possible mechanisms of hair loss in AA: (i) antibody dependent cell-mediated cytotoxicity which appears unlikely, given the lack of conclusive evidence about the presence of autoantibodies in the pathogenesis of AA; (ii) cell-mediated cytotoxicity against the follicular epithelium which is also an unlikely mechanism of hair loss in AA, as discussed before; and (iii) cytokines.

Whether AA is a Th1 or Th2 (or Tc1 or Tc2) disease has always been a subject of considerable debate. Accordingly, we studied the mRNA expression levels of main Th1-, Th2 as well as Th17-associated cytokines in the skin and lymphoid organs of AA affected mice relative to their normal haired littermates. Furthermore, given the important role of chemokines in leukocyte chemotaxis and cytokine induction, and our current limited knowledge about the relevance of these peptides to the AA pathogenesis, we sought to study their role by examining the gene expression profile of representative members of different subfamilies of chemokines, using the C3H/HeJ mouse model for AA.
Material and methods

Animals

We used C3H/HeJ mice as our model for alopecia areata (AA). Mice without hair loss were purchased from The Jackson Laboratories (Bar Harbor, ME). Induction of hair loss was achieved by grafting full-thickness alopecic skin from AA-affected mice onto the back of normal mice according to the techniques as described before (McElwee et al. 1998). Noticeable hair loss occurred in more than 95% of recipient mice by the twelfth week after grafting. Three to six months after grafting, AA-affected mice were sacrificed and skin samples were collected for reverse transcriptase-PCR. All normal control mice were age and sex matched. All animal studies were conducted with University of British Columbia ethics committee approval.

Real-time reverse transcriptase-PCR analysis for gene expression

Six AA affected mice and 6 normal haired littermates were sacrificed. Tissue samples from the skin, draining lymph nodes, spleen and thymus were excised, placed in RNA stabilizing reagent (RNAlater, Qiagen, Mississauga, Canada) and stored at -80 until used. Total RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen, Mississauga, Canada). Nine cytokines associated with different T cell subsets, and six representative members of the chemokine superfamily were chosen for analysis by RT-PCR. The sequences of the complete genome were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). A pair of oligonucleotide primers was designed for each sequence using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with the following criteria: 50-65°C melting temperature, 40-60% G+C content, 18-25bp primer length, and 75-250bp amplicon size. Primers were tested for their specificity for the target genes and redesigned when necessary. The primers’ sequences are listed in table 4.1.

Two micrograms of total RNA from each sample were subjected to reverse transcription using the Superscript first-strand cDNA synthesis kit (Invitrogen Life Technologies,
Carlsbad, CA) according to the manufacturer's protocol. The RT-PCR reactions were conducted using a total of 20 µL of reaction mixture (5 µL of cDNA, 10 µL of SYBR Green PCR Master Mix (Finnzymes, Burlington, Canada), 5 µL of each 5 µmol/L forward and reverse primers (Invitrogen Life Technologies, Carlsbad, CA) in an Opticon™ DNA Engine (MJ Research, MA, USA). The PCR program was initiated for 10 minutes at 95°C before 41 thermal cycles, each of 15 seconds at 94°C, 30 seconds at 60°C and 30 seconds at 72°C. β-actin was used as the reference gene for all analyses described. For data analysis, relative quantification was used to determine the ratio between the quantity of a target molecule in a sample (AA-affected) and in the calibrator (normal haired controls). Both the sample and the calibrator data were first normalized against variation in sample quality and quantity and normalized values, ΔC(t)s, were calculated. The ΔΔC(t) was then determined using the following formula: ΔΔC(t) = ΔC(t)sample-ΔC(t)calibrator. At the end, the expression level of the target gene normalized to the reference gene and relative to the calibrator was determined as 2^-ΔΔC(t). Melting curves for each PCR reaction were generated to ensure the purity of the amplification product. For statistical significance, the ΔCt values for AA affected mice and normal haired controls were subjected to student-T-test, which yielded the estimation of ΔΔCt. P-values were derived from testing the null hypothesis that ΔΔCt are equal to 0. Therefore, a small P-value (<0.05) indicates that the ΔΔCt is significantly different from 0, which demonstrates a significant effect in the mRNA expression level of target molecule in AA affected mice relative to their normal haired littermates (appendix 1).
Results

mRNA expression levels of nine Th1-, Th2-, and Th17-associated cytokines were compared between the skin, skin draining lymph nodes, spleens, and thymus of AA affected mice and normal haired controls. Interferon (IFN)-γ was dramatically upregulated in the skin of AA affected mice compared to controls (P-value<0.00001), pointing towards the potentially important role of this cytokine in the pathogenesis of AA. A modest increase in IFNγ expression was also revealed in the skin draining lymph nodes (P-value<0.05). Surprisingly, IFNγ was downregulated in the spleen of AA affected mice, although it did not prove statistically significant. IL-18, a potent inducer of IFN-γ production by T cells and natural killer (NK) cells, was found downregulated in the skin and all the lymphoid organs studied, but did not reach a statistical significance. IL-6 exhibited an elevated expression level in the skin of AA affected mice compared to normal haired controls, which was statistically significant (P-value<0.05) (Figure 4.1).

Intriguingly, the mRNA expression profile of some of the Th2-associated cytokines studied demonstrated significant changes between the AA affected and normal haired mice, as revealed by RT-PCR (Figure 4.2). The highest difference was found for IL-13, which showed more than 30 times increase in the AA affected skin compared with normal skin (P-value<0.001). Elevated levels were also revealed for IL-10 in both the skin and draining lymph nodes of AA affected mice (P-value<0.05). IL-4 increase in the skin and lymphoid organs of AA affected mice, however, did not meet statistical significance. The Th17-associated, pro-inflammatory cytokines IL-17 and IL-21 were also studied, and their mRNA levels were compared between the skin, skin draining lymph nodes, spleen, and thymus of mice with AA and normal haired littermates. No statistically significant change was found for these cytokines between the two groups, as revealed by RT-PCR (Figure 4.3).

Representative members of three main subclasses of chemokines were studied, and their mRNA levels were compared between AA affected and normal haired mice (Figure 4.4). A drastic change was revealed for macrophage inflammatory protein (MIP)1-β with more
than 800 times increase in the skin of AA affected mice compared with normal haired controls (P-value<0.000001). Regulated on activation normal T cell expressed and secreted (RANTES) and MIP1-α, two other members of C-C family of chemokines, also showed dramatically elevated mRNA levels in the skin of AA affected mice, relative to normal haired controls (P-values<0.000001). Also of note, among the three C-C chemokines mentioned above, RANTES was the only one for which an increased mRNA expression was displayed in the lymphoid organs of AA affected mice by RT-PCR (P-value<0.05).

mRNA expression levels of two members of C-X-C family of chemokines were examined by RT-PCR, and highly elevated levels for CXCL10 (P-value<0.00001) and moderately elevated levels for CXCL1 (P-value=0.07) were found in the AA affected mouse skin (Figure4.4). Moreover, while CXCL1 showed decreased mRNA expression in the skin draining lymph nodes and spleens of AA affected mice compared with normal haired controls, which were not statistically significant, CXCL10 levels were found significantly upregulated in the affected lymph nodes (P-value<0.05). The increased mRNA expression levels of the only member of CX3C family, namely CX3CL1, were only found statistically significant in the lymph nodes and spleens of AA affected mice, and not in the skin or thymus.
Discussion

AA occurs as a result of premature precipitation of anagen hair follicles into catagen and telogen (Eckert J et al. 1968). In the lesional skin, the majority of hair follicles are in either telogen or dystrophic anagen. The hair growth cycle is arrested in anagen III/IV, and this is associated with heavy inflammatory infiltration of hair follicles (Tobin DJ et al. 1995). Furthermore, it has been demonstrated that HF's can regrow hair once they are relieved from the deleterious effects of the immune system (Gilhar A et al. 1998). Accordingly, it is postulated that the aberrant hair growth cycle is the result of a direct insult from infiltrating cells and their products to the follicular epithelium.

We studied the role of Th1-, Th2-, and Th17-mediated responses in the pathogenesis of AA by examining the mRNA expression levels of associated cytokines in the skin and lymphoid organs of AA affected mice compared to normal haired controls, using quantitative RT-PCR. Furthermore, expression levels of representative members of the chemokine superfamily were studied to further elucidate mechanisms underlying leukocyte migration to the skin in mouse AA.

**Th1-associated cytokines**

RT-PCR revealed a highly upregulated mRNA expression level for IFN-γ in the skin of AA affected mice compared with normal haired littermates. This is consistent with what others have found in mouse and human AA by flow cytometry (Hoffmann R et al. 1994, and McElwee KJ et al. 2002) and points towards the crucial role of this cytokine and Th1-mediated responses in the pathogenesis of AA.

Functional studies on C3H/HeJ mice have further provided evidence for the central role of IFN-γ in the induction of AA. It was demonstrated that mice deficient for IFN-γ are completely resistant to AA induction when lesional skin grafts were transplanted from AA affected mice to IFN-γ knockout recipient littermates (Freyschmidt-Paul P et al.
The authors concluded that such a resistance to AA onset was directly a consequence of IFN-γ deficiency, and was not the result of counteractive immune responses since neither regulatory T cells nor Th2-associated cytokines were upregulated.

The proximal segment of anagen stage hair follicles in humans and rodents are believed to be sites of relative immune privilege with reduced MHC expression, and some have hypothesized that AA occurs as a result of failure in maintaining such an immune privilege (Paus R et al. 2005). This is supported by observation of elevated expression of MHC class I and II antigens in anagen stage hair follicles in AA and increased presence of activated infiltrating antigen presenting cells (Bröcker EB et al. 1987, McElwee KJ et al. 2002, and Messenger AG et al. 1985). IFN-γ, on the other hand, has been shown to induce expression of MHC class I and II antigens as well as intracellular adhesion molecule (ICAM)-1 in the follicular epithelium in *in vitro* organ cultures of human hair follicles (Mcdonagh AJ et al. 1993). Therefore, a role was suggested for IFN-γ in promotion of antigen presentation and the breakdown of HF immune privilege in AA.

IFN-γ has also been reported to accelerate AA development in C3H/HeJ mice. In one experiment, C3H/HeJ mice, with a genetic susceptibility to develop AA spontaneously, were shaved and injected with IFN-γ intravenously. Hair loss occurred on day 36 after depilation, which was associated with complete hair growth on day 46. A second wave of patchy hair loss was observed in IFN-γ-treated mice by day 86 which lasted until day 252. No hair loss was noted in the phosphate-buffered saline (PBS)-treated mice. Hair loss was associated with dystrophic anagen HF formation, intra- and peri-follicular CD8+ and CD4+ T cell infiltration, and expression of MHC I and II antigens on the follicular epithelium (Gilhar A et al. 2005).

Besides its pro-inflammatory properties and its potential role in antigen presentation and induction of AA, IFN-γ has been suggested as a potent inducer of catagen in human HF. When cultured with human scalp HF, IFN-γ rapidly inhibited hair elongation and proliferation, which was associated with increased apoptosis, and switched-off melanogenesis (Ito T et al. 2005). The hair growth inhibitory properties of IFN-γ may
explain the drastic upregulation of this cytokine in chronic mouse AA. However, whether increased expression of IFN-γ has an etiologic implication, or it is only an epiphenomenon in AA has yet to be answered.

IL-18 is a pro-inflammatory cytokine, produced mainly by antigen presenting cells, and is known for its ability to enhance production of IFN-γ by T cells and NK cells (Muhl H and Pfeilschifter J 2004). It also mediates Th1 cell chemotaxis into tissues and has been suggested to be involved in chronic inflammatory conditions such as rheumatoid arthritis (Komai-Koma M et al 2003). IL-18 has been reportedly implicated in skin diseases such as atopic dermatitis and psoriasis (Tanaka, T et al 2001, and Pietrzak A et al 2003), and shown to induce expression of MHC II and the chemokine CXCL10 in the keratinocytes (Wittmann M et al. 2005). Therefore, a role has been suggested for this cytokine in inflammatory skin diseases. We, accordingly, sought to examine the role of this cytokine in the inflammatory hair loss disease AA, using RT-PCR. mRNA expression levels of IL-18 were found downregulated in the skin and lymphoid organs of mice with AA compared to normal haired controls, although it was not statistically significant. Our data is not consistent with a role for IL-18 in the pathogenesis of AA in C3H/HeJ mice.

**Th2-associated cytokines**

IL-13, originally described as a potent inhibitor of inflammatory cytokine production, has proved to have a number of other effector functions as well. Along with IL-4, IL-13 is produced by Th2 cells, and is involved in allergic responses, IgE antibody production, killing of intestinal helminthes, mastocytosis and many more (Wynn TA 2001).

We found highly upregulated mRNA levels for IL-13 in the skin of AA affected mice compared with normal haired controls. This suggests a role for IL-13 and Th2-mediated responses in the pathogenesis of AA. However, we did not find a statistically significant difference in the mRNA levels of IL-4 between AA affected and normal haired mice. This is an intriguing finding, given the central role of IL-4 in the differentiation of Th0 to
Th2 cells (Nelms K et al. 1999). Nevertheless, independent effector functions have been proposed for IL-13, based on observation of impaired Th2 response generation reported in some studies utilizing IL-13 knockout mice. It is even suggested that in many situations IL-13 may play a more important role than IL-4 (Wynn TA 2003).

Little is known about the role of IL-13 in AA and other hair loss diseases. However, IL-13 and Th2-mediated response have been reportedly associated with a number of other skin disorders, including atopic dermatitis (Miraglia del Giudice M et al. 2006, and Katagiri K et al 1997). The frequency and severity of AA, on the other hand, appears to be associated with a greater frequency of allergies compared with the general population. Atopy is reported in 10 to 52.4% of patients with AA, and in up to 75% of patients it is associated with a more extensive and prolonged hair loss (Goh C et al. 2006, Tan E et al. 2002, Weitzner JM 1990, and De Weert J et al. 1984). In one recent pilot study, a 15-year-old patient with alopecia universalis was treated with Imiquimod, the compound well known for its ability to downregulate Th2 response, and a transient hair growth was observed (Letada PR et al. 2007). Taken together, Th2-mediated response in general, and IL-13 in particular, might be a determining component of the course and severity of the hair loss disease AA.

We previously showed that mast cells may potentially play an important role in the pathogenesis of AA. In addition to the bioactive mediators such as histamine, prostaglandins, and leukotrienes, mast cells secrete a large number of cytokines including IL-13 (Bradding P et al. 2006). IL-13, on the other hand, has been shown to be capable of promoting mast cell proliferation (Kaur D et al. 2006). Therefore, it does not appear unreasonable to speculate that mast cell-derived IL-13 may play a role in the hair loss disease mechanism.

Significantly elevated mRNA levels were found for IL-6 in the skin of AA affected mice compared with normal haired littermates. A pleiotropic cytokine with a wide range of biological activities, IL-6 is produced by various types of lymphoid and nonlymphoid cells, such as T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, and
mesangial cells. It induces growth of T cells and differentiation of cytotoxic T cells and macrophages, stimulates hepatocytes to produce acute-phase proteins, and acts as a growth factor for a variety of cells including keratinocytes (Tetsuji N et al. 2002).

Our data is consistent with a number of other studies on humans to show a significant association between IL-6 and AA. Enzyme-linked immunosorbent assay (ELISA) studies showed higher production levels of IL-6 by the peripheral blood mononuclear cells (PBMC) of children with patchy alopecia areata compared with normal controls (Shohat M et al. 2005). Moreover, immunohistological studies of the lesional scalp revealed an increased expression of IL-6 among the mononuclear peri-follicular infiltrate of hair follicles (Bodemer C et al. 2000). However, little is known about the functional significance of this cytokine in the pathogenesis of AA.

One possible explanation for the increased expression of IL-6 in the AA affected skin has been provided by a study of transgenic mice with overproduction of IL-6 in the skin epithelium. The mice exhibited a retarded hair growth, and this was not associated with leukocyte infiltration of hair follicles, suggesting a direct hair growth inhibitory effect for IL-6 (Turksen K et al 1992). Yu M, et al. further explored the hair growth inhibitory properties of the IL-6 cytokine family, and found among members of this family significant hair growth retardation effects (Yu M et al. 2008). Therefore, a role of IL-6 in chronic hair loss disease may be attributed to its potential hair growth inhibitory properties.

We found an elevated mRNA level for the anti-inflammatory cytokine IL-10 in the skin and draining lymph nodes of mice with AA compared with normal haired controls. IL-10 was initially described as a product of Th2 cells with an ability to inhibit activation and cytokine production of Th1 cells (Asadullah K et al. 2003). Furthermore, IL-10 has been suggested to play a role in peripheral tolerance and protection against organ-specific autoimmunity, since conditions such as inflammatory bowel disease, experimental autoimmune encephalomyelitis and T cell mediated skin diseases, including contact-
hypersensitivity responses, have been shown to be exaggerated in IL-10 deficient mice (Moore KW et al. 2001).

Studies of the role of IL-10 in AA have returned confusing results. The immunosuppressive effects of IL-10 in AA were first suggested when increased expression of this cytokine was found in the scalp samples of patients with AA following successful treatment with the immunomodulatory compound diphenylcyclopropenone (Hoffmann R et al. 1994). However, in a study on the C3H/HeJ mouse model for AA, Freyschmidt-Paul P, et al. demonstrated that mice deficient for IL-10 are less susceptible to the induction of AA (Freyschmidt-Paul P et al. 2002). The precise role of this cytokine in the pathogenesis of AA has yet remained to be fully understood.

**Th17-associated cytokines**

The newly emerged Th17 lineage is characterized by production of large amounts of the cytokine IL-17. Th17 cells implicated in a number of autoinflammatory disorders, including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and psoriasis (Weaver CT et al. 2007). IL-21 is produced by Th17 cells and has a pivotal role in the differentiation of Th0 to TH17 cells (Korn T et al. 2007). The potential utility of IL-21-targeted therapies for human autoimmune conditions has been recently suggested based on reports showing that neutralizing IL-21 in murine models of lupus or rheumatoid arthritis ameliorated disease severity (Deenick EK and Tangye SG 2007). However, we did not find any significant difference in the mRNA levels of these cytokines in the skin and lymphoid organs between AA affected and normal haired mice. Accordingly, our data is not consistent with a significant role for Th17-mediated responses in the pathogenesis of AA.
Chemokines

We found a striking association between expression of members of the CC family of chemokines and the pathogenesis of AA. mRNA expression of MIP-1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) were dramatically upregulated in the skin of AA affected mice compared to normal haired controls. Moreover, CXCL10 was identified as highly upregulated in the AA affected skin, suggesting this CXC chemokine may play an important role in recruiting leukocytes to the vicinity of hair follicles in mouse AA. Consistent with our data, Kuwano Y, et al. showed that the serum level of RANTES is significantly increased in AA patients, although they failed to demonstrate a significant change in the serum levels of MIP-1α and MIP-1β (Kuwano Y et al. 2007). One possible weakness of their study is that they have examined the serum levels rather than the focal expression profile of chemokines in AA affected skin. Given that chemokines usually act in a localised autocrine/paracrine manner, studying their focal expression may yield more accurate results.

Little is known about the functional role of chemokines in mouse and human AA. Activated keratinocytes have been shown to express high levels of chemokines, including RANTES, and CXCL10 (Albanesi C et al. 2001). Furthermore, it has been demonstrated that the development of hair loss in the lesional skin explants on SCID mice is associated with increased expression of RANTES by the follicular epithelium (Gilhar A et al. 2003). Additional abundant sources of chemokines are infiltrating monocytes, dendritic cells, and mast cells which secrete MIP-1α, MPI-1β, and RANTES, among others (Vissers JL et al. 2001, Sallusto F et al 1999, and Fischer M et al. 2006), suggesting a role for these cells in the process of leukocyte migration to the skin of AA affected mice.

Chemokines have been reported to play important roles in recruiting dendritic cells and effector T cells from the peripheral blood to the skin in chronic inflammatory skin diseases, including atopic dermatitis and psoriasis (Homey B 2005). Chemokines have
also been shown to be associated with the activation of leukocytes; MIP-1α, for instance, is involved in mast cells migration to the site of inflammation and serves as a costimulatory signal for mast cell degranulation (Miyazaki D et al. 2005).

Another potential source of chemokines and cytokines in the skin of AA affected mice could be CD8+ T cells. CD8+ T cells constitute a major proportion of the follicular inflammatory infiltrate in AA, while their cytotoxic role against hair follicles appears unlikely, as discussed before. However, the ability of these cells to secrete a battery of cytokines, such as IFN-γ and TNF-α (Walter U and Santamaria P 2005), and chemokines, such as RANTES and CXCL10 (Ejrnaes M et al. 2005), suggests a role for them in cytokine-induced hair growth inhibition and leukocyte migration to the skin of AA affected mice.

Taken together, the highly upregulated expression levels of chemokines point toward the significant role they may play in recruiting leukocytes to the skin of AA affected mice. This is encouraging for further research to study their functional relevance in the pathogenesis of AA, and suggests chemokine/receptor antagonists might be promising candidates in long-term management of patients with chronic, relapsing-remitting AA.

**Conclusion**

Our data is not consistent with AA as a mere Th1 cell-mediated autoimmune disease, and it is likely that both Th1- and Th2-mediated responses play significant pathogenic roles in chronic mouse AA. The following scenario is suggested as a possible mechanism of hair loss in AA: Due to as yet unknown events, and following the exposure of putative autoantigen(s) and their capture by antigen presenting cells, T cells are primed in the draining lymph nodes, and migrate to the skin. An array of cytokines is secreted by autoreactive T cells, which in turn boosts antigen presentation, T cell activation and differentiation, and further drives the inflammatory responses. In an attempt to balance the immune system, anti-inflammatory responses are initiated to compensate for the
increased expression of inflammatory mediators. Chemokines, such as MIP-1α, MIP-1β, RANTES, and CXCL10 are secreted by keratinocytes and the inflammatory infiltrate, which, in turn, recruits more inflammatory cells to the skin. The interaction of cytokines, such as IFN-γ and IL-6, with the follicular epithelium and their interference with normal hair growth cycling likely results in aberrant keratinocyte proliferation/differentiation, premature arrest of the hair growth cycle, and the formation of dystrophic anagen stage hair follicles, features that are characteristic of the hair loss disease AA.
### Tables and figures

Table 4.1 lists the genes studied and the sequences of primers used.

<table>
<thead>
<tr>
<th>target gene</th>
<th>forward primer sequence</th>
<th>reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>ACTGGCAAAAGGATGGTGAC</td>
<td>TGAGCTCATAGATGCTTGAG</td>
</tr>
<tr>
<td>IL-4</td>
<td>TCAACCCCCAGCTAGTGTC</td>
<td>TGTTCTCGGCTGTCAGG</td>
</tr>
<tr>
<td>IL-6</td>
<td>CACAAGTCGGAGAGGAGAC</td>
<td>CAGAATTGCGCTGCAAGA</td>
</tr>
<tr>
<td>IL-10</td>
<td>CCAAGCCTTATCGGAAATGA</td>
<td>TTTTCACAGGGGAAATCG</td>
</tr>
<tr>
<td>IL-13</td>
<td>GCTGAGCAACCATCACAAAGA</td>
<td>GGAATCCAGGCTACAGA</td>
</tr>
<tr>
<td>IL-17</td>
<td>TCTCTGATGCTGTGCTGCT</td>
<td>ACGTGGAACGGTTGAGTAG</td>
</tr>
<tr>
<td>IL-18</td>
<td>ACTTTGGCGGACTTCATGT</td>
<td>GGGGACTGGAACAAGCAGAG</td>
</tr>
<tr>
<td>IL-21</td>
<td>CGGCTCTGTATAGACTCTCG</td>
<td>GCCCTTTACATCTTGTAGA</td>
</tr>
<tr>
<td>RANTES</td>
<td>CCTCACCATACATCCTCAGC</td>
<td>AGAGTTAGGCAAAGCAGCA</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>CCCACTTTCTGCTTTTCTC</td>
<td>GTCTGCTTTTGGTCAGG</td>
</tr>
</tbody>
</table>
Figure 4.1 Th1-associated and other proinflammatory cytokines in AA mice. mRNA levels of Th1-associated and other proinflammatory cytokines in AA affected mice relative to normal haired controls are given by $2^{\Delta \Delta Ct}$ (n=6 each). For statistical significance, the $\Delta Ct$ values for AA affected and normal haired mice were subjected to student-T-test, which yielded the estimation of $\Delta \Delta Ct$. A P-value of less than 0.05, marked with an asterisk, indicates that the $\Delta \Delta Ct$ is significantly different from 0. AA, alopecia areata; Ct, threshold cycle; $\Delta Ct$, Ct(target) – Ct(reference); $\Delta \Delta Ct$, $\Delta Ct$(AA) - $\Delta Ct$(normal); IFN-$\gamma$, interferon $\gamma$. 
**Figure 4.2 Th2-associated cytokine gene expression in AA mice.** Expression of major Th2-associated cytokines in AA affected mice normalized to a reference gene and relative to normal haired controls is given by $2^{-\Delta \Delta Ct}$ (n=6 each). For statistical significance, the $\Delta Ct$ values for AA affected and normal haired mice were subjected to student-T-test, which yielded the estimation of $\Delta \Delta Ct$. A P-value of less than 0.05, marked with an asterisk, indicates that the $\Delta \Delta Ct$ is significantly different from 0. AA, alopecia areata; Ct, threshold cycle; $\Delta Ct$, Ct(target) – Ct(reference); $\Delta \Delta Ct$, $\Delta Ct$(AA) - $\Delta Ct$(normal).
Figure 4.3 Th17-associated cytokines. mRNA expression levels of Th17-associated cytokines are shown in the skin, draining lymph nodes, spleens, and thymus of AA affective mice relative to normal haired littermates. (n=6 each). Expression of the target gene normalized to the reference gene and relative to calibrator (control) is given by $2^{\Delta\Delta Ct}$. For statistical significance, the $\Delta Ct$ values for AA affected and normal haired mice were subjected to student-T-test, which yielded the estimation of $\Delta\Delta Ct$. A small P-value (<0.05), marked with an asterisk, indicates that the $\Delta\Delta Ct$ is significantly different from 0. AA, alopecia areata; Ct, threshold cycle; $\Delta Ct$, Ct(target) – Ct(reference); $\Delta\Delta Ct$, $\Delta Ct$(AA) - $\Delta Ct$(normal).
Figure 4.4 Chemokines. mRNA expression levels of several main chemokines in AA affected mice relative to their normal haired littermates are shown, as revealed by RT-PCR (n=6 each). For statistical significance, the ΔCt values for AA affected and normal haired mice were subjected to student-T-test, which yielded the estimation of ΔΔCt. A P-value of less than 0.05, marked with an asterisk, indicates that the ΔΔCt is significantly different from 0. AA, alopecia areata; Ct, threshold cycle; ΔCt, Ct(target) – Ct(reference); ΔΔCt, ΔCt(AA) - ΔCt(normal); MIP-1α, macrophage inflammatory protein-1α; MIP-1β, macrophage inflammatory protein-1 β; RANTES, regulated upon activation normal T cell expressed and secreted.
References:


Roitt I, Brostoff J, Male D (2001) Immunology, Sixth edition. MOSBY, Spain pp. 119-120


Chapter 5. Synthesis and future perspectives

Alopecia areata (AA) is usually regarded as an organ-specific autoimmune disease. The disease is characterized by antigen presentation, activation of autoreactive T cells, inflammatory infiltration of hair follicles composed mainly of CD4+ T cells, CD8+ T cells, Langerhans' cells, macrophages, NK cells, and cytokine production (Gilhar A and Kalish RS 2006). The resultant is premature arrest of the hair growth cycle, formation of dystrophic anagen stage hair follicles (HF), and massive shedding of hair which starts focally and spreads centrifugally (Tobin DJ et al. 1995). However, the exact mechanism of the inflammatory hair loss and the pathogenic role of different components of the immune system in the pathogenesis of AA have yet to be understood.

Two main hypotheses about the mechanisms of the immune response in AA include cell-mediated cytotoxicity, leading to increased apoptosis of the follicular epithelium and premature precipitation of anagen HFs to telogen, and cytokine-induced inhibition of the normal hair growth cycle (Hoffmann R 1999). We, accordingly, sought to further study the nature of the immune response against HFs, using C3H/HeJ mouse model for AA.

Cell-mediated cytotoxicity

Cell-mediated cytolysis of HFs has been proposed as a possible mechanism of hair loss in AA based on observations of infiltration of HFs with CD8+ T cells, and the beneficial effects of their depletion on HFs in the animal models for AA (McElwee KJ et al. 1996). However, few studies have so far shown a direct link between the putative cytotoxic cells or their products and degenerative keratinocytes and/or melanocytes in the lesional skin.

We hypothesized that if cell-mediated cytotoxicity against HFs is a component of the hair loss, increased expression of typical cytotoxic products in the lesional skin, as well as elevated apoptosis activity within the follicular epithelium would be expected to occur in AA. Accordingly, genes and products typical of cytotoxic cells, as well as the extent of
apoptosis activity, were studied in the skin and lymphoid organs of mice with AA relative to normal haired controls, using quantitative RT-PCR and immunohistochemistry.

Although highly elevated gene and protein expression was found for granzyme A, granzyme B, and FasL in AA affected skin, pointing toward the potential pathogenic role of these molecules in AA, their involvement in direct cytotoxicity against HF's appears unlikely. RT-PCR and immunohistochemical studies revealed limited gene and protein expression of perforin in the skin of AA affected mice compared with normal haired controls. In the absence of perforin, granzymes A and B are regarded incapable of inducing apoptosis in putative targets of cytotoxic cells (Pinkoski MJ et al. 1998). FasL+ cells were abundant, but scattered, in the skin of AA affected mice, and their expression pattern was not found comparable with infiltrating CD8+ cells. Moreover, the major cellular source of FasL was revealed to be mast cells. Taken together with the limited apoptosis activity within the AA affected HF's, as revealed by TUNEL studies of the C3H/HeJ mouse skin, cell-mediated cytotoxicity does not appear as a pathogenic component of hair loss in chronic mouse AA.

The unlikely involvement of cell-mediated cytotoxicity against HF's of AA affected C3H/HeJ mice, as discussed above, is a unique and yet intriguing finding, given the significant role which is regarded for CD8+ T cells in the pathogenesis of AA. CD8+ T cells are the major intrafollicular infiltrating cells in the lesional skin of both humans and rodents with AA (Sperling LC et al. 1995). Furthermore, it has been demonstrated that depletion of CD8+ cells partially restores hair growth in the Dundee experimental haldrats (DEBR) model for AA, and injection of C3H/HeJ mice with CD8+ cells, derived from their AA affected littermates, induces hair loss focally (McElwee KJ et al. 1996 and McElwee KJ et al. 2005). These findings suggest a pathogenic role for CD8+ cells in the induction and/or initiation of the inflammatory hair loss. Nevertheless, given that all mice included in our studies were 3 to 6 months past the experimental induction of hair loss, our data appears consistent with studies of chronic AA in humans; in chronic lesions, lymphocytes are usually absent in the peri- or intrafollicular region, further undermining
their direct involvement in the hair loss mechanism in chronic stage of AA (Wasserman D et al. 2007).

Although we have identified an increased presence of CD8+ cells in the intra- and perifollicular regions in AA affected mice, we fell short of further characterizing this population of infiltrating cells. The mAb 53-6.7 (anti-mouse CD8a), which is commonly used in immunohistological studies of CD8+ T cells, reacts to the α chain of CD8 (CD8a). CD8a is expressed on majority of T cytotoxic (Tc) cells, as well as Subsets of γδ TCR-bearing T cells, intestinal intraepithelial lymphocytes, and dendritic cells (MacDonald HR et al. 1990, Lefrancois L 1991, and Vremec D et al. 1992).

In addition to cytolysis of target cells, CD8+ T cells can execute their effector functions by secreting an array of cytokines, such as IFN-γ and TNF-α, and chemokines, such as CXCL10 and RANTES. This suggests a role for these cells in recruiting other inflammatory cells to the skin, and their potential involvement in the putative cytokine-induced inhibition of the hair growth in AA.

Combined Immunohistochemical staining with mAbs against CD8 α and β chains and/or TCR receptor would appear appropriate in order to further individualize CD8+ T cells. Furthermore, multi-color flow cytometric studies of the skin’s inflammatory population for the characteristic surface markers and/or products of T cells and other inflammatory cells can further characterize the infiltrating CD8+ cells. Such studies may also provide invaluable information about the activation status and/or effector functions of putative infiltrating CD8+ T cells. Finally, using the enzyme linked immunosorbent assay (ELISA) technique, the cytokine profile of isolated infiltrating CD8+ T cells can be studied in the skin of AA affected mice.
Mast cells

Another unique finding of this study is the presence of mast cells in the skin of AA affected C3H/HeJ mice, in a substantially increased number relative to normal haired controls. Moreover, skin mast cells were found considerably more activated in terms of producing granzyme B and FasL in AA affected mice, an observation which, in our best knowledge, had not been reported previously in the literature.

Although understanding the exact mechanism of mast cells' involvement in the pathogenesis of the hair loss disease entails more studies, several potential effector functions may be attributed to mast cells, based on our findings from RT-PCR and immunohistochemistry, AA's associations with atopy and stress, hair growth inhibitory properties of mast cells, and their potential immuno-protective effects.

Given the highly increased expression of granzyme B on the AA affected skin mast cells, and critical positioning of mast cells around the putative locations of blood vessels in the skin, a role for mouse mast cells may be defined in the recruitment of leukocytes to the site of inflammation in the AA affected skin. Granzyme B has been shown capable of inducing anoikis in endothelial cells, and mast cells' involvement in migration of leukocytes has been demonstrated in previous studies. To further assess the potential effects of mast cell-released granzyme B on the endothelial cell-to-cell contacts, in vitro cultures of endothelial cells from C3H/HeJ mice can be treated with various concentrations of granzyme B, derived from mouse skin mast cells' supernatants. If granzyme B plays a role in leukocyte extravasation, disorganizational changes would be expected to occur in endothelial cells (Pardo J et al. 2007). Moreover, AA affected C3H/HeJ mice may be treated with antibodies against granzyme B. If granzyme B plays a pathogenic role in AA, increased rate of hair regrowth would be expected to occur in granzyme B-deficient mice.
The association of atopic diseases such as atopic dermatitis and eczema with AA has been reportedly demonstrated in a number of studies. Given the pivotal role of mast cells in allergic responses, and their ability to release a wide variety of bio-active substances upon degranulation, a potential role for mast cells appear likely in determining the course and severity of AA. In this context, mast cell stabilizers, such as cromolyn sodium and nedocromil may be promising therapeutic options. When used properly, they are very effective in preventing mast cell degranulation, thus preventing the release of histamine and other constituents of mast cells’ granules (Cox JSG 1971).

Mast cells’ hair growth inhibitory properties have been shown in several studies. This is mediated in part by the ability of mast cells to produce the neuropeptide substance P (SP). However, other products of mast cells, including granzyme B, may play a role. The potential hair growth inhibitory effects of granzyme B can be further studied by treating organ-cultured HFs from humans and mice with different concentrations of granzyme B. Furthermore, the functional relevance of granzyme B in the inflammatory hair loss and activation status of mast cells can be studied by supplying C3H/HeJ mice with additional granzyme B. If granzyme B is to have a role in the mechanism of hair loss in AA, acceleration of HF regression (catagen) will be expected to occur.

The immuno-protective effects of mast cells have been proposed based on their association with infiltrating CD4+/CD8+ T regulatory (Treg) cells in skin grafts transplanted onto mice. Granzyme B, on the other hand, has been found as an essential mediator of Treg cells’ immunosuppressive functions. Taken together with increased presence of mast cells in the skin of AA affected mice, and their elevated production of granzyme B (part of our observations from histological and immunohistological staining of the mouse skin), functional studies are warranted to elucidate the effector function of mast cells in mouse AA; mast cells can be depleted in AA affected C3H/HeJ mice by treating them with compound 48/80 which has been shown to be very effective in depleting mast cells without significantly affecting other cell types (De-Matos IM et al. 2001). A disease protective role may be considered for mast cells if hair loss is aggravated upon treatment. In contrast, if mast cells are to play a disease enhancing role...
against the inflammatory hair loss, improvements in hair regrowth would be expected to occur.

**Cytokines in AA**

Several studies have found cytokines, both at the protein and mRNA levels, upregulated in the skin of mice and humans with AA. A consistent feature was the presence of Th1-associated cytokines, such as IFN-\(\gamma\). Here, we show that not only Th1-associated cytokines are upregulated, but also the expression of Th2-associated cytokines is increased in the skin of mice with AA relative to their normal haired littermates. In contrast, we did not find consistent with our data a role for Th17-associated cytokines in the pathogenesis of AA.

The clinical implication of such findings is the introduction of cytokines as promising targets in the treatment of AA, given the availability of an array of antibodies and antagonists to block the effects of cytokines (van de Vosse Eand van Agtmael MA 2007). A large body of evidence suggests that IFN-\(\gamma\) enhances the inflammatory hair loss process; thus, anti-IFN-\(\gamma\) antibodies appear as appropriate therapeutic candidates in AA (Skurkovich S et al. 2005).

IL-6 is another cytokine with potent hair growth inhibitory effects which is significantly upregulated in the skin of mice with AA, but its functional role has not properly been addressed yet. Therefore, functional studies using IL-6-knockout C3H/HeJ mice are recommended, where the susceptibility of mice to develop hair loss can be compared between the recipient IL-6\(+/-\) and IL-6\(-/-\) mice, following the transfer of skin grafts from the donor AA affected mice of the same species.

We found a substantial association between mRNA expression of members of the CC, and CXC families of chemokines and the pathogenesis of AA. While the role of chemokines in recruiting leukocytes have been demonstrated in other skin inflammatory
diseases, little is known about their role in the inflammatory hair loss. Nevertheless, our data is consistent with a significant role for chemokines, encouraging for further research to study their functional relevance in the pathogenesis of AA, using a variety of available chemokine/receptor antagonists.

A proposed model for AA

Due to as yet unknown events, in genetically susceptible individuals and under several environmental factors, an autoantigen(s) is exposed to the immune system. Skin residing dendritic cells and Langerhans' cells become activated and migrate to the skin draining lymph nodes to present antigens to T cells. T cells become primed and migrate to the skin where CD4+ T cells compose the main population of infiltrating perifollicular cells and CD8+ T cells mainly reside inside anagen stage hair follicles. CD4+ and CD8+ T cells, in turn, secrete a battery of cytokines including IFN-γ, TNF-α, IL-6, IL-13, and chemokines such as MIP-1α, MIP-1β, RANTES, and CXCL10. IFN-γ promotes antigen presentation by the follicular epithelium and antigen presenting cells and further boosts the immune response. Chemokines play an important role in recruiting leukocytes, including mast cells, to the skin.

Mast cells produce granzyme B and chemokines (e.g. MIP-1α), and play a significant part in recruiting more inflammatory cells to the site of inflammation. In addition, mast cells may be directly involved in the hair loss mechanism through their potential hair growth inhibitory properties, and their ability to secrete cytokines such as TNF-α and IL-6. Potentially, mast cells' derived granzyme B and other bio-active agents play important roles in determining the disease course and severity. The resultant of such inflammation is highly upregulated expression of proinflammatory cytokines, some of which (e.g. IFN-γ, TNF-α, and IL-6) have known hair growth inhibitory effects. Their interference with the normal hair growth cycle through their inhibition of normal keratinocytes' and/or melanocytes' proliferation and differentiation results in premature precipitation of anagen stage hair follicles into catagen and telogen. Clinically, the disease is manifested as
massive shedding of telogen hair follicles and appearance of alopecic patches which spreads to other parts of the body.

**Future directions**

This study has considerably contributed to our understanding of mechanisms of hair loss in AA. Evidence which has been shown here potentially undermines the role of cell-mediated cytotoxicity in the pathogenesis of AA, while suggesting important roles for mast cells, cytokines, and chemokines. However, many questions remain to be answered. Following is a list of next experiments which I would consider to perform if I were to pursue with this study:

1. In order to further characterize the peri- and intra-follicular infiltrating cells, I would conduct immunofluorescence and/or flow cytometric studies of the C3H/HeJ mouse skin using antibodies against characterizing surface markers, stimulatory and inhibitory receptors of CD4+ T cells, CD8+ T cells, CD4+/CD25+ Treg cells, CD8+ Treg cells, γδ T cells, NK cells, NKT cells, mast cells, dendritic cells, and Langerhans' cells.

2. The aforementioned studies could also be performed on human lesional scalp skin samples in order to extend the studies on mouse AA to humans.

3. Selective depletion of the aforementioned cells to reveal their functional effects in mouse AA.

4. Organ cultures of human and/or mouse hair follicles treated with granzyme B to study the potential hair growth inhibitory properties of granzyme B.

5. The potential pathogenic role of granzyme B could be further studied by injecting mice intraperitoneally with granzyme B.

6. Antagonizing granzyme B by treating mice with anti-granzyme B antibody could further elucidate the functional relevance of this molecule to the pathogenesis of AA.

7. Treatment of mice with inhibitors of mast cell degranulation like cromolyn sodium or nedocromil and antagonists to selected mast cell products (antihistamines, serotonin-antagonists).
8. Mast cell depletion using compound 48/80 to study the role of mast cells in the hair loss mechanism.

9. The role of cytokines such as IFN-γ, TNF-α, IL-6, IL-13, and chemokines such as MIP-1α, MIP-1β, RANTES, and CXCL10 could be further examined by treating AA affected C3H/HeJ mice with appropriate antibodies/antagonists.

**Conclusion**

Our data is not consistent with a role for cell-mediated cytotoxicity in the mechanism of hair loss in chronic mouse AA. Cytokines, on the other hand, may play a more significant role than thought before, not only by influencing the immune response, but also by exhibiting potential hair growth inhibitory properties. Intriguingly, mast cells are suggested as potential modulators of the immune response and/or hair growth cycling in C3H/HeJ mice. Finally, chemokines, with their putative abilities to recruit leukocytes to the site of inflammation, show a potential association with AA, and may be offered as promising therapeutic targets in AA.
References:


119


**APPENDICES**

**Appendix 1**

**Real time PCR data.** AA, alopecia areata; Ct, threshold cycle; ΔCt, Ct(target) − Ct(reference); ΔΔCt, ΔCt(AA) - ΔCt(normal); NL, normal.

<table>
<thead>
<tr>
<th>Target molecule</th>
<th>Tissue source</th>
<th>Average ΔCt AA</th>
<th>Average ΔCt Ni. Control</th>
<th>ΔΔCt μΔCt(AA)-μΔCt(NL)</th>
<th>Fold change 2^−ΔΔCt</th>
<th>Statistical Significance (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Granzyme A</strong></td>
<td>Skin</td>
<td>7.953</td>
<td>14.988</td>
<td>-7.036</td>
<td>131.194</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>8.389</td>
<td>10.619</td>
<td>-2.23</td>
<td>4.69</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>8.457</td>
<td>9.114</td>
<td>-0.657</td>
<td>1.577</td>
<td>0.595</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>7.159</td>
<td>10.438</td>
<td>-3.28</td>
<td>9.712</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Granzyme B</strong></td>
<td>Skin</td>
<td>5.275</td>
<td>16.096</td>
<td>-10.821</td>
<td>1808.975</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>8.623</td>
<td>10.627</td>
<td>-2.004</td>
<td>4.011</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>10.77</td>
<td>11.892</td>
<td>-1.122</td>
<td>2.177</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>11.633</td>
<td>12.58</td>
<td>-0.947</td>
<td>1.928</td>
<td>0.005</td>
</tr>
<tr>
<td><strong>Perforin</strong></td>
<td>Skin</td>
<td>7.745</td>
<td>9.157</td>
<td>-1.412</td>
<td>2.661</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>7.773</td>
<td>8.083</td>
<td>-0.31</td>
<td>1.24</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>8.033</td>
<td>8.208</td>
<td>-0.176</td>
<td>1.13</td>
<td>0.259</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>9.12</td>
<td>9.899</td>
<td>-0.778</td>
<td>1.715</td>
<td>0.031</td>
</tr>
<tr>
<td><strong>FAS</strong></td>
<td>Skin</td>
<td>9.874</td>
<td>11.046</td>
<td>-1.172</td>
<td>2.253</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>9.221</td>
<td>9.26</td>
<td>-0.039</td>
<td>1.027</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>9.641</td>
<td>10.05</td>
<td>-0.409</td>
<td>1.328</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>7.742</td>
<td>8.203</td>
<td>-0.461</td>
<td>1.377</td>
<td>0.127</td>
</tr>
<tr>
<td><strong>FAS L</strong></td>
<td>Skin</td>
<td>10.398</td>
<td>16.656</td>
<td>-6.258</td>
<td>76.548</td>
<td>4.392</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>11.34</td>
<td>11.699</td>
<td>-0.359</td>
<td>1.283</td>
<td>0.217</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>12.123</td>
<td>12.497</td>
<td>-0.374</td>
<td>1.296</td>
<td>0.132</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>11.615</td>
<td>12.36</td>
<td>-0.744</td>
<td>1.675</td>
<td>0.017</td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td>Skin</td>
<td>9.784</td>
<td>12.194</td>
<td>-2.41</td>
<td>5.315</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>9.224</td>
<td>9.582</td>
<td>-0.358</td>
<td>1.281</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>11.861</td>
<td>11.395</td>
<td>0.466</td>
<td>0.724</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>9.789</td>
<td>10.421</td>
<td>-0.633</td>
<td>1.551</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>TNFR1</strong></td>
<td>Skin</td>
<td>8.947</td>
<td>9.374</td>
<td>-0.427</td>
<td>1.344</td>
<td>0.475</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>7.788</td>
<td>7.691</td>
<td>0.097</td>
<td>0.935</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>7.795</td>
<td>7.695</td>
<td>0.1</td>
<td>0.933</td>
<td>0.682</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>8.653</td>
<td>8.851</td>
<td>-0.198</td>
<td>1.147</td>
<td>0.711</td>
</tr>
<tr>
<td><strong>TNFR2</strong></td>
<td>Skin</td>
<td>7.267</td>
<td>9.508</td>
<td>-2.241</td>
<td>4.729</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>5.813</td>
<td>5.838</td>
<td>-0.024</td>
<td>1.017</td>
<td>0.941</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>7.343</td>
<td>7.441</td>
<td>-0.098</td>
<td>1.07</td>
<td>0.565</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>8.776</td>
<td>8.887</td>
<td>-0.111</td>
<td>1.08</td>
<td>0.828</td>
</tr>
<tr>
<td>Target molecule</td>
<td>Tissue source</td>
<td>Average ΔCt AA</td>
<td>Average ΔCt NL Control</td>
<td>ΔΔCt</td>
<td>Fold change 2^−ΔΔCt</td>
<td>Statistical Significance (p-value)</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
<td>----------------</td>
<td>------------------------</td>
<td>------</td>
<td>----------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Skin</td>
<td>10.323</td>
<td>10.174</td>
<td>0.149</td>
<td>0.902</td>
<td>0.445</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>8.023</td>
<td>8.587</td>
<td>-0.563</td>
<td>1.478</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>10.275</td>
<td>10.998</td>
<td>-0.722</td>
<td>1.65</td>
<td>0.041</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>9.518</td>
<td>10.406</td>
<td>-0.887</td>
<td>1.85</td>
<td>0.019</td>
</tr>
<tr>
<td>TRAILR</td>
<td>Skin</td>
<td>10.369</td>
<td>9.055</td>
<td>1.314</td>
<td>0.402</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>10.627</td>
<td>10.665</td>
<td>-0.038</td>
<td>1.027</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>13.04</td>
<td>12.81</td>
<td>0.23</td>
<td>0.853</td>
<td>0.869</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>11.125</td>
<td>11.306</td>
<td>-0.181</td>
<td>1.134</td>
<td>0.77</td>
</tr>
<tr>
<td>TRAMP</td>
<td>Skin</td>
<td>14.416</td>
<td>13.989</td>
<td>0.427</td>
<td>0.744</td>
<td>0.466</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>12.622</td>
<td>9.739</td>
<td>2.883</td>
<td>0.136</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>12.711</td>
<td>12.358</td>
<td>0.353</td>
<td>0.783</td>
<td>0.588</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>12.036</td>
<td>12.278</td>
<td>-0.243</td>
<td>1.183</td>
<td>0.624</td>
</tr>
<tr>
<td>CD16</td>
<td>Skin</td>
<td>6.562</td>
<td>7.945</td>
<td>-1.382</td>
<td>2.607</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>7.488</td>
<td>7.801</td>
<td>-0.313</td>
<td>1.243</td>
<td>0.107</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>8.126</td>
<td>7.854</td>
<td>0.272</td>
<td>0.828</td>
<td>0.251</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>9.077</td>
<td>9.403</td>
<td>-0.327</td>
<td>1.254</td>
<td>0.153</td>
</tr>
<tr>
<td>CD56</td>
<td>Skin</td>
<td>11.54</td>
<td>9.963</td>
<td>1.577</td>
<td>0.335</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>13.494</td>
<td>13.609</td>
<td>-0.115</td>
<td>1.083</td>
<td>0.357</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>17.089</td>
<td>16.493</td>
<td>0.596</td>
<td>0.662</td>
<td>0.116</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>14.7</td>
<td>15.884</td>
<td>-1.184</td>
<td>2.273</td>
<td>0.015</td>
</tr>
<tr>
<td>CD94</td>
<td>Skin</td>
<td>8.958</td>
<td>10.282</td>
<td>-1.324</td>
<td>2.504</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>7.649</td>
<td>6.895</td>
<td>0.754</td>
<td>0.593</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>11.133</td>
<td>9.246</td>
<td>1.887</td>
<td>0.27</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>8.49</td>
<td>9.471</td>
<td>-0.981</td>
<td>1.974</td>
<td>0.001</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Skin</td>
<td>9.756</td>
<td>11.242</td>
<td>-1.486</td>
<td>2.802</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>8.396</td>
<td>9.193</td>
<td>-0.797</td>
<td>1.737</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>11.072</td>
<td>10.577</td>
<td>0.495</td>
<td>0.71</td>
<td>0.182</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>8.528</td>
<td>9.67</td>
<td>-1.142</td>
<td>2.207</td>
<td>0.001</td>
</tr>
<tr>
<td>Gp49B1</td>
<td>Skin</td>
<td>8.333</td>
<td>10.046</td>
<td>-1.713</td>
<td>3.278</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>7.766</td>
<td>8.162</td>
<td>-0.395</td>
<td>1.315</td>
<td>0.174</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>9.625</td>
<td>8.162</td>
<td>1.446</td>
<td>0.367</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>10.442</td>
<td>11.264</td>
<td>-0.822</td>
<td>1.768</td>
<td>0.023</td>
</tr>
<tr>
<td>CD244</td>
<td>Skin</td>
<td>11.597</td>
<td>11.312</td>
<td>0.285</td>
<td>0.821</td>
<td>0.199</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>10.644</td>
<td>10.988</td>
<td>-0.344</td>
<td>1.269</td>
<td>0.308</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>10.257</td>
<td>9.744</td>
<td>0.513</td>
<td>0.701</td>
<td>0.071</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>13.007</td>
<td>13.236</td>
<td>-0.229</td>
<td>1.172</td>
<td>0.318</td>
</tr>
<tr>
<td>Target molecule</td>
<td>Tissue source</td>
<td>Average ΔCt</td>
<td>Average ΔCt</td>
<td>ΔΔCt</td>
<td>Fold change</td>
<td>Statistical Significance (p-value)</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------</td>
<td>-------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA Control</td>
<td>Nl. Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD161 Skin</td>
<td>11.377</td>
<td>10.503</td>
<td>0.874</td>
<td>0.546</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CD161 Lymph Node</td>
<td>10.932</td>
<td>10.596</td>
<td>0.335</td>
<td>0.793</td>
<td>0.249</td>
<td></td>
</tr>
<tr>
<td>CD161 Spleen</td>
<td>13.648</td>
<td>13.513</td>
<td>0.135</td>
<td>0.911</td>
<td>0.313</td>
<td></td>
</tr>
<tr>
<td>CD161 Thymus</td>
<td>13.273</td>
<td>13.647</td>
<td>-0.374</td>
<td>1.296</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>CD49b Skin</td>
<td>6.926</td>
<td>7.638</td>
<td>-0.712</td>
<td>1.638</td>
<td>0.108</td>
<td></td>
</tr>
<tr>
<td>CD49b Lymph Node</td>
<td>10.618</td>
<td>9.82</td>
<td>0.798</td>
<td>0.575</td>
<td>0.219</td>
<td></td>
</tr>
<tr>
<td>CD49b Spleen</td>
<td>10.867</td>
<td>10.614</td>
<td>0.253</td>
<td>0.839</td>
<td>0.521</td>
<td></td>
</tr>
<tr>
<td>CD49b Thymus</td>
<td>11.767</td>
<td>12.255</td>
<td>-0.488</td>
<td>1.402</td>
<td>0.321</td>
<td></td>
</tr>
<tr>
<td>Ly49g Skin</td>
<td>13.803</td>
<td>16.916</td>
<td>-3.112</td>
<td>8.649</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Ly49g Lymph Node</td>
<td>12.409</td>
<td>12.373</td>
<td>0.036</td>
<td>0.975</td>
<td>0.911</td>
<td></td>
</tr>
<tr>
<td>Ly49g Spleen</td>
<td>13.744</td>
<td>13.341</td>
<td>0.403</td>
<td>0.756</td>
<td>0.106</td>
<td></td>
</tr>
<tr>
<td>Ly49g Thymus</td>
<td>10.929</td>
<td>11.665</td>
<td>-0.736</td>
<td>1.665</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>IFN-γ Skin</td>
<td>9.638</td>
<td>18.724</td>
<td>-9.086</td>
<td>543.381</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>IFN-γ Lymph Node</td>
<td>11.955</td>
<td>13.132</td>
<td>-1.177</td>
<td>2.261</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>IFN-γ Spleen</td>
<td>14.469</td>
<td>14.287</td>
<td>0.182</td>
<td>0.881</td>
<td>0.397</td>
<td></td>
</tr>
<tr>
<td>IFN-γ Thymus</td>
<td>12.424</td>
<td>12.95</td>
<td>-0.525</td>
<td>1.439</td>
<td>0.105</td>
<td></td>
</tr>
<tr>
<td>IL-10 Skin</td>
<td>12.714</td>
<td>13.789</td>
<td>-1.075</td>
<td>2.106</td>
<td>0.042</td>
<td></td>
</tr>
<tr>
<td>IL-10 Lymph Node</td>
<td>11.925</td>
<td>14.073</td>
<td>-2.148</td>
<td>4.433</td>
<td>0.012</td>
<td></td>
</tr>
<tr>
<td>IL-10 Spleen</td>
<td>12.497</td>
<td>14.005</td>
<td>-1.508</td>
<td>2.844</td>
<td>0.037</td>
<td></td>
</tr>
<tr>
<td>IL-10 Thymus</td>
<td>14.356</td>
<td>14.897</td>
<td>-0.541</td>
<td>1.455</td>
<td>0.049</td>
<td></td>
</tr>
<tr>
<td>IL-17 Skin</td>
<td>13.742</td>
<td>12.516</td>
<td>1.226</td>
<td>0.428</td>
<td>0.099</td>
<td></td>
</tr>
<tr>
<td>IL-17 Lymph Node</td>
<td>12.649</td>
<td>12.159</td>
<td>0.491</td>
<td>0.712</td>
<td>0.228</td>
<td></td>
</tr>
<tr>
<td>IL-17 Spleen</td>
<td>18.013</td>
<td>18.18</td>
<td>-0.166</td>
<td>1.122</td>
<td>0.411</td>
<td></td>
</tr>
<tr>
<td>IL-17 Thymus</td>
<td>17.651</td>
<td>17.564</td>
<td>0.086</td>
<td>0.942</td>
<td>0.402</td>
<td></td>
</tr>
<tr>
<td>IL-18 Skin</td>
<td>9.606</td>
<td>8.407</td>
<td>1.199</td>
<td>0.435</td>
<td>0.045</td>
<td></td>
</tr>
<tr>
<td>IL-18 Lymph Node</td>
<td>11.229</td>
<td>11.073</td>
<td>0.156</td>
<td>0.898</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>IL-18 Spleen</td>
<td>9.757</td>
<td>9.753</td>
<td>0.003</td>
<td>0.998</td>
<td>0.495</td>
<td></td>
</tr>
<tr>
<td>IL-18 Thymus</td>
<td>11.964</td>
<td>11.829</td>
<td>0.136</td>
<td>0.91</td>
<td>0.329</td>
<td></td>
</tr>
<tr>
<td>IL-21 Skin</td>
<td>16.045</td>
<td>16.245</td>
<td>-0.201</td>
<td>1.149</td>
<td>0.437</td>
<td></td>
</tr>
<tr>
<td>IL-21 Lymph Node</td>
<td>11.925</td>
<td>11.593</td>
<td>0.331</td>
<td>0.795</td>
<td>0.264</td>
<td></td>
</tr>
<tr>
<td>IL-21 Spleen</td>
<td>14.735</td>
<td>15.39</td>
<td>-0.655</td>
<td>1.574</td>
<td>0.084</td>
<td></td>
</tr>
<tr>
<td>IL-21 Thymus</td>
<td>14.333</td>
<td>14.567</td>
<td>-0.235</td>
<td>1.177</td>
<td>0.249</td>
<td></td>
</tr>
<tr>
<td>IL-4 Skin</td>
<td>17.414</td>
<td>18.171</td>
<td>-0.757</td>
<td>1.69</td>
<td>0.147</td>
<td></td>
</tr>
<tr>
<td>IL-4 Lymph Node</td>
<td>18.91</td>
<td>18.05</td>
<td>0.86</td>
<td>0.551</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>IL-4 Spleen</td>
<td>17.225</td>
<td>16.976</td>
<td>0.249</td>
<td>0.842</td>
<td>0.397</td>
<td></td>
</tr>
<tr>
<td>IL-4 Thymus</td>
<td>16.541</td>
<td>17.055</td>
<td>-0.515</td>
<td>1.429</td>
<td>0.233</td>
<td></td>
</tr>
<tr>
<td>Target molecule</td>
<td>Tissue source</td>
<td>Average ΔCt AA</td>
<td>Average ΔCt NL</td>
<td>ΔΔCt</td>
<td>Fold change</td>
<td>Statistical Significance (p-value)</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
<td>----------------</td>
<td>----------------</td>
<td>-------</td>
<td>-------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>IL-6</td>
<td>Skin</td>
<td>14.279</td>
<td>16.71</td>
<td>-2.431</td>
<td>5.393</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>17.016</td>
<td>17.717</td>
<td>-0.701</td>
<td>1.626</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>18.107</td>
<td>17.511</td>
<td>0.596</td>
<td>0.662</td>
<td>0.487</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>15.911</td>
<td>15.658</td>
<td>0.253</td>
<td>0.839</td>
<td>0.521</td>
</tr>
<tr>
<td>IL-13</td>
<td>Skin</td>
<td>7.71</td>
<td>12.707</td>
<td>-4.996</td>
<td>31.919</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>15.085</td>
<td>14.786</td>
<td>0.299</td>
<td>0.813</td>
<td>0.628</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>10.332</td>
<td>11.37</td>
<td>-1.038</td>
<td>2.053</td>
<td>0.098</td>
</tr>
<tr>
<td>MIP1-α</td>
<td>Skin</td>
<td>7.676</td>
<td>12.442</td>
<td>-4.766</td>
<td>27.214</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>10.978</td>
<td>11.188</td>
<td>-0.21</td>
<td>1.157</td>
<td>0.274</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>10.405</td>
<td>11.221</td>
<td>-0.816</td>
<td>1.761</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>11.785</td>
<td>12.008</td>
<td>-0.222</td>
<td>1.166</td>
<td>0.235</td>
</tr>
<tr>
<td>MIP1-β</td>
<td>Skin</td>
<td>6.448</td>
<td>16.105</td>
<td>-9.657</td>
<td>807.349</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>12.505</td>
<td>13.242</td>
<td>-0.738</td>
<td>1.668</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>13.167</td>
<td>14.003</td>
<td>-0.836</td>
<td>1.786</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>13.737</td>
<td>14.193</td>
<td>-0.456</td>
<td>1.371</td>
<td>0.051</td>
</tr>
<tr>
<td>RANTES</td>
<td>Skin</td>
<td>5.393</td>
<td>11.05</td>
<td>-5.657</td>
<td>50.464</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>0.563</td>
<td>1.599</td>
<td>-1.037</td>
<td>2.052</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>6.78</td>
<td>6.926</td>
<td>-0.156</td>
<td>1.114</td>
<td>0.278</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>5.307</td>
<td>5.932</td>
<td>-0.625</td>
<td>1.542</td>
<td>0.006</td>
</tr>
<tr>
<td>CXCL1</td>
<td>Skin</td>
<td>9.9</td>
<td>11.07</td>
<td>-1.17</td>
<td>2.251</td>
<td>0.071</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>12.264</td>
<td>11.333</td>
<td>0.931</td>
<td>0.525</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>13.074</td>
<td>12.955</td>
<td>0.119</td>
<td>0.921</td>
<td>0.393</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>14.042</td>
<td>14.167</td>
<td>-0.125</td>
<td>1.09</td>
<td>0.364</td>
</tr>
<tr>
<td>CXCL10</td>
<td>Skin</td>
<td>3.503</td>
<td>9.037</td>
<td>-5.534</td>
<td>46.328</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>7.252</td>
<td>8.289</td>
<td>-1.037</td>
<td>2.051</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>8.123</td>
<td>6.498</td>
<td>1.625</td>
<td>0.324</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>8.462</td>
<td>8.795</td>
<td>-0.333</td>
<td>1.26</td>
<td>0.145</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>Skin</td>
<td>7.677</td>
<td>8.108</td>
<td>-0.431</td>
<td>1.348</td>
<td>0.124</td>
</tr>
<tr>
<td></td>
<td>Lymph Node</td>
<td>9.418</td>
<td>9.973</td>
<td>-0.554</td>
<td>1.469</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>11.368</td>
<td>12.368</td>
<td>-1</td>
<td>2</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>8.49</td>
<td>8.962</td>
<td>-0.472</td>
<td>1.387</td>
<td>0.066</td>
</tr>
</tbody>
</table>
**ANIMAL CARE CERTIFICATE**

<table>
<thead>
<tr>
<th>Application Number:</th>
<th>A05-1714</th>
</tr>
</thead>
<tbody>
<tr>
<td>Investigator or Course Director:</td>
<td>Kevin McElwee</td>
</tr>
<tr>
<td>Department:</td>
<td>Medicine, Department of</td>
</tr>
<tr>
<td>Animals:</td>
<td>Mice C3H/HeJ 160</td>
</tr>
<tr>
<td>Start Date:</td>
<td>January 1, 2006</td>
</tr>
<tr>
<td>Approval Date:</td>
<td>November 19, 2007</td>
</tr>
<tr>
<td>Funding Sources:</td>
<td></td>
</tr>
<tr>
<td>Funding Agency:</td>
<td>Canadian Dermatology Foundation</td>
</tr>
<tr>
<td>Funding Title:</td>
<td>Natural Killer (NK) Cell Dysfunction in Human and Rodent Model</td>
</tr>
<tr>
<td>Unfunded Title:</td>
<td>N/A</td>
</tr>
</tbody>
</table>

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093