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of the University of Lübeck

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Abnormal interactions between perifollicular mast cells and CD8+ T-cells may contribute to the pathogenesis of alopecia areata

Dissertation

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Submitted by

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I would like to dedicate this thesis to my wonderful family, my special boyfriend, my friends, my colleagues, and to ...

Table of contents

List of abbreviations

AA	Alopecia areata
ABC	Avidin-biotin-complex
APM	Arrector pili muscle
AP	Alkaline phosphatase
BSA	Bovine serum albumin
c-Kit	CD117, stem cell factor receptor
CTS	Connective tissue sheath
DP	Dermal papilla
FITC	Fluorescein isothiocyanate
HB	Hair bulb
HF	Hair follicle
HLA	Human leukocyte antigen
HM	Hair matrix
MHC	Major histocompatibility complex
mMCP6	Murine mast cell protease-6
ICAM-1	Intercellular adhesion molecule-1
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon-γ
IHC	Immunohistochemistry
IL	Interleukin
IP	Immune privilege
IR	Immunoreactivity
IRS	Inner root sheath
L	Ligand
LFA-1	Lymphocyte function-associated antigen
MC	Mast cell
min	Minute
PD	Programmed cell death
ORS	Outer root sheath
PAR-2	Protease-activated receptor-2
NGF	Nerve growth factor
PFD	Perifollicular dermis
RT	Room temperature
SCID	Severe combined immunodeficiency
SCF	Stem cell factor, Kit ligand
SG	Sebaceous glands
SP	Substance P
TGF	Transforming growth factor
TNFα	Tumour necrosis factor-α

1 Introduction

1.1 Background and project overview

Alopecia areata (AA) is an organ-specific, T-cell-dependent autoimmune disease characterized by hair loss. In AA, the typical inflammatory cell infiltrate around growing hair follicles (HF) may result from the collapse of the constitutive immune privilege (IP) of the HF, leading to ectopic presentation of intrafollicular autoantigens via major histocompatibility complex (MHC) class I followed by a CD8+ T-cell-mediated autoimmune attack on the HF. Mast cell (MC) activities are implicated in the control of hair growth, may play a role in HF-IP maintenance, and modulate acquired immunity, including antigen-specific CD8+ T-cell (auto-)immune responses. Moreover, an increased number and activities of MCs has been described in AA.

Therefore, the current project aims to dissect for the first time, the role that perifollicular MCs, and their interactions with CD8+ T-cells may play in AA pathogenesis.

Unfortunately, none of the routinely used MC-deficient or MC-overexpressing mouse models develop classical AA lesions, and it is not possible yet to selectively eliminate or functionally modulate MCs exclusively in established AA mouse models. For these reasons, the investigation of the role of MC-CD8+ T-cell interactions in AA pathobiology is currently restricted to descriptive phenomenological studies. However, in order to keep the analyses as instructive as possible and to generate data of maximal clinical relevance, extensive qualitative and quantitative (immuno-)histomorphometry investigations of markers that permit one to gauge changes in MC functions immunohistologically, namely during their interactions with CD8+ T-cells, were performed in human AA lesions *in situ* (compared with non-lesional and healthy control skin).

In addition, organ cultures of normal human scalp HF, as well as of healthy and AA scalp skin, were carried out to investigate the effects of MC secretagogues and MC stabilizers on perifollicular MC-CD8+ T-cell interactions *in vitro.* Finally, MC and CD8+ T-cell interactions *in situ* were also examined in three complementary *in vivo* models, i.e. ageing C3H/HeJ AA mice, which spontaneously develop AA-like hair loss lesions, grafted C3H/HeJ mice that develop AA lesions in an accelerated manner, and in a newly established humanized mouse model where AA lesions are experimentally induced in healthy human scalp skin transplanted onto immunocompromised mouse skin.

Taken together, these studies provide important new insights into the role of perifollicular MCs and MC-CD8+ T-cell interactions in this common autoimmune disease.

1.2 Hair follicle biology

The skin is the biggest organ of the human body and is composed of epidermis, dermis and subcutis **(Figure 1.1)**. It contains several appendages among which the HFs and their associated sebaceous glands (SG) (Heath and Carbone 2013, Di Meglio et al. 2011, Sterry at al. 2006).

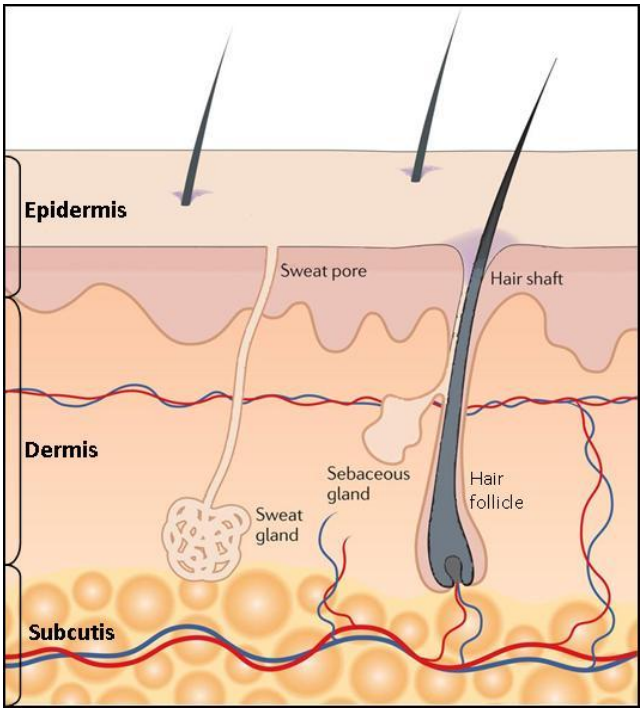

Figure 1.1: Human scalp skin structure. The human scalp skin is composed by epidermis, dermis and subcutis. It contains appendages such as sweat glands, HFs and SG and an array of structures such as nerves, blood and lymphatic vessels. Figure modified after Grice and Segre 2011.

HFs are complex mini-organs whose main function is to produce a pigmented hair shaft (HS), which plays an important role in physical protection of the skin from environmental damage, thermoregulation, dispersion of secreted substances on the skin surface and social communication (Patzelt et al. 2013, Schneider et al. 2009, Stenn and Paus 2001, Paus and Cotsarelis 1999).

In human skin, the HF develops during embryogenesis thanks to a bidirectional cross-talk between cutaneous ectoderm and mesenchyme which is tightly regulated by multiple growth factors and their receptors, growth factor antagonists, adhesion molecules and intracellular signal transduction components as well as many different transcription factors (Plikus et al. 2012, Shimomura and Christiano 2010, Schneider et al. 2009, Schmidt-Ullrich and Paus 2005). Once the HF is formed, it continuously self-renews during a process called HF cycling

(Jing et al. 2014, Shimomura and Christiano 2010, Schneider et al. 2009, Stenn and Paus 2001).

The HF is a highly sensitive tactile organ which registers even slight HS movements. In fact, the HFs is highly innervated by cutaneous nerves which contain neuromediators and neuropeptides, such as substance P (SP) or calcitonin gene-related peptide (CGRP) that can influence follicular keratinocytes and the HF cycle (Samuelov et al. 2012, Peters et al. 2007, Paus et al. 2006, Botchkarev et al. 2004, Paus et al. 1997, Paus et al. 1994b).

1.2.1 Hair follicle anatomy

Mature anagen VI HFs have a complex structure composed by ectoderm- or mesoderm-derived tissue compartments that are arranged in the shape of an inverted-wine glass (hair bulb (HB)) that encloses an onion-shaped structure (dermal papilla (DP)) **(Table 1.1)**. The HF's epithelial compartments, from the outermost to the innermost layer, are the outer root sheath (ORS), the inner root sheath (IRS) and the HS and the hair matrix (HM) **(Table 1.1.** and **Figure 1.2A-B)**. The ORS represents the continuation of the epidermal basal layer. The IRS is further divided into four parts, the companion layer, Henle's layer, Huxley's layer and the IRS cuticle and reaches the insertion of the SG duct **(Table 1.1** and **Figure 1.2A-B)**. The HS is constituted by the cuticle, cortex and medulla **(Table 1.1** and **Figure 1.2A-B)**. A substantial basement membrane surrounds the ORS, which in turn is enclosed by a mesenchyme-derived layer, the connective tissue sheath (CTS) (**Figure 1.2A-B**) (Shimomura and Christiano 2010, Schneider et al. 2009, Paus and Foitzik 2004, Stenn and Paus 2001).

Glossary of hair follicle anatomy.

Arrector pili muscle	Tiny smooth muscle that connects the hair follicle with the dermis. When contracted the arrector pili causes the 'raising' of the hair.
Bulb	Thickening of the proximal end of the hair follicle. Contains rapidly proliferating, rather undifferentiated matrix cells (transient amplifying cells), melanocytes and outer root sheath cells.
Bulge	Convex protrusion of the outer root sheath in the most distal permanent portion of the hair follicle, just below the sebaceous gland and at the insertion site of the muscle arrector pili. Contains the hair follicle stem cells.
Club hair	Fully keratinized, dead hair (telogen follicle) formed during catagen and telogen.
Dermal papilla	Mesodermal signaling center of the hair follicle consisting of closely packed specialized mesenchymal fibroblasts. Framed by the enlarged bulb matrix in anagen.
Hair canal	Tubular connection between the epidermal surface and the most distal part of the inner root sheath. Contains the hair shaft.
Hair germ	Also called 'hair placode' depending on the developmental stage. Bud-like thickening in the fetal epidermis consisting of elongated keratinocytes, which at the distal end are in touch with numerous aggregated specialized dermal fibroblasts, the dermal condensate.
Hair peg	Column of keratinocytes growing into the dermis during embryonic hair follicle development (developmental stages 3-5). The concave proximal end starts to encase the dermal condensate, the future dermal papilla.
Hair shaft	The hair *per se*, composed of trichocytes, which are terminally differentiated hair follicle keratinocytes. It is composed of the medulla, the central part with loosely connected keratinized cells and large air spaces, and the cortex, which is the bulk of the hair shaft, consisting of keratinized cells, keratin filaments, and melanin granules in pigmented hairs.
Infundibulum	Most proximal part of the hair follicle relative to the epidermis, extending from the sebaceous duct to the epidermal surface. Includes the hair canal and the distal Outer root sheath.
Inner root sheath	A multilayered, rigid tube composed of terminally differentiated hair follicle keratinocytes, surrounded by the outer root sheath.
Isthmus	Middle part of the hair follicle extending from the sebaceous duct to the bulge.
Outer root sheath	The outermost layer of the hair follicle. Merges proximally with the basal layer of the interfollicular epidermis and distally with the hair bulb.
Pelage hairs	Pelage hair covers most of the body's surface, and at the molecular level it is the most extensively studied hair type in mice. Divided into four types: the large primary monotrich or guard hairs, the secondary intermediate awl and auchene hairs, and the secondary downy zigzag hairs.
Sebaceous gland	Acinar gland composed of lipid-filled sebocytes, localized close to the insertion of the arrector pili muscle. Secretes sebum to the epidermal surface via a holocrine mechanism. Sebum helps making hair and skin waterproof. Together with the hair follicle and the arrector pili muscle it forms the pilosebaceous unit.

Table 1.1: Hair follicle-associated terms: Definition. Table from Schneider et al. 2009.

Sagittally, the upper part of the HF is composed by the ORS infundibulum, which is the opening of hair canal to the skin surface, and the isthmus which is delimited by the arrector pili muscle (APM) **(Table 1.1** and **Figure 1.3A)**. In detail, the insertion of the SG duct divides the infundibulum part from the isthmus **(Table 1.1)**, which is, in turn, separated from the suprabulbar region by the insertion of the APM **(Figure 1.3A)** (Schneider et al. 2009, Paus and Foitzik 2004).

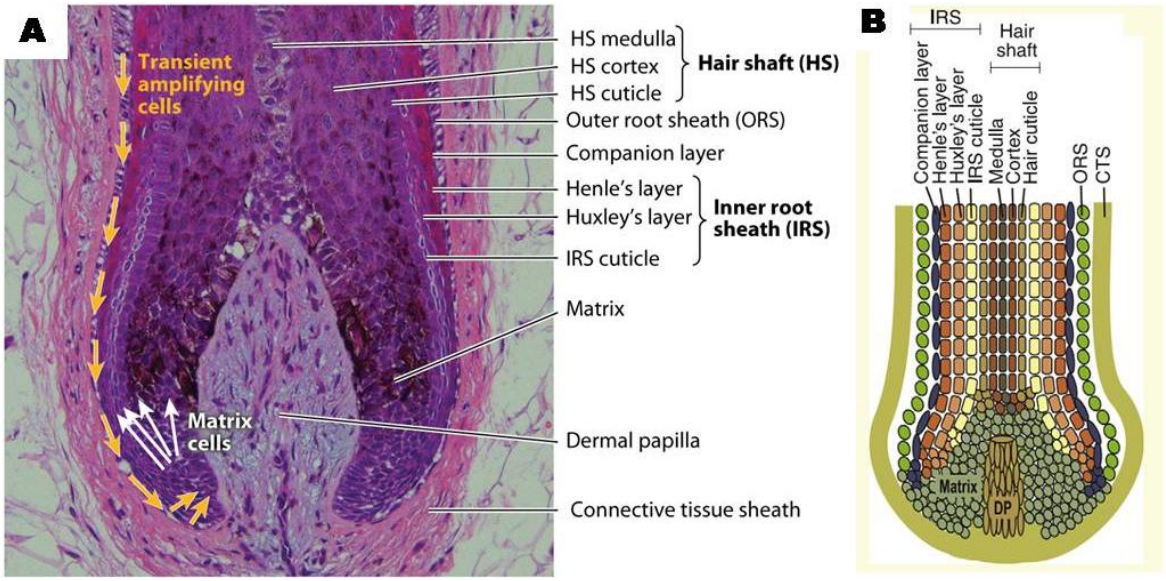

Figure 1.2: Histomorphology of the lower hair follicle (anagen hair bulb) and the concentric layers of the hair follicle epithelium.
High magnification image of the HF bulb (A) and schematic drawing (B) showing the different HF cell layers. Starting from the periphery, ORS, the companion layer and the IRS which is composed of Henle's and Huxley´s layers and the IRS cuticle. The central compartment is the HS which comprises the HS cuticle, cortex and medulla, all of which are formed by terminally differentiated trichocytes, i.e. HF keratinocytes that richly express hair keratins (Moll at al. 2008, Langbein and Schweizer 2005). The HF is finally enveloped by a mesenchyme layer, the CTS. Connective tissue sheath (CTS), hair shaft (HS), inner root sheath (IRS), outer root sheath (ORS). Figures modified after Shimomura and Christiano 2010 and Schneider et al. 2009.

The APM is a muscle structure which orients obliquely the HF to the epidermal surface **(Figure 1.3A,B)**. Normally, 3-4 terminal HFs share a single APM composing the so-called follicular unit. It is also responsible for the 'standing up' of hair shafts, during stress, anger or anxiety because it is under adrenergic control (Paus and Peker 2003).

The lower follicle which is the cycling part of the HFs, is constantly modified during the HF cycle and contains the suprabulbar and bulbar areas **(Figure 1.3A,C).** The mesenchyme-derived tissue with onion-like structure placed in the centre of an anagen hair bulb is the DP, which is composed by an aggregate of highly specialized, inductive fibroblasts, collagen bundles, nerve fibers, capillaries and melanocytes **(Figure 1.3C)**. Within the HB resides one of the most proliferative cells of the human body, the HM keratinocytes **(Figure 1.3C)** which differentiate into trichocytes of all epithelial HF cell layers with the exception of the ORS. During further progress they express different sets of hair keratins to produce the HS

(Shimomura and Christiano 2010, Schneider et al. 2009, Langbein and Schweizer 2005, Moll et al. 2008, Panteleyev et al. 2001, Stenn and Paus 2001). The HB harbours also the pigmentary unit constituted by active melanocytes (c-Kit positive) which transfer the melanin to the pre-cortical matrix keratinocytes (Tobin et al. 2011, Slominski et al. 2005).

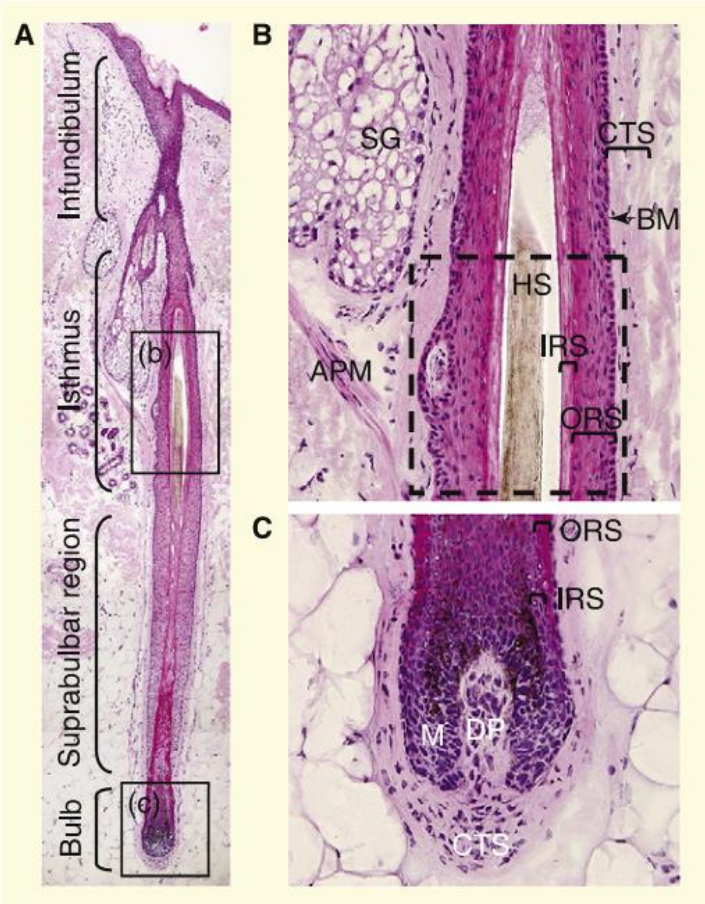

Figure 1.3: Histology of an anagen VI hair follicle.
Histochemically stained human HF section showing the permanent (infundibulum, isthmus) and anagen-associate (suprabulbar and bulb) regions (A). Higher magnification of the HF stem cell niche (bulge) which is located within the isthmus under the SG and whose lower limit is indicated by the insertion of the APM (B). Higher magnification showing the bulbar region, i.e. the HS production machinery of the HF, which is mainly inhabited by highly proliferating HM keratinocytes and HF melanocytes of the HF pigmentary unit (Paus 2011, Tobin 2011) (C). Arrector pili muscle (AMP), basement membrane (BM), connective tissue sheath (CTS), dermal papilla (DP), hair shaft (HS), inner root sheath (IRS), matrix (M), outer root sheath (ORS), sebaceous gland (SG). Figure modified after Schneider et al. 2009.

The stem cell niche, were the multipotent epithelial HF stem cells reside, is also called HF bulge ("Wulst") (Purba et al. 2014, Schneider 2011) and is located below the SG duct at the insertion point of the APM where the ORS sometimes forms a "trochanter"-like protrusion (Lecardonnel et al. 2013, Leishman et al. 2013, Myung and Ito 2012, Plikus et al. 2012, Kloepper et al. 2008, Tiede et al. 2007, Cotsarelis 2006) **(Figure 1.3B)**. These slow-cycling stem cells are responsible of the constant renewal of the HFs during the hair cycle (Purba et al. 2014, Myung and Ito 2012) and are located in a tissue niche that enjoys relative IP (Harries et al. 2013, Meyer et al. 2008).

Apart from terminal HFs in the scalp, in the human body other hair type can be found with different sizes, diameter and anagen duration: lanugo, vellus and miniaturized HFs as well as other specialized hair types such as eyebrow and eyelash HFs. Lanugo hairs are fine filaments typical of the prenatal period and are shed in uterus or during the first weeks of life.

Vellus hairs which populate most of the body are very short, non-pigmented and usually non-medullated; they lack of the APM and can show large SG. Instead, miniaturized follicles derive from terminal-to-vellus hair conversion which produces HFs that still retain the APM (Yazdabadi et al. 2012).

1.2.2 Hair follicle cycle

As a (mini-)organ, the HF is a unique organ in the mammalian body since it undergoes extensive, lifelong, cyclic transformations. During this process (hair cycle) the HF passes through an active growth phase (anagen) during which a pigmented HS is produced, a regression phase (catagen), which represents apoptosis-mediated organ involution, and a stage of relative quiescence (telogen) in which the club hair becomes enveloped by the permanent part of the HF epithelium (Gilhar et al. 2012, Shimomura and Christiano 2010, Schneider et al. 2009, Stenn and Paus 2001) **(Figure 1.4)**.

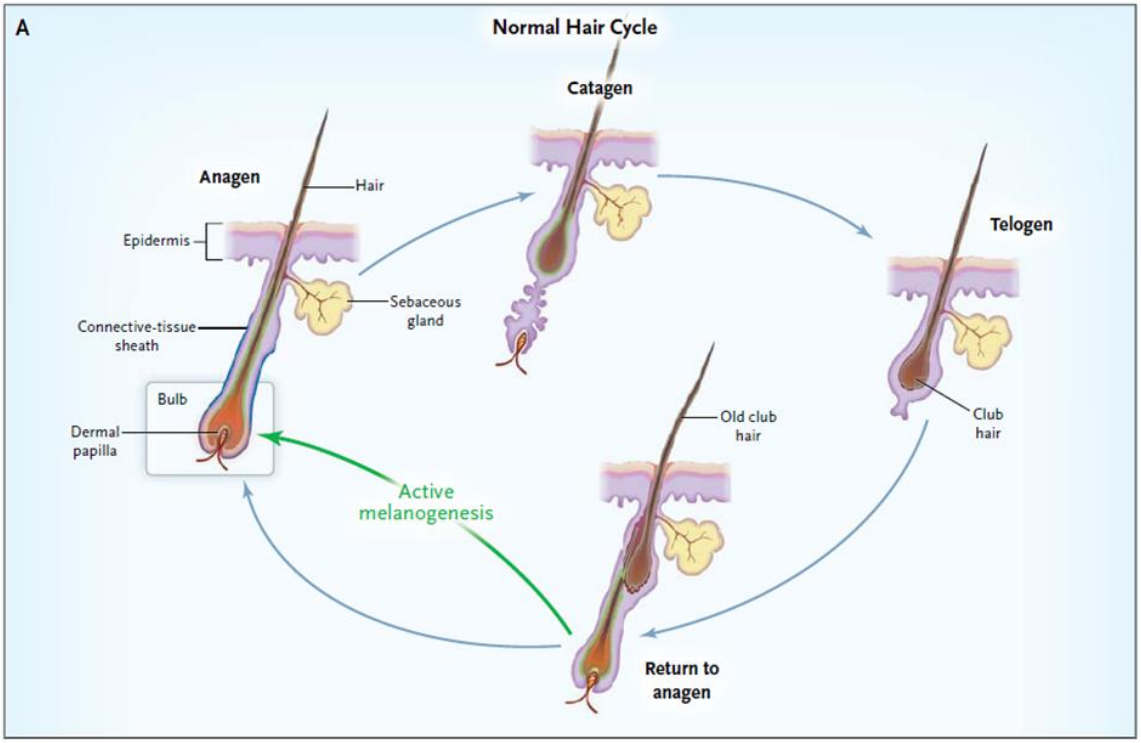

Figure 1.4: The hair follicle cycle.
The HF cycle starts immediately after HF morphogenesis with the catagen phase. In humans, the first catagen occurs already *in utero*. In catagen, the lower part of the HF epithelium regresses by precisely regulated apoptosis (Shimomura and Christiano 2010, Schneider et al. 2009, Stenn and Paus 2001, Lindner et al. 1997) and the hair bulb moves upwards to the bulge region. During catagen the melanin production stops. This causes the depigmentation of the lower part of the HS called at this point, club hair **(Table 1.1)**. The HF enters then into the telogen phase, characterized by resting regarding proliferation and biochemical activity, in which the DP is close to the bulge. At this stage, follicle stem cells in the secondary hair germ and/or in the bulge region can generate the new HF. The anagen phase is composed by 6 stages (anagen I-IV) in which transient amplifying cells in the HM differentiate into at least 8 different cell lines to produce mature HSs and the DP grows deep into the hypodermis. During these steps, the melanogenesis (i.e. pigmentation) is stimulated by induced melanocyte differentiation from precursors. Figure from Gilhar et al. 2012.

Actually, the telogen HF is much less “quiescent” than frequently hypothesized, in fact this cycle phase is characterized by an increase of cholesterol and steroid metabolism and

changes in the skin immune status and circadian clock function (Geyfman et al. 2012). During telogen, the HF possesses a tightly cluster of follicular papilla cells called hair germ, which is implicated in anagen induction. It is in fact recently proposed, that these cells are responsible of the formation of some HF layers, such as the IRS and HS and to activate the bulge epithelial stem cells which in turn produce the ORS. However, contrary to this hypothesis (called "hypothesis of HF predetermination"), in the old theory called "bulge activation hypothesis" hair germ cells activate bulge stem cells to produce all HF layers (Myung and Ito 2012, Schneider et al. 2009, Panteleyev et al. 2001, Stenn and Paus 2001).

The HS final length is determined by the anagen duration and depends of the body site. The anagen phase of scalp HFs is 1-6 years, while the catagen phase lasts only few weeks and the telogen phase for 2-4 months. The human HF clock is synchronized until shortly after birth, later on the cycling activity of human HFs become independent from each other (Al-Nuaimi et al. 2013, Al-Nuaimi et al. 2012, Paus and Foitzik 2004). In human adult individual, about the 85-90% of scalp HFs are in anagen. The cycle is regulated by an array of genes, molecular mediators, hormones and neuropeptides (Al-Nuaimi et al. 2013, Al-Nuaimi et al. 2012, Paus 2007, Stenn and Paus 2001).

The proliferation, differentiation, and apoptosis of neuroectodermal and immune cell populations in or around the HFs together with the HF mesenchyme, perifollicular vascular system and follicular innervations are also strongly associated to the HF cycle (Al-Nuaimi et al. 2012, Paus and Foitzik 2004, Stenn and Paus 2001, Paus et al. 1998).

Dysfunctions of the HF cycle are associated with several important HF diseases, including AA **(Table 1.2)** (D`Ovidio 2014, Ito 2013, Gilhar et al. 2012, Schneider et al. 2009).

Frequent diseases of hair growth.	
Alopecia	Abnormal hair loss; androgenetic alopecia is baldness caused by miniaturization of genetically predisposed follicles; alopecia areata (patchy hair loss) is thought to be caused by an autoimunne reaction to anagen follicles; scarring or cicatricial alopecia, caused by destruction of hair follicles after inflammation and other causes.
Anagen effluvium	Abrupt hair shedding caused by interruption of hair growth, for instance in patients undergoing chemotherapy.
Hirsutism	Excessive hair growth in androgen-dependent areas in women.
Hypertrichosis	Excessive hair growth beyond the normal pattern.
Miniaturization	Conversion of large terminal hairs into small vellus hairs.
Telogen effluvium	Poorly defined thinning of scalp hair, mostly associated with physical or psychological stress.

Table 1.2: Hair follicle cycle disorders.
Table from Shneider et al. 2009.

1.2.3 Hair follicle immune system

The human HF possesses an immune system which is tightly connected with the one of the skin (Health and Carbone 2013, Di Meglio et al. 2011), and is similar to the immune system of pelage (Paus et al. 1999) and vibrissae (Bertolini et al. 2013) HFs. Its composition strongly changes during the HF cycle and vice versa, perifollicular immuno-cells (macrophages and MCs) can influence the hair growth (Paus et al. 2007, Paus et al. 2005, Christoph et al. 2000, Paus et al. 1999).

The HF perifollicular dermis (PFD) and CTS harbour many immunocytes, such as Langerhans cells **(Figure 1.5A)**, macrophages **(Figure 1.5B)**, MCs **(Figure 1.5C)**, T-cells **(Figure 1.5D,E)**, B-cells and natural killer (NK) cells (Christoph et al. 2000).

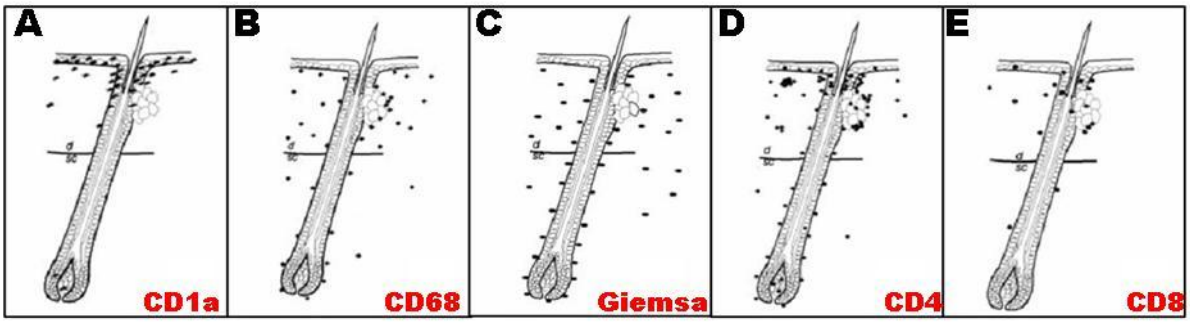

Figure 1.5: Perifollicular immunocytes of the hair follicle immune system.
Langerhans cells, detected by CD1a, are rare around the HFs but their number is high in the basal layer of the epidermis and in the distal ORS. The expression of CD1a decreases towards the HF bulb, which shows only rare Langerhans cells (A). High number of macrophages, detected by CD68, is found in the HF PFD, CTS and infundibulum. However, macrophages are almost absent in the proximal HF epithelium and HB (B). The PFD and CTS are colonized by abundant MCs, here revealed by Giemsa, which are absent in the DP and in the epithelium (C). Few CD4+ and CD8+ T-cells inhabit the PFD and CTS, mostly in the upper dermis. Within the epithelium, the maximal number of T-cells is found in the distal ORS. However, CD8+ T-cells disappear in the proximal HF epithelium and in the DP, while CD4+ T-cells only decline (D,E). Figure modified after Christoph et al. 2000.

The HF epithelium is also populated by immunocytes, however their expression is mostly restricted to the infundibulum **(Figure 1.5A,B,D,E**), and the few Langerhans cells that can be detected ultrastructurally or by CD1a immunohistology in human anagen hair bulbs are functionally impaired (Ito et al. 2008b, Meyer et al. 2008, Paus et al. 2005, Christoph et al. 2000). In fact, the HF bulge and anagen VI bulb are characterized by a special immune protection environment, which is further explained below.

1.2.4 Hair follicle immune privilege

The first functional evidence that the HF harbours IP sites derived from the experiment of Billigham, in which ear skin epidermis from black guinea pig was allotransplanted onto the skin of genetically incompatible white guinea pigs. The transplanted skin quickly lost its pigmentation, however after 100 days black hairs appeared within the skin area onto which previously black epidermis had been allotransplanted. This important study suggests that

epidermal donor melanocytes escaped from rejection moving into the HB of recipient HFs **(Figure 1.6)** (Billigham and Silvers 1971a,b).

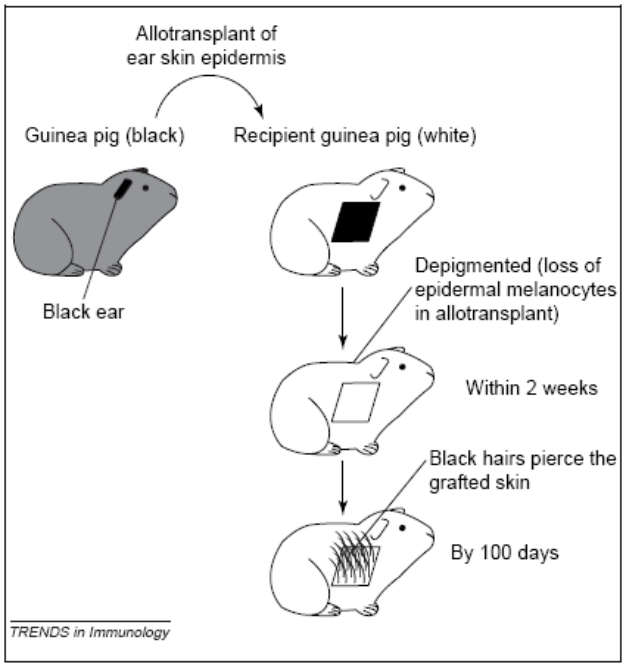

Figure 1.6: Billigham's experiment suggesting the existence of a relative hair follicle immune privilege.
The recipient HB provides a special milieu that permits transplanted allogeneic cells (namely donor melanocytes) to escape the attack by host immune system. Figure from Paus et al. 2005.

Apart of this old landmark study, recent studies further suggest that the HF bulge (Meyer et al. 2008, Rosemblum et al. 2006) and hair bulb (Paus et al. 1998, Paus at al. 1994a) exhibit relative IP (Wang et al. 2014, Breitkopf et al. 2013, Alli et al. 2012), confirming previous results based on (immuno-)histomorphometry analyses (McElwee et al. 2013, Gilhar et al. 2012, Bertolini et al. 2012, Kinori et al. 2011, Ito et al. 2008b, Meyer et al. 2008, Paus et al. 2005).

In fact, as other mammalian tissue (central nervous system, anterior eye chamber, feto-trophoblast, testis) these two HF regions meet most of the criteria (Murphy 2012, Playfair and Chain 2009, Kindt et al. 2007) defined for the establishment and maintenance of IP **(Table 1.3**). These criteria mainly encompass the following items (Stein-Streilein and Caspi 2014, Bertolini et al. 2013, McElwee et al. 2013, Muldoon et al. 2013, Niederkorn 2013, Stein-Streiler 2013, Gilhar et al. 2012, Kinori et al. 2011, Ito et al. 2008b, Meyer et al. 2008, Paus et al. 2005, Ito et al. 2004):

- Downregulation or absence of classical MHC class I expression, resulting in the incapacity (or greatly reduced capacity) to present (auto)antigens to cognate CD8+ T-cells.
- Downregulation or absence of expression of β2-microglobulin (since this molecule is needed to stabilize MHC class I molecules and to allow the latter to properly function as antigen presenting molecules (Li et al. 2013, Zoete et al. 2013, Murphy 2012,

Playfair and Chain 2009, Kindt et al. 2007), the few MHC class I that may still be expressed cannot properly present (auto-)antigen.

- Local production of potent immunosuppressants such as transforming growth factor β1 (TGFβ1), TGFβ2, interleukin 10 (IL-10), α-melanocyte-stimulating hormone (α-MSH).
- Functional impairment of antigen-presenting functions of dendritic cells, for example by absence of MHC class II expression.
- Suppression of natural killer cell function by secretion of inhibitory molecules (e.g. macrophage migration inhibitory factor (MIF)) and by down-regulation of endogenous agonists (e.g. MHC class I polypeptide–related sequence A (MICA), ULBP3) of activating NK cell receptors (NKG2D); as NK cells are primed to recognize and eliminate MHC class I-negative cells.
- Absence of lymphatic vessels.
- Reduction of effective immune cell trafficking by erection of extracellular matrix barriers.
- Expression of nonclassical MHC class I molecules (such as the MHC class Ib molecules human leukocyte antigen (HLA)-G, HLA-E in humans), which are involved in the suppression of NK cell- and cytotoxic T cell-dependent lysis.
- Expression of Fas Ligand (FasL) in order to delete autoreactive, Fas-expressing T-cells.

Anagen hair bulb	Anterior eye chamber	Central nervous system	Feto-trophoblast
Absence or low expression of ß2-microglobulin and MHC class I molecules	Absence of conventional MHC class I molecules	Absence of conventional MHC class I molecules	Absence of conventional MHC class I molecules
Reduced number of Langerhans cells which are functionally impaired (no MHC class II expression !)	Absence of MHC class II expression	Absence of MHC class II expression	Absence of MHC class II expression
Absence of conventional lymphatic drainage	Lack of conventional lymphatic drainage	Absence of conventional lymphatic drainage	
Expression of nonclassical, MHC class Ib	Expression of nonclassical, MHC class Ib molecules		Expression of nonclassical MHC class Ib molecules
Expression of TGF-ß, α-MSH, IGF-1, CGRP, MIF, IDO	Expression of TRAIL, IDO, VIP, TGF-ß	Immunosuppressive factors:TGF-ß, α-MSH, VIP, CGRP, FasL and MIF	Soluble immunosuppressive factors :TGF- , α-MSH, VIP, CGRP, Fasl, MIF, Galectin-1, and IDO
Restricted immune cells trafficking through hair follicle connective tissue and basement membrane	Efficient blood-retina barrier	Blood brain barrier	
(Presence of Treg cells and suppressor CD8 T-cells?)			Presence of Treg cells and suppressor CD8 T-cells
		Production of gangliosides and MIF ligand for a receptor restricted to myeloid cells (CD200-Cd200L), with the potential to deliver inhibitory signals	Ligand for a receptor restricted to myeloid cells (CD200-CD200L), with the potential to deliver inhibitory signals
Local synthesis of cortisol			Local synthesis of cortisol?

Table 1.3: Key mechanisms underlying the relative immune privilege of selected human tissues.
Calcitonin gene-related peptide (CGRP), indoleamine 2,3-dioxygenase (IDO), insulin like-growth factor (IGF), major histocompatibility (MHC), melanocyte stimulating hormone (α-MSH), macrophage migration inhibitory factor (MIF), TNF-releated apoptosis inducing ligand (TRAIL), transforming growth factor (TGF), vasoactive intestinal peptide (VIP). Modified after Gilhar et al. 2012.

Specifically, human HF bulge and anagen VI bulb are deficient of both MHC class I and II and β2-microglobulin **(Figure 1.7)**. Moreover, the few Langerhans cells which reside in the HB are MHC class II negative (Breitkopf et al. 2013, Meyer et al. 2008, Christoph et al. 2000, Paus et al. 1993).

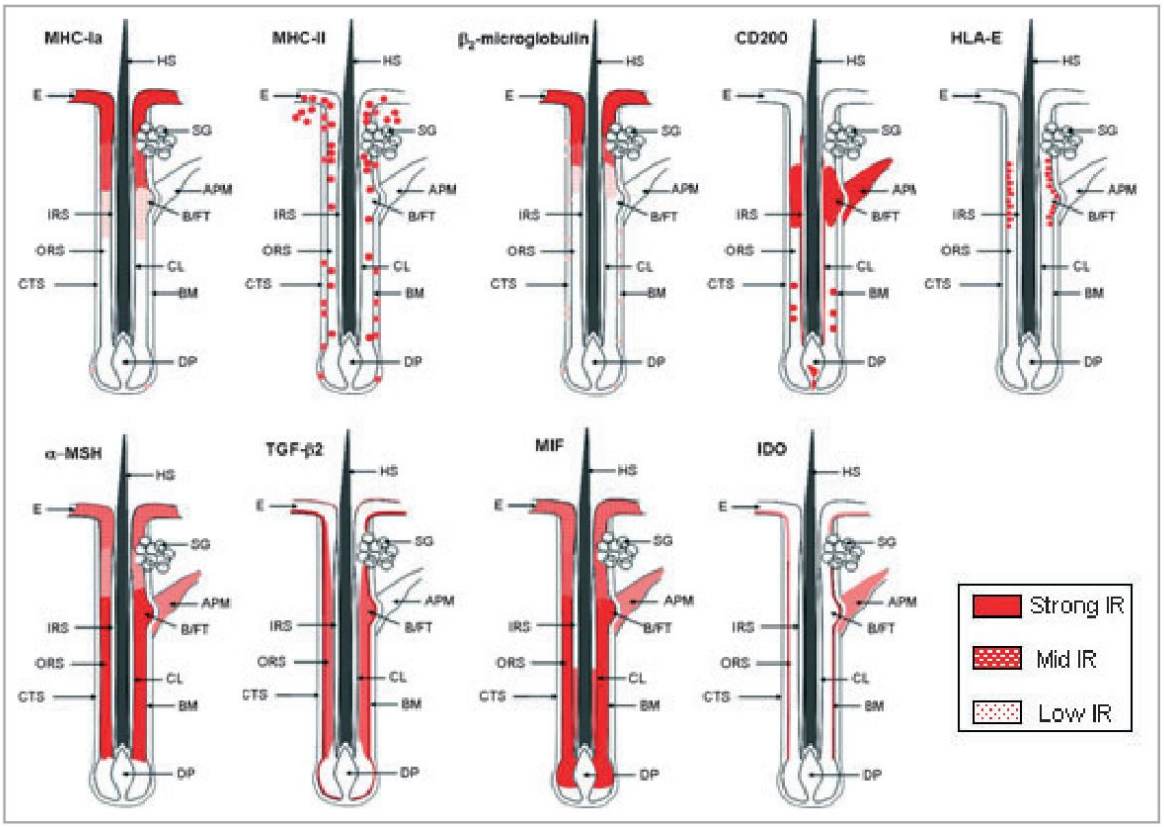

Figure 1.7: The expression pattern of hair follicle-immune privilege-associated molecules.
MHC class I and β2-microglobulin molecules and MHC class II+ cells are down-regulated in the HF bulge and almost absent in the HF bulb. The few Langerhans cells which populate the HB are MHC class II negative. The "no danger signal" CD200 is strongly up-regulated in the HF bulge and HLA (human leucocyte antigen)-E positive cells can be found in the HF bulge ORS. Immunosuppressors such as α-MSH (melanocyte stimulating hormone), TGFβ1/2 (transforming growth factor) and MIF (macrophage migration inhibitory factor) are expressed in the HF bulge and anagen VI bulb. The IDO (indoleamine 2,3-dioxygenase) enzyme activity is presented in the HF bulge. Arrector pili muscle (APM), bulge/follicular trochanter (B/FT), basement membrane (BM), companion layer (CL), connective tissue sheath (CTS), derma papilla (DP), epidermis (E), hair shaft (HS), inner root sheath (IRS), outer root sheath (ORS), sebaceous gland (SG). Figure from Meyer et al. 2008.

During the growth stage, the HF epithelium strongly expresses adrenocorticotropic hormone and α-MSH **(Figure 1.7)**, which derives from the cleavage of the pro-opiomelanocortin polypeptide (Ito 2010, Meyer et al. 2008, Slominski et al. 1992), and stimulates intrafollicularly cortisol generation and immunosuppressive activity (Ito et al. 2008b). In addition, the "IP guardians" TGFβ-1 (Foitzik et al. 2000, Bertolini et al. 2014) and TGFβ-2 **(Figure 1.7)** (Meyer et al. 2008) are expressed in the HF ORS which prevent the attack of natural killer and CD8+ T-cells in tissue characterized by down-regulation MHC class I (Lee et al. 2014, Wahl et al. 2006).

Moreover, under physiological conditions, the HF epithelium expresses low level of NK cell–stimulating ligands, such as MICA or ULBP3/6 (Petukhova et al. 2010, Ito et al. 2008a).

Recently, it was shown that the epithelium and mesenchyme of human anagen HFs express also programmed cell death (PD)-ligand (L)1. Moreover, the expression of PD-L1 in cultured dermal sheath cup cells mediates the hypo-responsiveness from allogeneic T cells and inhibited T-cell activation and proliferation by inducing apoptosis of activated T cells (Wang et al. 2014).

In addition, the number of CD4+ and mostly CD8+ T-cells is sharply reduced in the bulge and in the HB epithelium of anagen VI HFs **(Figure 1.5D,E)** (Ito et al. 2008b, Christoph et al. 2000).

In the human bulge, additional mechanism are present since this stem cell niche also upregulates indoleamine 2,3-dioxygenase (IDO) enzyme which catabolises tryptophan, limiting T-cell proliferation **(Figure 1.7)** (Meyer et al. 2008) and CD200 **(Figure 1.7)** (Meyer et al. 2008), the ‘no danger signal’ that inhibits immunocytes and downregulates antigen presenting cell activity (Meyer et al. 2008, Rosemblum et al. 2006).

The function of the IP in mammalian tissues is to protect important human organs such as brain and eyes from autoaggressive inflammation (Ichiryu and Fairchild 2013). It has been hypothesized that same is valid for the HFs (Paus et al. 1993) which might need to create a special immunoinhibitory environment to preserve the HF cycle and the cyclic production of melanogenesis-related autoantigens, which may be highly immunogenic and often are a target of autoimmune response in human skin (e.g. vitiligo) (Tobin 2014, McElwee et al. 2013, Paus and Bertolini 2013, Sandoval-Cruz et al. 2011, DiPreta et al. 2000). Interestingly, the HF represents one of the most frequent targets of immune-mediated diseases in man (Gilhar et al. 2012, Paus et al. 2005): collapse of the HF bulge IP and the subsequent destruction of epithelial HF stem cells are involved in scarring alopecia (e.g. lichen planopilaris, lupus erythematosus) (Harries et al. 2013, Harries and Paus 2010), while collapse of the hair bulb IP is considered to play a key factor in the AA pathogenesis (McElwee et al. 2013, Paus and Bertolini 2013, Gilhar et al. 2012, Paus et al. 1993). This underscores not only the necessity of establishing a relative HF-IP in order to preserve a functional HS factory, i.e. the HB, but also HF epithelial stem cells in the bulge, the guarantors of HF cycling as such (Harries et al. 2013, Paus and Bertolini 2013, Harries and Paus 2010, Meyer et al. 2008, Cotsarelis 2006).

1.3 Alopecia areata

AA is one of the most common human organ-specific autoimmune disorders, involving about the 1,7% of the population, characterized by a collapse of the HF-IP, a CD8+ T-cell-driven response to MHC class I-presenting autoantigen(s) and a premature anagen termination (D`Ovidio 2014, Alkhalifah 2013, McElwee et al. 2013, Bertolini et al. 2012, Gilhar et al. 2012).

1.3.1 Clinical features

AA is rather easy to identify since it appears as circumscribed round patches of hair loss without macroscopically visible skin inflammation, most commonly in scalp skin and the beard area **(Figure 1.8A)**. AA can develop and involve the whole scalp, alopecia totalis (AT) **(Figure 1.8B),** or the entire body, alopecia universalis (AU), including the eyebrows, eyelashes, or pubic hair **(Figure 1.8C)** (D`Ovidio 2014, Alkhalifah 2013, Price 2013, Gilhar et al. 2012, Alkhalifah et al. 2010a, Harries et al. 2010). AA can manifest also with other phenotype such as band-like distribution-ophiasis pattern in which hair loss can be limited to the periphery of the scalp **(Figure 1.8D)**, or conversely, it can spare the periphery of the scalp-sisapho (ophiasis inversus). Spontaneous remission is noted in patchy AA, but rarely in AU or AT (D`Ovidio 2014).

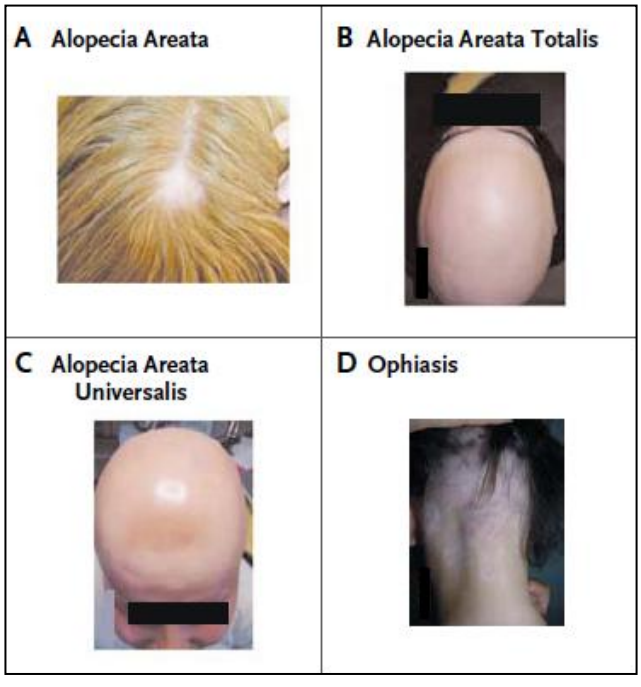

Figure 1.8: Clinical manifestations of alopecia areata.
AA develops as round patches (A) and progresses involving the whole scalp, alopecia totalis (B) or the whole body, alopecia universalis (C). Ophiasis is a further clinical manifestation. Figure from Gilhar et al. 2012.

The diagnosis is normally made by physical observation and the use of a videodermoscopy which reveals yellow dots (hair canal may be devoid of hairs or contain miniaturized, cadaverized, or dystrophic hairs) and ‘exclamation point’ hairs at the patches edges, which

are small and broken HSs **(Table 1.4)** (D`Ovidio 2014, Haliasos et al. 2013, Gilhar et al. 2012, Miteva and Tosti 2012, Alkhalifah et al. 2010a).

Diagnostic Tool	Diagnostic Findings
Family history	Atopy, thyroid disease, or other autoimmune disorders may be associated with alopecia areata; a family history of any of these disorders may therefore be diagnostic
Physical examination	
Hair and skin	Most characteristic diagnostic finding is the presence of circumscribed, hairless patches or large alopecic areas in otherwise normal-appearing skin areas; pigmented hair is preferentially attacked and lost in active disease, whereas regrowth is frequently characterized by tufts of white hair; sudden pseudowhitening of hair is observed in a rare, rapidly progressing, diffuse variant form of alopecia areata
Nails	Nail changes, if present, are usually characterized by pitting; onychodystrophy is less common
Eyes	Ocular abnormalities include lens opacities and abnormalities of retinal pigment epithelium[43]
Dermoscopy	Yellow dots (i.e., keratotic plugs in follicular ostia) are often seen in alopecia areata[32] but are not specific for the diagnosis
Cadaver hairs	Comedo-like cadaver hairs (black dots) may also be present
Exclamation-mark hair	Distal segment of the hair shaft is broader than its proximal end, resembling an exclamation mark
Follicular ostia	Openings in the hair follicles through which the hair fiber emerges from the skin; these ostia are well preserved in alopecia areata, in contrast to the findings in scarring alopecia
Pull test*	A positive pull test at the margins of alopecic lesions that produces telogen ("club") or dystrophic anagen hairs supports a clinical working diagnosis
Laboratory tests	None of the available tests will confirm the diagnosis, but thyroid-function tests and tests for thyroid antibodies may be advisable because of the increased association between alopecia areata and thyroid autoimmunity[29]; abnormal results of thyroid-function tests, the presence of thyroid autoantibodies, or both further support a clinical or histologic working diagnosis of alopecia areata
Histologic examination†	Biopsy specimens should be obtained only if the clinical diagnosis is in doubt; on histologic examination, a dense, peribulbar lymphocytic infiltrate is seen in acute alopecia areata

Table 1.4: Alopecia areata: Diagnostic criteria. Table from Gilhar et al. 2012.

AA affects not only the HF, but also other ectodermal skin appendages, for example the nail apparatus (D`Ovidio 2014, Alkhalifah 2013, Gilhar et al. 2012, Alkhalifah et al. 2010a), which also harbours a site of relative IP (Ito et al. 2005c), and (rarely) the retina pigment epithelium (Ayuso et al. 2011, Pandhi et al. 2009).

In many cases, AA is associated with other autoimmune (e.g. thyroid disease, vitiligo, diabetes mellitus) and chronic inflammatory diseases (e.g. atopy, psoriasis) (D`Ovidio 2014, Alkhalifah 2013, Ishak and Piliang 2013, Bertolini et al. 2012, Gilhar et al. 2012, Alkhalifah et al. 2010a).

1.3.2 Histological features

AA development is related to the alteration of the HF cycle due to inflammatory events, particularly those with an autoimmune background. In general, if the infiltration around the HF lasts for a long time, HFs undergo anagen arrest, catagen induction and HF involution, followed by conversion of terminal HF into tiny, depigmented vellus HFs (D`Ovidio 2014, Alkhalifah 2013, Gilhar et al. 2012, Cetin et al. 2009, Alexis et al. 2004, Whiting 2003, Kossard 2001, Messenger et al. 1986) **(Figure 1.9** and **1.10)**. This can be appreciated also by histopathology of AA lesional skin **(Figure 1.9)**. Therefore, each step of this process in AA development is characterized by a different histopathological picture.

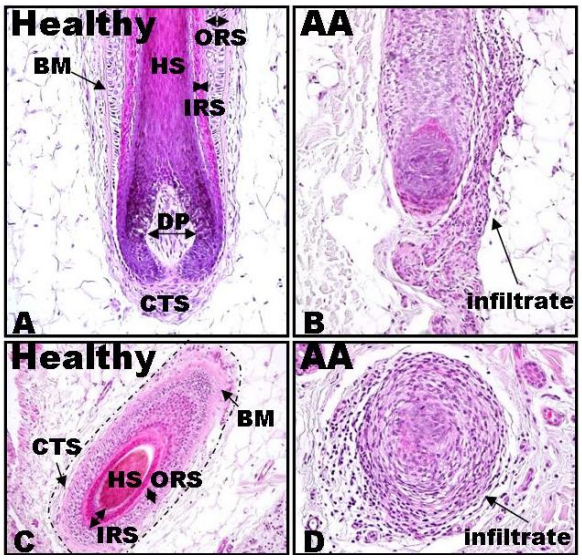

Figure 1.9: Histopathology of acute alopecia areata. Histochemical identification of a healthy scalp HF bulb (A) and the corresponding in AA skin, the HF bulb is surrounded by a massive infiltrate (B). Trasversal view of a healthy HF bulb (A) and an AA affected HF bulb. Basement membrane (BM), connective tissue sheath (CTS), dermal papilla (DP), hair shaft (HS), inner root sheath (IRS), outer root sheath (ORS). These figures, some of which taken during the current thesis project, were provided by M. Bertolini for incorporation into Gilhar et al. 2012.

In the acute stage, the HF is surrounded by an inflammatory cell infiltrate "swarm of bees" containing preferentially lymphocytes **(Figure 1.9)** (Gilhar et al. 2012, Alkhalifah et al. 2010a, King et al. 2008, Whiting 2003). Most of these T-cells are CD4+ T-cells which localize preferentially around the hair bulb (peribulbar infiltrate) while CD8+ T-cells seem to be the first to infiltrate the follicular epithelium (intrafollicular infiltrate) (Gilhar et al. 2012, Cetin et al. 2009, Alexis et al. 2004; Whiting 2003, Bodemer et al. 2000, Perret et al. 1984).

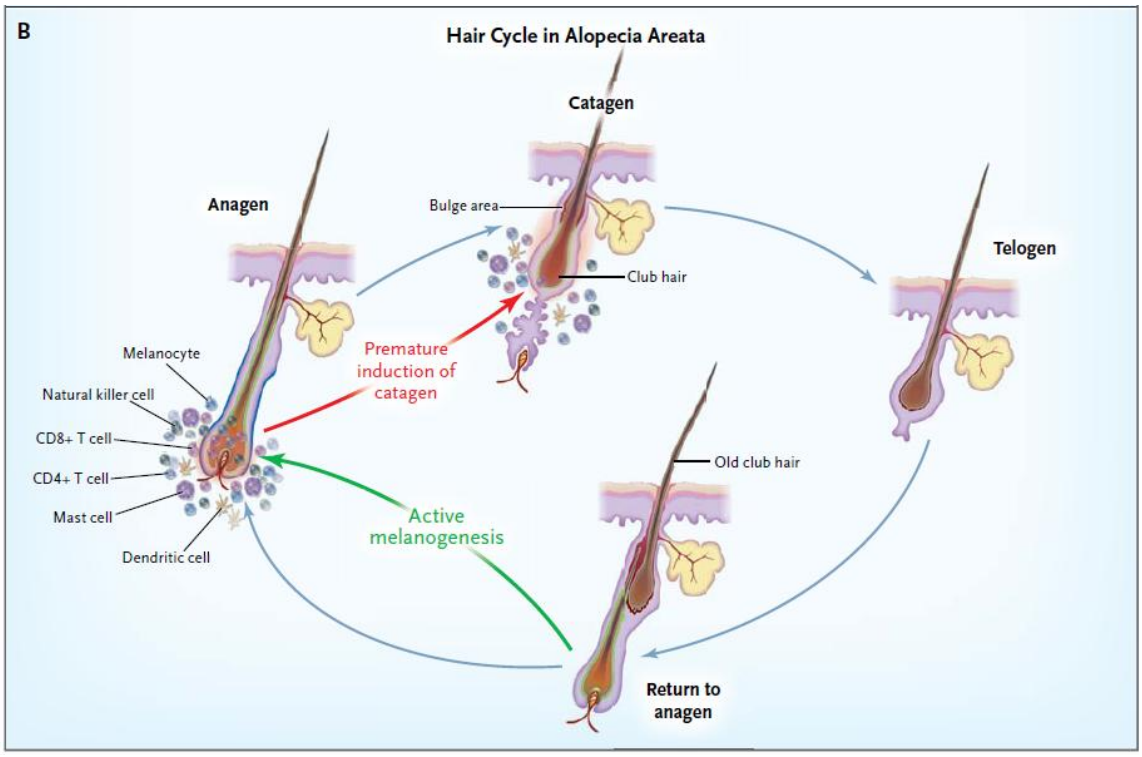

Figure 1.10: Abnormal hair follicle cycle in alopecia areata.
The first episode in AA is an inflammatory infiltrate around anagen HF bulb which causes the sudden arrest of the anagen phase and a premature induction of catagen. The HFs continue their cycle through the telogen phase and return in anagen. This is possible only if the inflammatory environment does not persist, in contrary case an abnormal cycle will start promoting the conversion to miniaturized HFs. Figure from Gilhar et al. 2012.

Although the inflammatory infiltrate attacks mostly the peri- and intrabulbar HF area (Gilhar et al. 2012, Alkhalifah et al. 2010a, Whiting 2003) the damage can sometimes also reach the infundibulum (Karashima et al. 2013, Zhang et al. 2013b). The inflammatory environment promotes anagen arrest and catapults the HF into catagen **(Figure 1.10)**. In fact, in the subacute stage, the number of catagen HFs increases and few telogen HFs can be seen (Alkhalifah et al. 2010a, Whiting 2003). In most of the cases, in the absence of inflammation, telogen hairs may revert to terminal hair which contributes to the spontaneous remission of the disease **(Figure 1.10)**. In contrast, if the inflammatory condition persists when the HFs re-enter in the growth cycle, they are suddenly pushed again in catagen. This phenomenon starts the conversion to miniaturized hairs (vellus) which causes the entry into the chronic phase (Alkhalifah et al. 2010a, Whiting 2003). During this process, the HB and follicular epithelium suffer of important changes which promote the production of dystrophic anagen HFs (Alkhalifah et al. 2010a, Horenstein and Simon 2007). During recovery, vellus HFs can also switch to terminal hairs (Whiting 2003). The conversions telogen-to-anagen or vellus-to-anagen are possible because the inflammatory cell attack spares the stem cells in hair bulge (Gilhar et al. 2012, Kossard 2001).

Apart from T-cells, the inflammatory infiltrate is composed by NK cells and some plasma cells around the HF (in healthy HFs normally absent) and high number of Langerhans cells and MCs both in peribulbar and perivascular areas (Bertolini et al. 2014, Zhang et al. 2013b, Petukhova et al. 2010, Cetin et al. 2009, Ito et al. 2008a, Bodemer et al. 2000, D'Ovidio et al. 1988, Ranki et al. 1984, Finzi and Landi 1964, Baccaredda-Boy and Giacometti 1959). Eosinophils and macrophages can also appear in the inflammatory mechanisms in about 40% of the cases (Zhang et al. 2013b, Alkhalifah et al. 2010a, Alexis et al. 2004, Whiting 2003).

In AA, the HF epithelium prominently presents immunophenomenological evidence for IP collapse (D`Ovidio et al. 2014, Paus and Bertolini 2013): increase expression of MHC and class I and II antigens (Mcdonagh et al. 1993, Bröcker et al. 1987), intercellular adhesion molecule (ICAM)-1 (Mcdonagh et al. 1993) and NKG2D ligands expression (Breitkopf et al. 2013, Wang et al. 2013a, Alli et al. 2012, Petukhova et al. 2010, Ito et al. 2008a).

The role of immunomodulatory neuropeptides such as CGRP (Kinori et al. 2012, Ito 2010), SP (Cetin et al. 2009, Peters et al. 2007, Siebenhaar et al. 2007, Toyoda et al. 2001) and of the neurotrophin, nerve growth factor (NGF) (Peters et al. 2007), all of which are key players in the induction of neurogenic skin inflammation (Paus et al. 2008, Arck et al. 2006, Arck et al. 2005) is of particular interest in AA pathogenesis, since in some patients the initial

episode or disease aggravation seems to be connected to specific stressful events (Taheri et al. 2012, Paus and Arck 2009). SP and NGF, released after inducing stress, inhibit murine HF keratinocytes proliferation, induces apoptotic processes, cause catagen regression and triggers MC-dependent neurogenic inflammation (Peters et al. 2007).

Moreover, during the onset of AA, C3H/HeJ mice reveal an increase of SP-immunoreactive nerve fibres and an up-regulation of SP-degrading enzyme (NEP) in the late AA stage (Siebenhaar et al. 2007, Toyoda et al. 2001). SP treatment in mice or in organ healthy scalp skin culture promotes HF catagen involution, MC degranulation, CD8+ T-cells recruitment around HFs as well as up-regulation of NGF and its apoptosis- and catagen-promoting receptor (p75NTR) (Peters et al. 2007; Siebenhaar et al. 2007). In fact, the HF epithelium, CD8+ T-cells and macrophages all express the SP receptor, neurokinin 1 receptor (NK1R). However, SP can act also independently of its receptor, e.g. in MCs (Siebenhaar et al. 2007). Moreover, SP treatment induces HF-IP collapse *in vitro* (Peters et al. 2007). These lines of evidence clearly support the role of neurogenic inflammation in AA development (Ito 2010, Paus and Arck 2009).

1.3.3 Immunopathogenesis of alopecia areata

The immunopathogenesis of AA and the relevant HF autoantigen(s) remain to be clarified. However, the best mechanism proposed in AA field concern the “IP collapse hypothesis” of AA pathogenesis **(Figure 1.11)** (D`Ovidio et al. 2014, McElwee et al. 2013, Paus and Bertolini 2013, Bertolini et al. 2012, Gilhar et al. 2012, Paus et al. 2005, Paus et al. 1993).

According to this AA pathogenesis scenario, the existence of autoreactive CD8+ T-cells alone is insufficient for the development of AA. For the disease to occur, the normal HF-IP, which is predominantly based on the down-regulation of MHC class I molecules in the epithelium of anagen HFs, needs to collapse (D`Ovidio et al. 2014, McElwee et al. 2013, Paus and Bertolini 2013, Bertolini et al. 2012, Gilhar et al. 2012, Gilhar 2010, Paus et al. 2005, Paus et al. 1993). Skin microtrauma, stress-induced neurogenic inflammation, infection, bacterial superantigens and/or other as yet unknown factors can trigger the release of MHC class I-up-regulating cytokines such as interferon (INF)-γ or immunomodulatory neuropeptides such as SP. This provokes HF-IP collapse by inducing ectopic overexpression of MHC class I in the HB so that previously sequestered, anagen-associated HF autoantigen(s) can now be presented (D`Ovidio et al. 2014, McElwee et al. 2013, Gilhar et al. 2012, Peters et al. 2007, Paus et al. 2005, Ito et al. 2004). In some genetically predisposed individuals with autoreactive CD8+ T-cells, the recognition of these peptides with the help of co-stimulatory signals and CD4+ T-cells then induces a cytotoxic CD8+ T-cell attack on hair matrix keratinocytes. This process is believed to activate secondary immune response

(CD4+ T-cells, Langerhans cells, and macrophages) which amplifies the damage in the HF bulb, resulting in the clinical variants of AA (D`Ovidio et al. 2014, McElwee et al. 2013, Gilhar et al. 2012, Ito 2010, Paus et al. 1993) **(Figure 1.11)**.

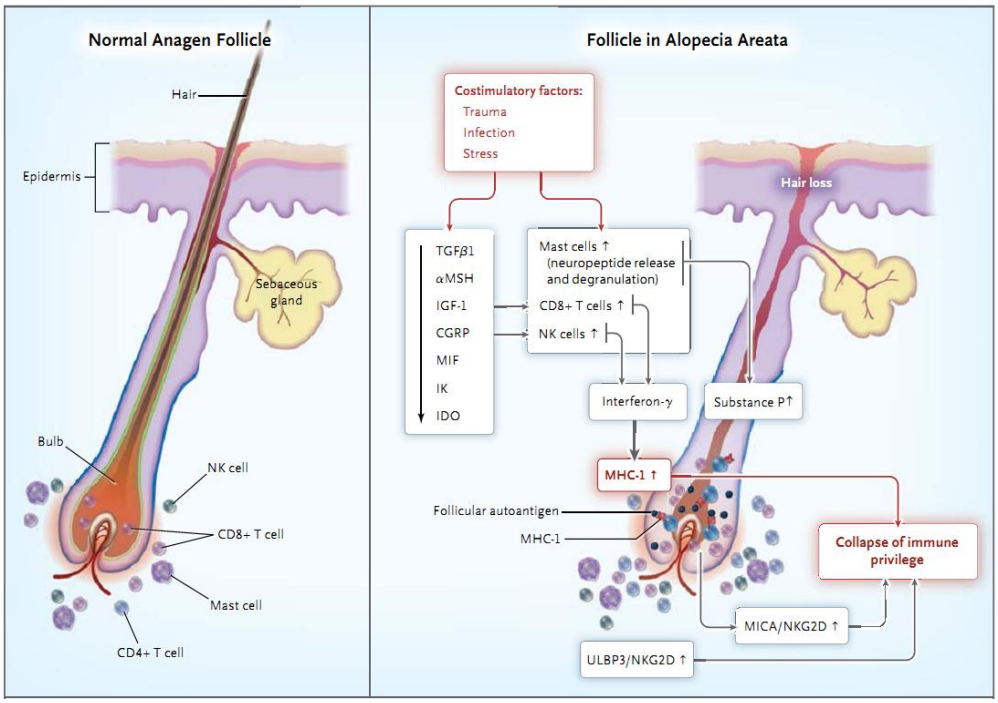

Figure 1.11: Proposed immunopathogenesis for alopecia areata.
In normal anagen HFs few MCs, CD4+ T-cells and rare CD8+ T-cells and NK cells surround the IP protected hair bulb. In AA, due to unknown reason (maybe micro-trauma, infection, stress..) in some predisposed individuals the HF-IP collapse exposing melanogenesis-related antigens to autoreactive CD8+ T-cells. This triggers the inflammatory process which promotes catagen induction. Calcitonin gene–related peptide (CGRP), indoleamine 2,3-dioxygenase (IDO), insulin-like growth factor 1 (IGF-1), down-regulator of HLA II (IK), major histocompatibility complex (MHC), MHC class I polypeptide–related sequence A (MICA), migration inhibitory factor (MIF), α melanocyte–stimulating hormone (αMSH), natural killer (NK), activating receptor for NK cells and subgroups of T-lymphocytes (NKG2D), and transforming growth factor β1 (TGFβ1). Figure from Gilhar et al. 2012.

Since keratinocytes are able to present antigen to CD8+ T-cell (Kim et al. 2009), it is plausible that HF keratinocytes can present follicular autoantigens via ectopic MHC class I expression and co-expression with β2-microglobulin.

The specific HF autoantigen(s) for autoreactive CD8+ T-cells in AA patients remain(s) to be identified, but several observations (reported below) suggest that they are anagen-associated HF, most likely melanogenesis-related peptides (Paus et al. 2005, Gilhar et al. 2002, Gilhar et al. 2001, Paus et al. 1993), but definitive evidence for this is still missing. In fact, clinically, the inflammatory attack in AA occurs only during anagen, when the melanogenesis is active and seems to spare white hairs preferentially (D`Ovidio 2014, McElwee et al. 2013, Gilhar et al. 2012, Paus et al. 1993). Experimentally, injection of melanogenesis-associated peptides or scalp T-cells activated by incubation with a homogenate from pigmented human scalp HFs induces hair loss in human scalp grafts on immunocompromised mice (Gilhar et al. 2002, Gilhar et al. 2001).

1.3.4 Genetic component

As most other autoimmune diseases, AA also has a strong genetic component **(Table 1.5)** (D`Ovidio 2014, Jabbari et al. 2013, McElwee et al. 2013, Petukhova et al. 2011, Petukhova et al. 2010).

Table 2. Clues to the genetic aetiology of alopecia areata

Genetic clue	Details
Family history	In all reports, there is a high frequency of a positive family history in first-degree relatives of affected patients, ranging from 10–42% (30% in our group), more obvious if the disease starts at a younger age. This and the equal frequency in men and women, hint to a dominant genetic disease. One small study found a very high concordance of 55% in monozygotic twins. Such a high concordance rate is not seen in any other autoimmune diseases.
Susceptibility genes	The various HLA associations, for example, an increase in the appearance of DRB1*04 alleles and a significant decrease in the appearance of DRB1*03 alleles in patients with AA, may be one genetic component predisposing to AA. Recently, following the advances in mapping of complex disorders, genome-wide scans of patients with AA provided evidence for several susceptibility loci, some of which lie outside the HLA loci. These include genomic regions containing genes that control regulatory T cells, cytotoxic T-lymphocyte-associated antigen 4, IL-13 and the *ULBP* gene cluster, which encodes activating ligands of the natural killer cell receptor NKG2D. In addition, the R620W variant of PTPN22 was found to be more common in patients with AA, and the presence of the *filaggrin* gene mutations was found to be associated with a more severe form of AA when it occurred in conjunction with atopic dermatitis. Case–control studies have shown that single nucleotide polymorphisms (SNPs) in Foxp3, NOTCH4 and ICOSLG genes were associated with AA. Additionally, SNPs in some immune-related genes (including HLA-DMB, PMS2 and TLR1) have been found to be associated with alopecia universalis using exomic sequencing. Over the years, polymorphisms in other genes have been reported to be associated with AA. They are summarized in Fig. 3.
Animal model based on genetics	The C3H/HeJ mouse develops adult onset disease that resembles AA in adult humans. The disease in mice develops spontaneously, based on four different genetic susceptibility loci. One could postulate that AA in humans could also develop spontaneously, without the need for any external trigger, taking into account the complex gene susceptibility.
Differential expression of genes	The age of onset and the different expression patterns may be attributable to different expression of genes, some of which might be defected, during different time periods. Indeed, HFs are known to express different genes depending on hair cycle phase.

Table 1.5 Evidence for a genetic component in alopecia areata pathogenesis. Table from McElwee et al. 2013.

The most severe cases of AA result in patients with positive family history (Jabbari et al. 2013, Blaumeiser et al. 2006) and monozygotic twins have a risk of 55% to develop both AA (Jabbari et al. 2013, Scerri et al. 1992).

AA presents susceptible genes commonly involved in autoimmune diseases, i.e HLA locus (Jabbari et al. 2013, Petukhova et al. 2011, Petukhova et al. 2010, Martinez-Mir et al. 2007), cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) (Jabbari et al. 2013, Muto et al. 2011), the autoimmune regulator (AIRE) gene (Wengraf et al. 2008) **(Table 1.6)**.

Perhaps, the most important contribution to understand the connection between AA and genetic factors has been provided by recent genome-wide association studies which enlighten not only polymorphisms in genes related to the immune system, e.g. IL-2/IL-21, fundamental cytokines for T-cell proliferation, ULBP3-6 and MICA, ligands for the NKG2D expressed on NK cells and CD8+ T-cells, but also to the HF related genes, e.g. peroxiredoxins 5 (PRDX5), enzymes responsible for quenching reactive oxygen species, and syntaxins 17 (STX17) important for vesicular trafficking and membrane fusion in AA patients **(Table 1.6)** (Jabbari et al. 2013, McElwee et al. 2013, Petukhova et al. 2011, Petukhova et al. 2010, Martinez-Mir et al. 2007).

Region	Genes in region	Function	Strongest association (*p* value)	Maximum odds ratio	Other autoimmune diseases that have demonstrated association to the locus
2q33.2	CTLA4	T cell proliferation	3.55×10^{-13}	1.44	T1D, RA, CeD, MS, SLE, GD
	ICOS	T cell proliferation	4.33×10^{-08}	1.32	T1D, RA, CeD, MS, SLE
4q27	IL21	T-, B- and NK-cell proliferation	4.27×10^{-08}	1.34	T1D, RA, CeD, PS
	IL2	T cell proliferation	3.04×10^{-04}	1.37	T1D, RA, CeD, MS, CD, SLE, PS, GD
6q25.1	ULBP6	NKG2D activating ligand	4.49×10^{-13}	1.61	none
	ULBP3	NKG2D activating ligand	4.43×10^{-17}	1.65	none
9q31.1	STX17	Premature hair graying	3.60×10^{-07}	1.33	none
10p15.1	IL2RA	T cell proliferation	1.74×10^{-12}	1.41	T1D, MS, GD
11q13	PRDX5	Antioxidant enzyme	4.14×10^{-07}	1.33	MS, CD
12q13	CDK2	Cyclin-dependent kinase	1.75×10^{-07}	1.32	T1D
	RAB5B	RAS oncogene	9.29×10^{-09}	1.33	T1D
	SUOX	Sulfite oxidase	8.41×10^{-09}	1.32	T1D
	Eos (IKZF4)	T cell proliferation	3.21×10^{-08}	1.34	T1D, SLE
	ERBB3	Epidermal growth factor receptor	1.27×10^{-07}	1.32	T1D, SLE
6p21.32 (HLA)	MICA	NK cell activation	1.19×10^{-07}	1.44	T1D, RA, CeD, UC, PS, SLE
	NOTCH4	T cell differentiation	1.03×10^{-08}	1.61	T1D, RA, MS
	C6orf10		1.45×10^{-10}	2.36	T1D, RA, PS
	BTNL2	T cell proliferation	2.11×10^{-20}	2.70	T1D, RA, UC, CD, SLE, MS
	HLA-DRA	Antigen presentation	2.93×10^{-21}	2.62	T1D, RA, CeD, MS
	HLA-DQA1	Antigen presentation	3.60×10^{-17}	2.15	T1D, RA, CeD, MS, SLE, PS, CD, UC, (
	HLA-DQA2	Antigen presentation	1.38×10^{-35}	5.43	T1D, RA
	HLA-DQB2	Antigen presentation	1.73×10^{-13}	1.60	RA
	HLA-DOB	Antigen presentation	2.07×10^{-09}	1.72	SLE

Table 1.6: Table showing the genes with susceptible loci for alopecia areata, their significance and association with other autoimmune diseases.
Type 1 diabetes (T1D), rheumatoid arthritis (RA), celiac disease (CeD), multiple sclerosis (MS), systemic lupus erythematosus (SLE), Graves disease (GD), psoriasis (PS), Crohn's disease (CD), ulcerative colitis (UC), generalized vitiligo (GV). Table from Petukhova et al. 2011.

However, while these genetic elements of AA pathobiology may be important to determine an individual's personal risk to develop AA and may greatly impact on the course of the disease and its response to therapy, the fact that an AA-like hair loss phenotype can be experimentally induced even in normal human skin (derived from individuals without a personal or family history of AA) by the injection of selected peripheral blood immunocyte populations from healthy patients (Gilhar et al. 2013a) (details: see below, chapter 1.3.6), questions the notion that genetic elements are the decisive factor that determines whether or not a patient develops AA lesions in a defined skin region (McElwee et al. 2013)

1.3.5 Alopecia areata management

Although AA is not a life threatening disease, it has substantial negative impact on social interactions and on the psychology of the patients (Gilhar et al. 2012, Harries et al. 2010). However, AA often shows spontaneous remission, most frequently in single patchy AA in patients without a family history of AA or atopy (Gilhar et al. 2012). On the contrary, multifocal AA, and especially AT or AU are very difficult to treat, and routinely require a combination of several therapeutic approaches, e.g. along the lines indicated in **Figure 1.12**. The treatment of AA is depending on many factors: age of the patients, severity and duration of the disease **(Figure 1.12)** (Rossi and Calvieri 2014, Alkhalifah 2013, Ito 2012, Messenger et al. 2012, Miteva and Tosti 2012, Gordon and Tosti 2011, Hordinsky 2011, Harries et al. 2010). In any case, the - clinically overall disappointing - forms of AA treatment that are currently available are only symptomatic, of unpredictable efficacy, can neither reliably prevent disease recurrence nor do they appear to alter the course of AA. In addition, some of these AA management strategies are painful and/or stressful for the affected patients and carry the risk of significant undesired effects (Rossi and Calvieri 2014, Harries et al. 2010).

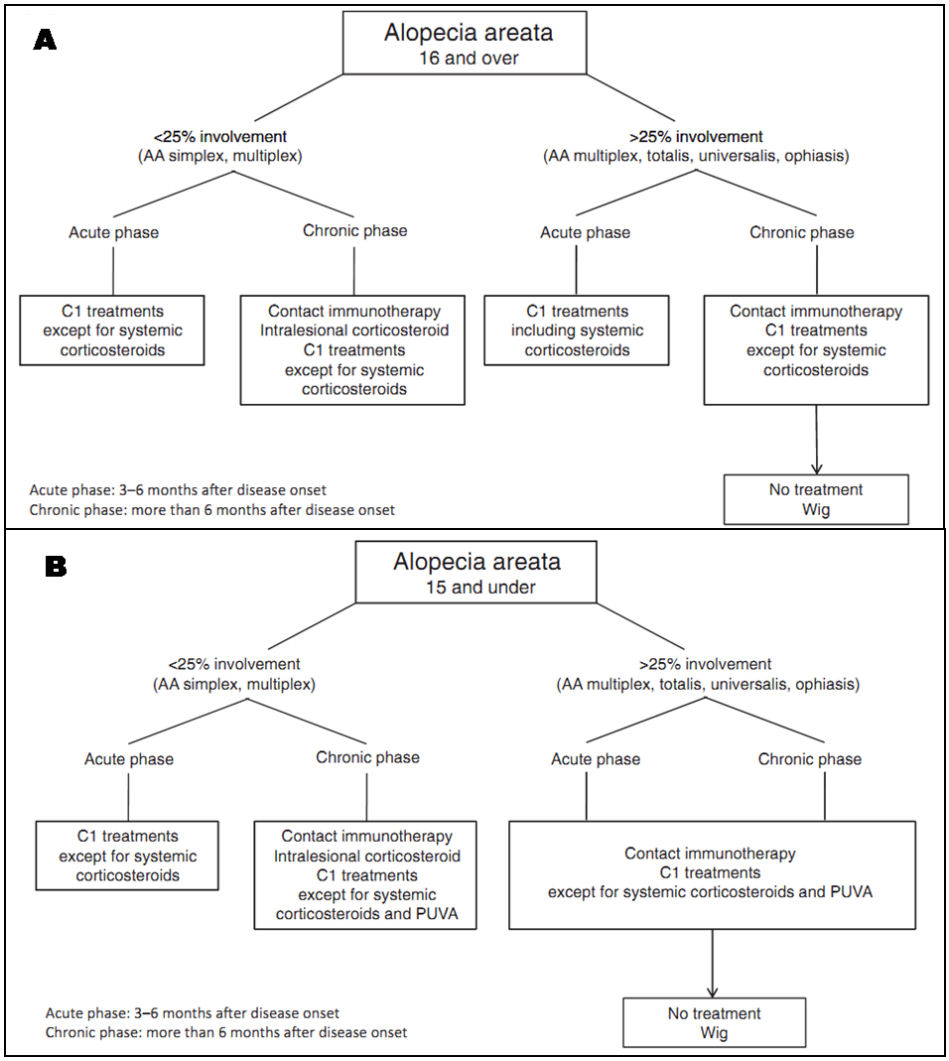

Figure 1.12: Treatment strategies for alopecia areata patients.
Treatment plan for patients of 16 years old or more (A) and of 15 years old or younger (B) according to the Japanese dermatological association. C1 treatments: systemic corticosteroids, psoralen, UV-A therapy (PUVA) and minoxidil. Modified after Ito 2012.

The management of AA is restricted to immunosuppressive drugs (e.g. corticosteroids, cyclosporine), or immuno-deviation strategies as contact immunotherapy (e.g. with DCP) or irritant contact dermatitis therapy (with dithranol), or photochemotherapy (PUVA)). The topically applied hair growth-promoting potassium channel opener, minoxidil, can also help namely if administered in combination with other therapies (Rossi and Calvieri 2014, Alkhalifah 2013, Ito 2012, Messenger et al. 2012, Miteva and Tosti 2012, Gordon and Tosti 2011, Hordinsky 2011, Alkhalifah et al. 2010b, Harries et al. 2010). Moreover, although larger controlled studies are needed to confirm these pilot observations. Recently, antihistamines have also been reported to be of benefit for some AA patients (Inui et al. 2007, Ito et al. 2013b, Ohyama et al. 2010).

1.3.6 Animal models for alopecia areata

AA is not restricted to the human condition but affects also other mammalian species (King et al. 2008). In AA research the most interesting animal models are ageing or C3H/HeJ mice

and the Dundee experimental bald rat (McElwee et al. 2013, Silva and Sundberg 2013, Wang et al. 2013, Ohyama et al. 2010, King et al. 2008, McElwee and Hoffmann 2002, McElwee et al. 1998a,b) as well as a newly established humanized mouse model of AA (Mcelwee et al. 2013, Gilhar et al. 2013a,b).

Approximately 20% of ageing C3H/HeJ mice spontaneously develop AA-like phenotype, routinely after 4-6 or more months **(Figure 1.13)**, with female C3H/HeJ mice being more susceptible than male mice. The lesions start to appear in the ventral side and develop until the back. Affected HFs reveal an inflammatory infiltrate similar to the one of human, however they are characterized of a ratio 2:1 for CD8+ T-cells and CD4+ T-cells and are localized from the hair bulb until the SG (Ohyama et al. 2010, King et al. 2008, McElwee et al. 1998a). Higher percentage of Dundee experimental bald rats also develops spontaneously AA in older age (King et al. 2008, McElwee et al. 1998b).

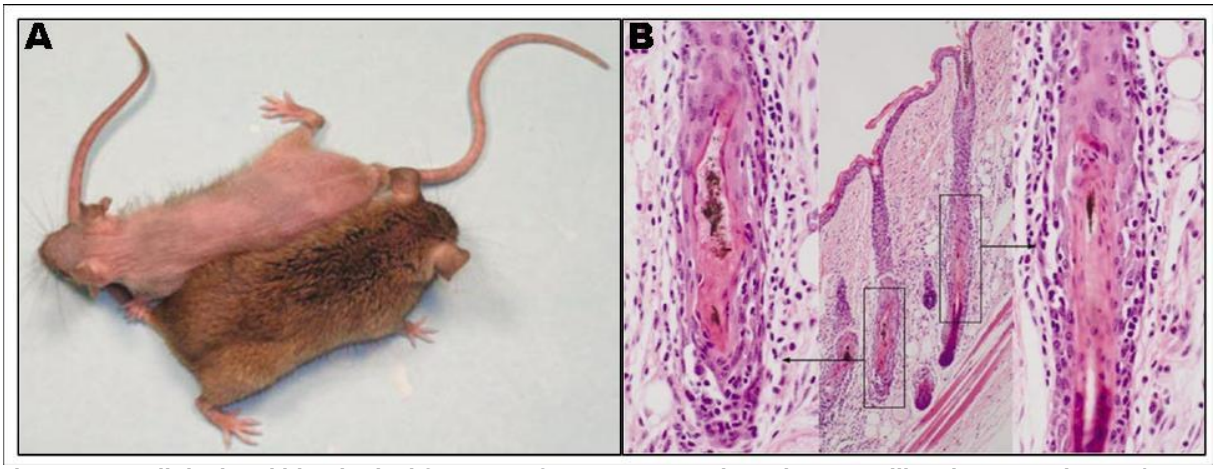

Figure 1.13: Clinical and histological features of spontaneous alopecia areata-like phenotype in C3H/HeJ mice.
C3H/HeJ affected mouse, showing the clinical appearance of spontaneous AA lesions, non-affected C3H/HeJ mouse (A). Histological features of AA lesional HFs in ageing C3H/HeJ mice showing inflammatory infiltrate extended until the dermis. Figure from King et al. 2008.

However, researchers have established a procedure by which lesional skin from older mice affected by AA is transplanted onto young, healthy mice (C3H/HeJ grafted model) **(Figure 1.14)**. This accelerates the disease development and bring the susceptibility up to 100% (Silva and Sundberg 2013, Wang et al. 2013, McElwee 1998a,b).

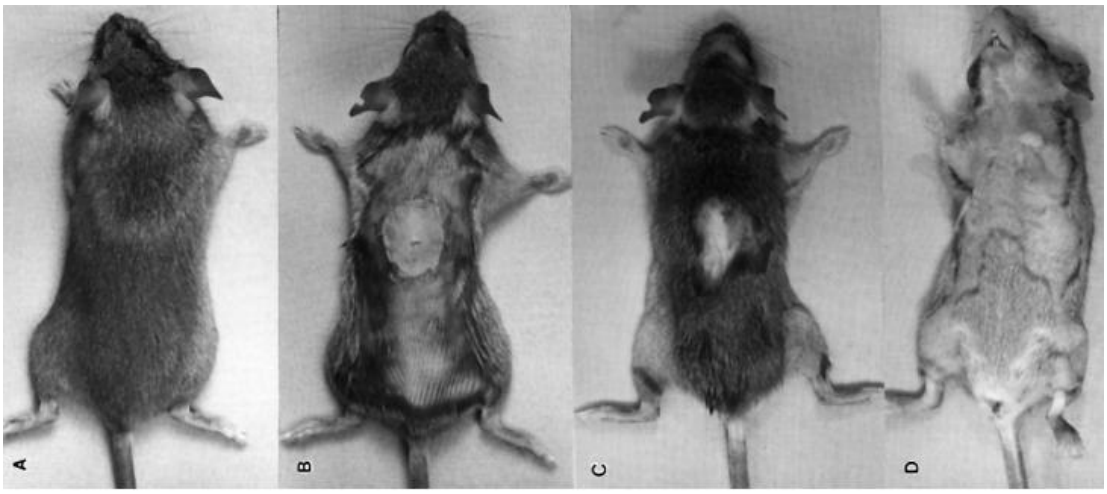

Figure 1.14: C3H/HeJ grafted mouse model of alopecia areata.
The lesion skin extracted from AA affected donor C3H/HeJ mice is transplanted in the back of young (6-8 weeks old) healthy C3H/HeJ mice. After 90 days, hair loss is localized in the skin transplant and in different site of the mouse body. After 100 days, the ventral exposes symmetric hair loss. Figure from McElwee et al. 1998a.

Recently, a new humanized-mouse model was established which permit not only to further dissect important mechanisms of AA pathogenesis but also the pre-clinical screening of drugs **(Figure 1.15)**. Moreover, the employment of this model seems to overcome the disadvantages of the C3H/HeJ model because the AA-like phenotype obtained in this model is identical to the one seen in AA patients and severe combined immunodeficiency (SCID) mice can easily cross-breed with other mouse mutants **(Table 1.7)** (McElwee et al. 2013, Gilhar et al 2013a,b).

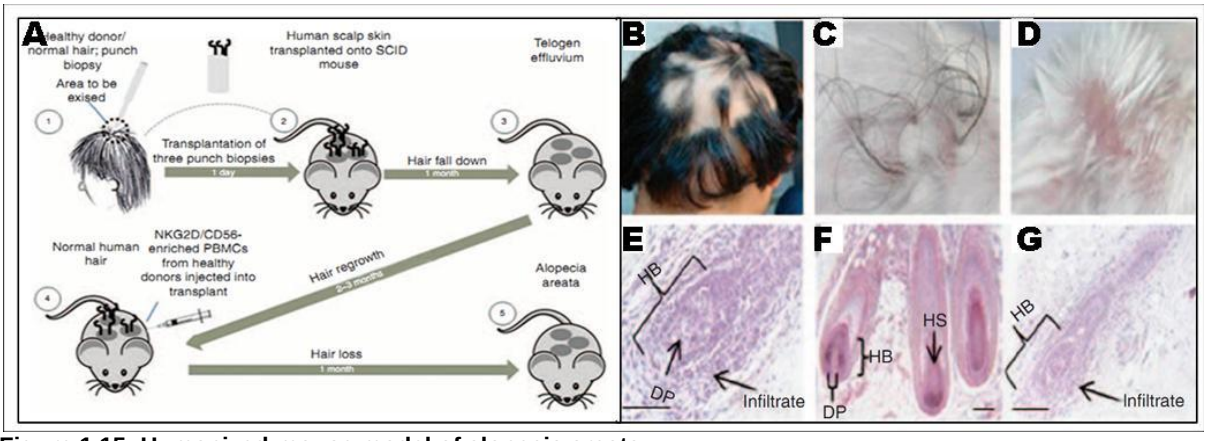

Figure 1.15: Humanized-mouse model of alopecia areata.
Healthy scalp skin from healthy donors is transplanted on the back of beige SCID mice. After 1 month, the skin graft reveals hair loss due to telogen effluvium caused by the transplantation. However, the HFs regrowth in the skin graft after 2-3 months. At this point, autologus or allogeneic peripheral blood mononuclear cells (PBMCs) previously stimulated with high dose of IL-2 and enriched for CD56+/NKG2D+ cells, are injected subcutaneously into the transplanted human skin. This promotes AA-like phenotype, including up-regulation of MHC class I and II, ICAM-1, MICA and inflammation around HFs, and hair loss in the human skin graft (A). Comparison of the clinical features of a AA patients (B), skin graft on SCID mice previously injected with phytohaemagglutinin (PHA) treated PBMCs and enriched for CD56+/NKG2D+ cells (control) (C), skin graft on SCID mice previously injected with IL-2 treated PBMCs and enriched for CD56+/NKG2D+ cells (AA-like) (D). Note that black hairs are present in control human skin grafts (C) but absent in AA-like lesional in transplanted humans scalp skin (D). Histological features of human AA in a biopsy taken from an AA patient (E), control (F) and AA-like phenotype (G) on SCID mice. Activating receptor for NK cells and subgroups of T-lymphocytes (NKG2D), hair bulb (HB), hair shaft (HS), dermal papilla (DP). Figure modified from Gilhar et al. 2013a.

Mouse Model	Characteristic	Advantages	Disadvantages	Refs
Ageing C3H/HeJ	-Patchy hair loss develop spontaneously in up to 20% of female mice by 12 months age - Often initial lesions expand in all body - The immuno cell infiltration is composed of more CD8+ T-cells compared to CD4+ T-cells and it is localized from the bulbar to the SG of anagen HFs - Telogen HFs are spared from inflammation	- Easy to handle	- Female are more susceptible to develop AA compared to male mice - The immuno cells infiltration contains more CD8+ T-cells compared to CD4+ T-cells - HF inflammation is not localized only around the bulb - Difficult to cross-bread with other mouse strains - The contribution to the disease pathogenesis of the mutation in the toll-like receptor 4 gene is still not understood	- Alikhalifah 2010a -King et al. 2008 -McElwee and Hoffmann et al. 2002 - McElwee et al. 1998b
Grafted C3H/HeJ	- The grafting of AA affected skin obtained from older donors into young (7-8 w) C3H/HeJ mice accelerates the disease development - Histological and clinical features identical to ageing C3H/HeJ mice	-The grafting increases the success that C3H/HeJ mice develop AA up to 100% - The develop of AA begin after 10 w after surgery	- See ageing C3H/HeJ	- Alikhalifah 2010a -King et al. 2008 -McElwee and Hoffmann et al. 2002 - McElwee et al. 1998b
Dundee experimental bald rats	- Patchy hair loss develop spontaneously in up to 40% of female rats by 5-8 months age -Focal AA and diffuse AA possible - Peribulbar infiltration around HFs composed by T-cells, macrophages, dendritic cells and other immunocytes	- The ratio (2:1) of CD4+ T-cells and CD8+ T-cells in the inflammatory infiltrate is similar to human	-Female are more susceptible to develop AA compared to male rats - Expression of lesions can be very heterogeneous	- Alikhalifah 2010a -King et al. 2008 -McElwee and Hoffmann et al. 2002 - McElwee et al. 1998b
Humanized-mouse	- AA-lesions experimentally induced in previously healthy human skin transplanted onto SCID mice and injected with autologus or allogeneic IL-2 treated PBMCs and enriched for CD56+/NKG2D+ cells - Histological and clinical features almost identical to that seen in patients with AA	- Possible the cross-breading with other mouse mutant strains - Human skin is affected in this model - Pre-clinical research possible - Histological and clinical features almost identical to that seen in patients with AA	- The model is only recently established	-Gilhar et al. 2013a,b

Table 1.7: Animal model for alopecia areata: Characteristics, advantages and disadvantages.

1.3.7 Alopecia areata as a model for autoimmune diseases

AA pathogenesis shares similar mechanisms with other important autoimmune diseases, such as multiple sclerosis, autoimmune uveitis, type 1 diabetes. In fact, these pathological conditions are mediated by an abnormal T cell-dependent response and reveal evidence of IP collapse in the affected tissues (Horai et al. 2013, Muldoon et al. 2013, van Belle et al. 2011, von Herrath and Homman 2004). Therefore, given that HFs are abundant in the human body (total number: ca. 5 million (Lien et al. 2014)) and relatively easy to obtain, AA is a regrettably under-utilized, but very instructive and accessible excellent general model in which common mechanisms in T cell-dependent autoimmune diseases can be dissected (McElwee et al. 2013, Bertolini et al. 2012, Gilhar et al. 2012, Kinori et al. 2011, Paus et al. 2005).

1.4 CD8+ T-cells

T-lymphocytes are responsible for the cell-mediated immune response of the adaptative immune system and are classified on the bases of the T-cell receptor (TCR) expression in

the surface in CD4+ T-, CD8+ T-, γδ T- and NKT-cells. Classical CD4+ or CD8+ T-cells, which express αβTCR receptor, develop in the bone marrow, undergo negative and positive selection in the thymus and migrate into peripheral tissues via the bloodstream **(Figure 1.16)** (Carvalheiro et al. 2013, Li et al. 2013, Murphy 2012, Playfair and Chain 2009, Kindt et al. 2007).

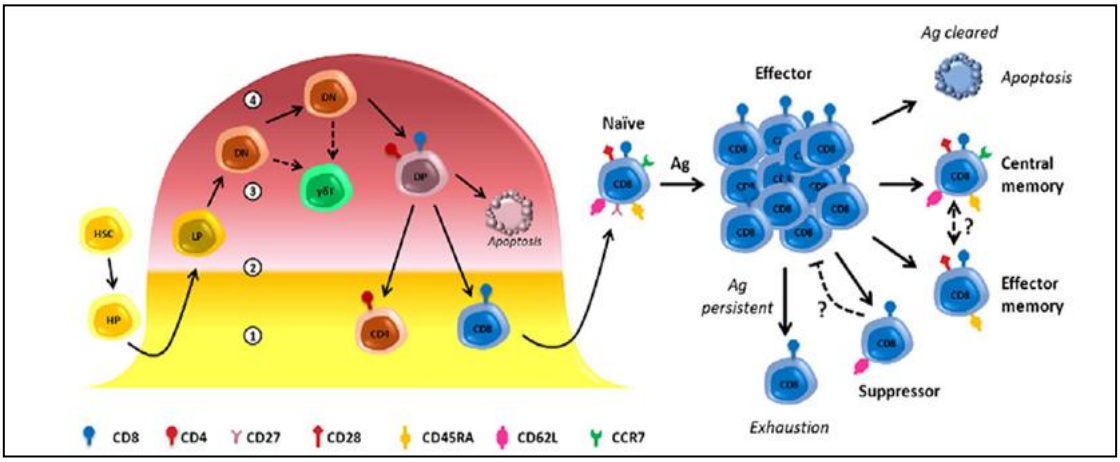

Fig 1.16: Development and differentiation of CD8+ T-cells.
The thymus is composed of medulla (1), cortico-medullary junction (2), cortex (3) and subcapsular zone (4). CD8+ T-cells precursors derive form hematopoietic stem cells (HSC) of the bone marrow and reach the thymus via the blood vessels (hematopoietic precursors, HP). Here, the passage through the cortico-medullary junction promotes their differentiation into lymphoid progenitors (LP). In the cortex, they become double-negative T-cells (DN) and they acquired a fully formed TCR in the subcapsular zone and CD4 and CD8 receptors, becoming double-positive (DP), in the cortex. After positive and negative selections they become single positive cells (CD8), they migrate in the medulla and enter the bloodstream and reach the lymphoid second organs where they reside as naïve T-cells. Here, once primed they become effector cells (cytotoxic T-cells, Tc) which proliferate and migrate into the peripheral tissue to exert their cytotoxic properties. When the antigen is cleared, most of them undergo apoptosis and the few that survive differentiate in memory T-cells. If the antigen persists, as in chronic status, CD8+ T-cells suffer of exhaustion due to the over activation. However, suppressor CD8 T-cells can be also found which release TGFβ1 and IL-10. Figure from Carvalheiro et al. 2013.

1.4.1 Biology of CD8+ T-cells

CD8+ T-cells are small lymphocytes with very little cytoplasm **(Figure 1.17A)** (Murphy 2012, Playfair and Chain 2009). The cell surface of CD8+ T-cells is characterized by the expression of an αβTCR receptor, which recognizes intracellularly generated peptides presented by MHC class I molecules and the co-receptor αβCD8, which weakly binds the MHC class I molecule **(Figure 1.17B)** (Li et al. 2013, Zoete et al. 2013, Murphy 2012, Playfair and Chain 2009, Kindt et al. 2007). Other cell surface receptors (e.g., CD28, 4-1BB, OX40, CD30, lymphocyte function-associated antigen (LFA)-1, protease-activated receptor (PAR)-2, PD-1, CD200R) that are intimately involved in the regulation of T-cell signalling complement the cell surface repertoire CD8+ T-cells (Carvalheiro et al. 2013, Murphy 2012, Springer et al. 2012, Shi et al. 2011, Croft 2010; Ishii et al. 2010, Fischer et al. 2006, Nakae et al. 2006, Keir et al. 2008, Watts 2005). MHC class I molecules are highly expressed by all the nucleated cells of the body, with the notable exception of cells that reside within a tissue area that enjoys relative IP (Dyer et al. 2013, Leone et al. 2013, Kinori et al. 2011). Therefore, all cells

expressing MHC class I are able to present antigens to CD8+ T-cells, once activated (Zoete et al. 2013, Murphy 2012, Playfair and Chain 2009, Kindt et al. 2007). However, since their effector functions are so destructive, CD8+ T-cells often require extra co-stimulatory signals in order to become activated; these co-stimulatory signals (such as IL-2, 4-1BBL, OX40L) are routinely provided by antigen- presenting cells or CD4+ T-cells **(Figure 1.17C)** (Murphy 2012, Hivroz et al. 2012, Springer et al. 2012, Playfair and Chain 2009, Kindt et al. 2007).

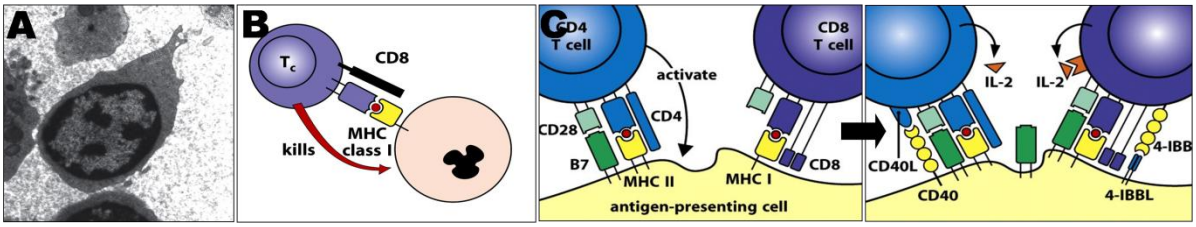

Figure1.17: CD8+ T-cell phenotype and activation.
Transmission electron micrograph of a CD8+ T-cell (A) and schematic drawing explaining how CD8+ T-cells recognize the complex MHC class I:peptide via TCR and CD8 co-receptor (B). However, most of CD8+ T-cells need special stimuli from antigen presenting cells and help from CD4+ T-cells (C). Pictures from Murphy 2012.

Human CD8+ T-cells are currently subdivided in at least, six groups **(Figure 1.16)**: naïve, effector or cytotoxic, central memory, effector memory, resident memory and suppressor (Carvalheiro et al. 2013, Health and Carbone 2013, Masopust and Schenkel 2013, Mueller et al. 2013, Playfair and Chain 2009, Kindt et al. 2007). Naïve CD8+ T-cells are mostly localized in secondary lymphoid organs and circulate via the lymphatic system (Masopust and Schenkel 2013, Mueller et al. 2013, Murphy 2012, Playfair and Chain 2009). They do not have encountered their cognate antigen yet. When they are primed by a suitable MHC class I: peptide complex, they proliferate, release IL-2 (which stimulates T-cell proliferation) and thus differentiate into effector T-cells (Carvalheiro et al. 2013, Health and Carbone 2013, Masopust and Schenkel 2013, Mueller et al. 2013, Murphy 2012, Playfair and Chain 2009, Kindt et al. 2007).

The physiological role of cytotoxic effector CD8+ T-cells is to directly kill cells on whose surface they recognize epitopes presented via MHC class I, such as cells infected by viruses or expressing certain tumour antigens **(Figure 1.17B)** (Cavalheiro et al. 2013, Leone et al. 2013, Masopust and Schenkel 2013, Murphy 2012, Willing and Friese 2012, Playfair and Chain 2009, Kindt et al. 2007). The effector molecules released by CD8+ T-cells are cytotoxins which are stored in pre-formed granules, such as perforin, granzymes (serine proteases), granulysin and serglycin (proteoglycans) (Murphy 2012, Playfair and Chain 2009, Kindt et al. 2007). Moreover, they release *de novo* synthesized cytokines depending on their subset: Tc1 (INFγ) and Tc2 (IL-4 and IL-5) (Cavalheiro et al. 2013, Murphy 2012, Playfair and Chain 2009, Kindt et al. 2007).

Once the antigen is eradicated, most of the effector CD8+ T-cells die by apoptosis, while some of them become memory CD8+ T-cells. Memory effector CD8+ T-cells are mostly localized in non-lymphoid peripheral tissue, where they are ready to become fully functional effector cells and to release INFγ, IL-4 or IL-5 **(Figure 1.18)** (Cavalheiro et al. 2013, Krzysiek et al. 2013, Mueller et al. 2013, Murphy 2012, Playfair and Chain 2009, Kindt et al. 2007).

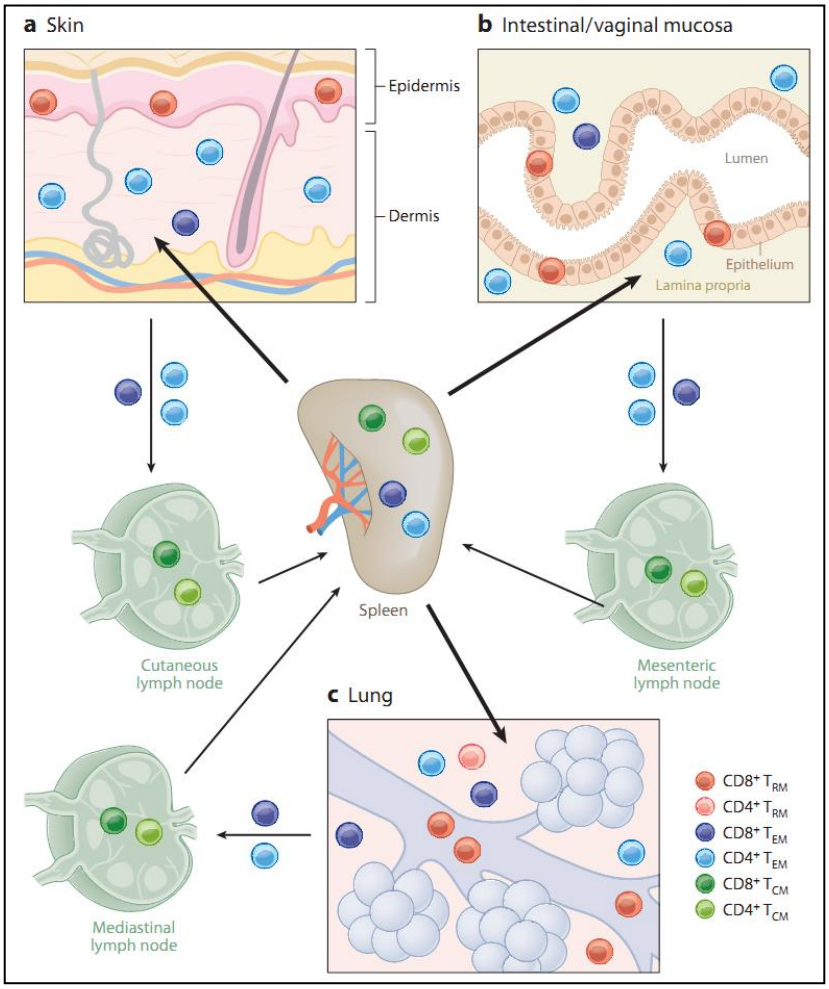

Figure 1.18: Localization of memory CD8+ T-cells. Effector memory CD8+ T-cells (T_{EM}) settle mostly in the tissue and they are immediately activate by a second encountering of the antigen while centre memory CD8+ T-cells (T_{CM}) circulate between peripheral lymphoid organs and are slower to re-activate. Resident memory CD8+ T-cells (T_{RM}) reside in particular peripheral tissues. Figure from Mueller et al. 2013.

Human central memory CD8+ T-cells circulate more than naïve T lymphocytes through the peripheral lymphoid organs but are slower to differentiate in effector cells than effector memory cells **(Figure 1.18)** (Carvalheiro et al. 2013, Masopust and Schenkel 2013, Mueller et al. 2013, Murphy 2012). Memory CD8+ T-cells which constantly reside in defined tissue compartments and protect the tissue during pathogens invasion, e.g. in the epidermis or HF infundibulum, are now called resident memory cells **(Figure 1.18)** (Carvalheiro et al. 2013, Health and Carbone 2013, Krzysiek et al. 2013, Masopust and Schenkel 2013, Mueller et al. 2013, Murphy 2012).

Some CD8+ T-cells are able to produce IL-10 and TGFβ1 and express FoxP3. These represent suppressor CD8+ T-cells (Carvalheiro et al. 2013, Murphy 2012, Mellor et al. 2011).

1.4.2 CD8+ T-cell in autoimmune diseases

While the antigens presented via MHC class I to CD8+ T-cells can be virally encoded or tumour antigens, they often represent a self-peptide which stimulates an abnormal autoreactive CD8+ T-cell-mediated immuno response. Such autoreactive CD8+ T-cell responses are critically involved in the pathogenesis of several autoimmune diseases, such as rheumatoid arthritis **(Figure 1.19)** (Carvalheiro et al. 2013, Alzabin and Williams 2011), type 1 diabetes **(Figure 1.20)** (Coppieters et al. 2013, Padgett et al. 2013, van Belle et al. 2011, Bluestone et al. 2010, von Herrath and Homann 2004) and multiple sclerosis **(Figure 1.21)** (Luo et al. 2014, Willing and Friese 2012, Sayed et al. 2010, Friese and Fugger 2009).

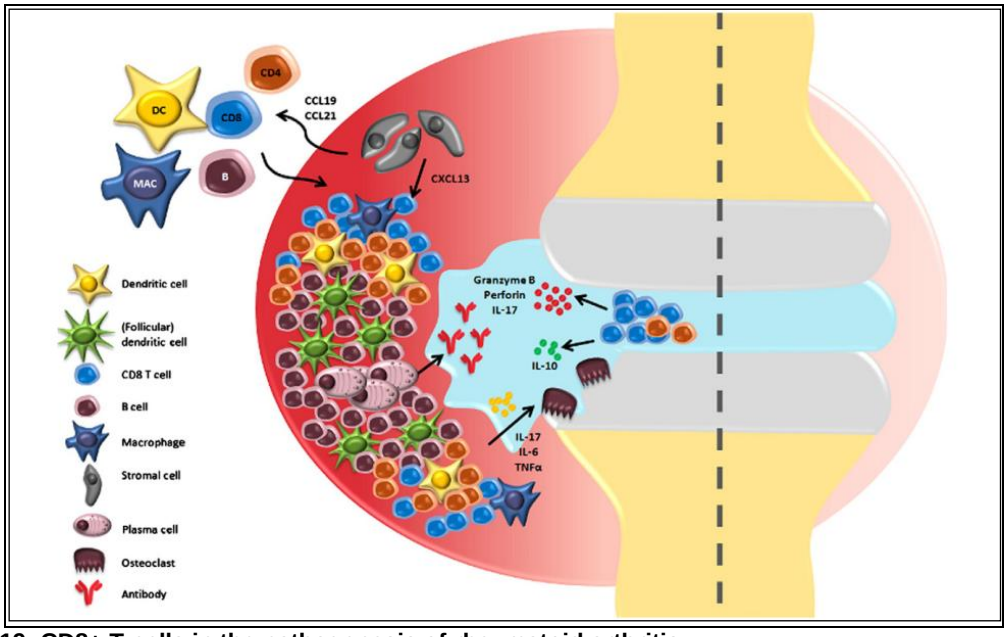

Figure 1.19: CD8+ T-cells in the pathogenesis of rheumatoid arthritis.
CD8+ T-cells are abundant in the inflammatory infiltrate in the synovial fluid of rheumatoid arthritis patients. These represent both Tc1 and suppressor CD8+ T-cells, with the latter presumably attempting to counterbalancing the excessive pro-inflammatory response. Tc1 cells release IL-6, IL-17, TNFα, granzyme, perforin which promote bone degradation by activating osteclasts. In the synovial membrane one can detect aggregates of T-cells or even follicular structures, which are reminiscent of germinal centres of secondary lymphoid organs. Figure from Carvalheiro et al. 2013.

CD8+ T-cells play either an effector role, e.g. in type 1 diabetes **(Figure 1.20)** (Padgett et al. 2013, van Belle et al. 2011, Bluestone et al. 2010, von Herrath and Homann 2004) or in multiple sclerosis **(Figure 1.21)** (Willing and Friese 2012, Sayed et al. 2010, Friese and Fugger 2009) or they amplify the inflammation by releasing pro-inflammatory cytokines, e.g. in rheumatoid arthritis **(Figure 1.19)** (Carvalheiro et al. 2013, Alzabin and Williams 2011).

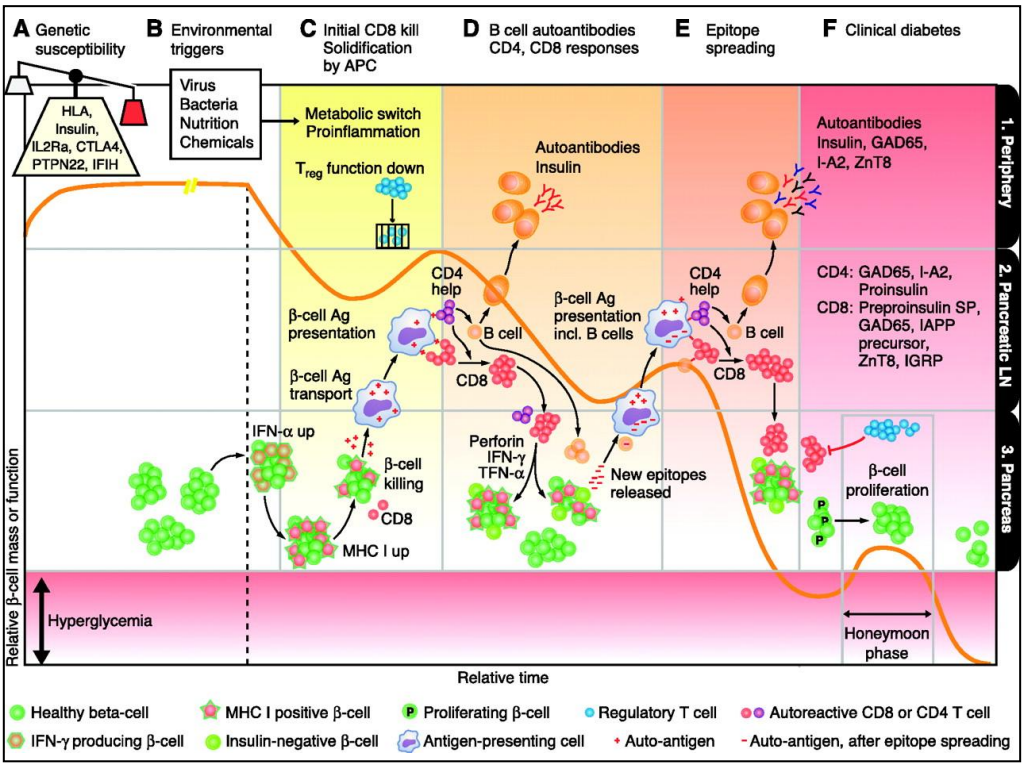

Figure 1.20: CD8+ T-cells in the pathogenesis of type 1 diabetes
The trigger event of type 1 diabetes is still unknown but it derives by a concurrence of genetic susceptibility and environmental factors. The first episode is the collapse of the IP by up-regulation of IFNγ and MHC class I in β cells of pancreas. CD8+ T-cells kill human islet β cells upon MHC class I:peptide complex recognition, by releasing perforins and granzymes and in some cases up-regulating the Fas ligand. This damage further increases the autoreactive response and results in the exposure of additional self antigens. Figure from van Belle et al. 2011.

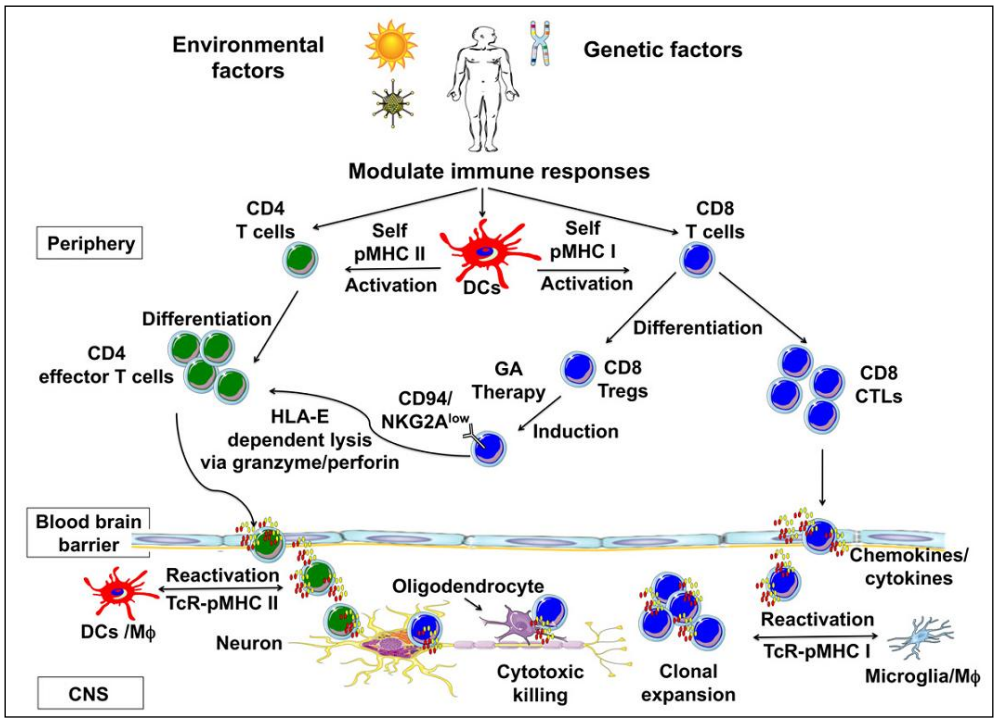

Figure 1.21: CD8+ T-cells in the pathogenesis of multiple sclerosis
Several factors (genetic, environmental) might trigger the disease which is characterized by a strong central nervous system inflammation, along with a defective blood brain barrier and a collapse of central nervous system IP. B cells, dendritic cells, microglia and macrophages present the antigen to T-cells. CD8+ T-cells kill glial cells, exposing the axons, transect axons, cause vascular permeability and activate oligodendrocyte death. Figure from Willing and Friese 2012.

1.4.3 CD8+ T-cells, hair follicles and alopecia areata

Human skin is populated by many T-cells, with most the CD8+ T-cells found in the epidermis and few residing in and around the HF (Health and Carbone et al. 2013, Di Meglio et al. 2011, Christoph et al. 2000). Namely, high numbers of CD8+ T-cell inhabit the infundibulum and their number start to decline at the level of the isthmus. CD8+ T-cells are seen only very rarely in the proximal and HB epithelium. The HF CTS shows a similar CD8+ T-cells distribution of the epithelium **(Figure 1.5)** (Christoph et al. 2000). In mouse HFs, CD8+ T-cells are rare in the HF epithelium, CTS and dermis (Bertolini et al. 2013, Paus et al. 1998).

CD8+ T-cells have long been a focus of AA research (e.g. (Gilhar et al. 2013a, Ito et al. 2013a, Alli et al. 2012, McElwee et al. 2005, Yano et al. 2002, Gilhar et al. 1998, Becker et al. 1996, McElwee et al. 1996, Paus et al. 1993, Kalish et al. 1992). Many studies suggest their key role in the pathogenesis of AA. First of all, CD8+ T-cell depletion abrogates AA onset in a rat model of AA **(Table 1.8)** (McElwee et al. 1996). In rodent AA models, CD8+ T-cells are the key pathogenesis-initiating immunocytes supported by CD4+ T-cells by which is determined the level of inflammatory HF damage **(Table 1.8)** (McElwee et al. 2005). Cell transfer studies in an excellent preclinical model for the study of human AA (i.e. human scalp skin from AA patients transplanted onto immunocompromised SCID mice) have clearly established that AA lesions can be induced just by transfer of human CD8+ T-cells **(Table 1.8)** (Gilhar et al. 2002, 1998) which recognize MHC class I-presented autoantigen(s). However, maximal induction of AA lesions in this model requires not only CD8+ T-cell exposure to HF-associated autoantigen(s), but also the help of CD4+ T-cells **(Table 1.8)** (Gilhar et al. 2002, Gilhar et al. 2001, Gilhar et al. 1998).

In C3H/HeJ mice, the injections of IL-4 or anti- IFNγ-specific monoclonal antibodies induced hair growth and a significant decrease of CD8+ T-cells around the HFs affected by AA **(Table 1.8)** (Nakamura et al. 2008). The treatment with the contact sensitizer, diphencyprone (DPCP), in AA C3H/HeJ mice and Dundee rat promote hair regrowth and the immunohistology showed a decrease of CD8+ T-cell numbers **(Table 1.8)** (Shapiro et al. 1999). A recent genome wide association study is in line with the concept that in AA, the autoimmune destruction is due to overstimulation of CD8+/NKG2D+ cytotoxic T-cells by the abnormal expression of ULBP3, a NKG2D ligand, in the HF CTS of AA patients (Petukhova et al. 2010). Moreover, a recently published new mouse model of AA **(Table 1.8)** impressively confirmed the key role of CD8+ T-cells in AA pathogenesis generating transgenic mice with HF-specific CD8+ T-cells (Alli et al. 2012). Similar phenotype and HF-attack by CD8+ T-cells occurs in C57BL/6 mice after administration of antibody-targeted IL-2 (therapy for melanoma) (Becker et al. 1996). The newly established humanized-mouse

model for AA bases on the injection of IL-2 stimulated NKG2D+/CD56+ immunocytes, many of which are CD8+, into healthy human skin transplanted on (SCID) mice **(**for additional lines of evidence see **Table 1.8)** (Gilhar et al. 2013a,b).

Animal model	Experiment
Dundee experimental balding rat	The depletion of CD8+ T-cells prevents the development of AA-like phenotype (McElwee et al. 1996).
	Hair regrowth was observed on the treated animals (C3H/HeJ mice and Dundee rat) . Immunohistochemical analyses revealed reduction of intrafollicular CD8+ T cells infiltrates after the successful treatment with the contact sensitizer, diphencyprone (DPCP) (Shapiro et al. 1999).
C3H/HeJ mice	Subcutaneous injections of CD8+ T cells from affected animals into normal C3H/HeJ mice induce patchy hair loss after several days, while injection of CD4+ T cells did not induce local hair loss, but activated the mice immune system to promote multiple patches of hair loss after several weeks, further supporting the concept that CD8+ T cells are the effector cells while CD4+ T cells provide a helper function (McElwee et al. 2005).
	Injections of IL-4 or anti- IFN -specific monoclonal antibodies induced hair growth, and the histological feature showed a significant decrease of CD8+ T cells around the hair follicles in C3H/HeJ mice with alopecia areata (Nakamura et al. 2008).
	Substance P treatment to the skin of alopecia areata affected mice leads to a significant increase of mast cell degranulation and to accelerated hair follicle regression (catagen), accompanied by an increase of CD8+ along with impressive increase of granzyme B and NK-1R expression by these cells (Siebenhaar et al. 2007).
C57BL/6 mice	Transgenic mice expressing selected T cell receptor chains which promote the development of hair follicle-specific CD8+ T cells that attack the hair follicle in an alopecia areata-like manner (however, unlike in aloepcia areata, hair follicle destruction also occurs) (Alli et al. 2012).
	Induction of alopecia areata –like lesions following immunotherapy for melanoma (antibody-targeted interleukin-2 administration). The histological features demonstrated the presence of dense CD8+ T cells infiltrates around the hair follicles. Passive transfer of the CD8+ T cells to naïve mice led to the development of alopecia areata-like lesions (Becker et al. 1996).
SCID mouse model	Injection of autologous CD8+ T cells to human scalp explants on the mice, not only caused hair loss but also induced expression of ICAM1, HLA-DR, and HLA-A, HLA-B, and HLA-C on follicular epithelium. CD4+ T cells were observed around the hair follicles, whereas perifollicular infiltrates were composed of CD8+ T cells, as is the case with the naturally occurring disease. Although the transfer of CD8+ T cells alone can induce alopecia areata lesions, optimal induction of hair loss requires transfer of both CD4+ and CD8+ T cells (Gilhar et al. 2002, 2001, 1998).
	Injection of human PBMCs stimulated with high dose of IL-2 and enriched for CD56+/NKG2D+ cells, mostly CD8+, to human scalp explants on the mice, caused hair loss but also induced expression of ICAM1, HLA-DR, and HLA-A, HLA-B, and HLA-C on follicular epithelium (Gilhar et al. 2013a,b).

Table 1.8: Evidence from animal models that CD8+ T-cells are key effector cells in alopecia areata. Intercellular Adhesion Molecule 1 (ICAM), human leucocyte antigen (HLA). Table modified after Gilhar et al. 2012.

1.5 Mast cells

1.5.1 Biology of mast cells

MCs were visualized histochemically in the human tissue for the first time by Paul Ehrlich in the 1878 as large mononuclear cells that contain characteristic metachromatic granules in their cytoplasm **(Figure 1.22)** (Melo et al. 2014, Wernersson and Pejler 2014, Beaven 2009, Dvorack 2005).

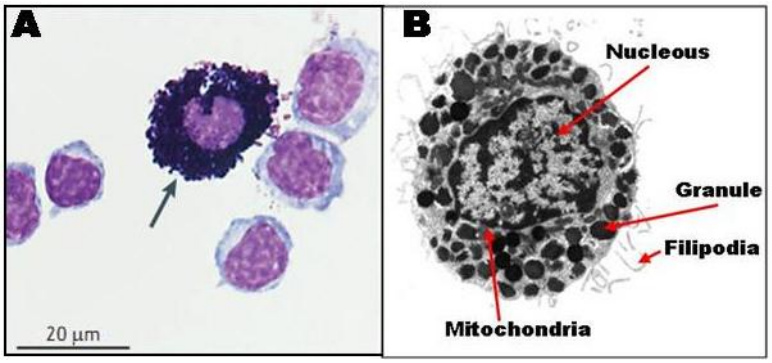

Figure 1.22: Ultrastructure of human mast cells.
Histochemical and transmission electron micrograph pictures of a MC showing many granules in the cytoplasm, centrioles and microvilli on its surface. Figure modified after Wernersson and Pejler et al. 2014 and Dvorak 2005.

MCs develop from pluripotent hematopoietic cells (CD34+) in the bone marrow from the same precursor of granulocytes, monocytes, megakaryocytes and erythrocytes **(Figure 1.23)**. It is not clear yet if they share most of this process with basophils, deriving from a common basophil/MC progenitors, or if they derive from a distinct MC precursor population that has differentiated from a common myeloid progenitor **(Figure 1.23)** (Frenzel and Hermine 2013, Voehringer 2013, Gurish and Austen 2012, Katz and Austen 2011, Galli et al. 2011, Beaven 2009, Galli 2005). MITF is thought to be the crucial transcription factor which determinates the MC fate (Qi et al. 2013, Voehringer 2013), and the appropriate stimuli is given by IL-3, IL-4 and stem cell factor (SCF) (Kritas et al. 2014b, Voehringer 2013, Gurish and Austen 2012, Galli et al. 2011, Beaven 2009, Bishoff 2007, Galli 2005).

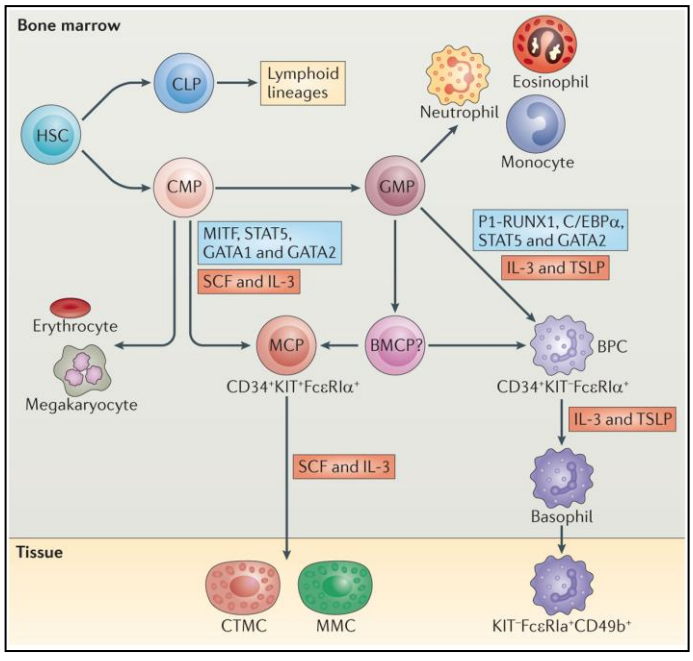

Figure 1.23: Development of mast cells.
Hematopoietic stem cells (HSCs) differentiate in common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). MCs progenitors (MCPs) may derive either directly from CMPs or from a granulocyte/monocyte progenitors (GMPs) which later on differentiate in basophil/MC progenitors (BMCP). Through the bloodstream immature MC precursors reach the tissue where they mature and differentiate. Depending on the tissue, MCs can differentiate in connective tissue MCs (CTMC) or mucosal MCs (MMC). C/EBPα, CCAAT/enhancer-binding protein-α; FcεRIα, high-affinity receptor for IgE; GATA, GATA-binding protein; IL-3, interleukin-3; MITF, microphthalmia-associated transcription factor; P1-RUNX1, distal promoter-derived runt-related transcription factor 1; SCF, stem cell factor; STAT5, signal transducer and activator of transcription 5; TSLP, thymic stromal lymphopoietin. Figure from Voehringer 2013.

Non-granulated MC progenitors circulate in the blood stream and migrate to tissues, where they differentiate and mature under the influence of local signals arising from the relevant microenvironment such as SCF, IL-3, and IL-9 (Dahlin and Hallgren 2014, Kritas et al.

2014b, Frenzel and Hermine 2013, Voehringer 2013, Taketomi et al. 2013, Gurish and Austen 2012, Beaven 2009, Galli 2005, Metcalfe et al 1997, Yong 1997).

However, in some tissue, notably in the CTS of murine vibrissae and human scalp HFs, MC precursors get deposited (Dahlin and Hallgren 2014), and from these intrafollicular resident MC precursors mature MC can differentiate and proliferate under organ culture conditions, i.e. in the absence of bone marrow and a functional vasculature (Sugawara et al. 2012, Ito et al. 2010, Gurish and Boyce 2006, Kumamoto et al. 2003). It has been proposed that pathological increase in the number of intracutaneous or mucosal MCs in human skin or upper airway mucosa, for example during atopic dermatitis, chronic urticarial or allergic asthma, may result at least in part from such resident peripheral MC progenitors (Sugawara et al. 2013, Sugawara et al. 2012).

The receptor for SCF is c-Kit, a tyrosine kinase membrane receptor (CD117) **(Figure 1.24)**. The stimulation of c-Kit is fundamental not only in the regulation and migration of MC progenitors but also in the proliferation, survival and maturation of mature MCs (Voehringer 2013, Gurish and Austen 2012, Gilfillan and Beaven 2011, Galli 2005, Metcalfe et al. 1997, Yong 1997).

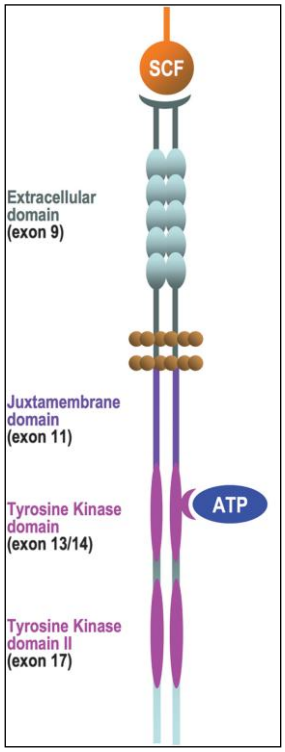

Figure 1.24: C-Kit structure
C-Kit receptors is composed by an extracellular ligand-binding region containing five immunoglobulin-like repeats, necessary for the binding of SCF, a transmembrane sequence, an autoinhibitory juxtamembrane domain. The intracellular tyrosine kinase domains are an ATPbinding pocket and a kinase activation loop. Figure from Pittoni et al. 2011.

Depending on the peripheral tissue where they reside, MC progenitors differentiate either in connective tissue MCs (CTMCs) or mucosal MCs (MMCs), both in human and mouse **(Figure 1.25)**. CTMCs are mainly localized in the dermis of skin, submucosal connective

tissues of the respiratory tract, joint synovial, and peritoneum while MMCs in mucosa of the gastrointestinal tract, in the lamina propria of the respiratory tract and in the small intestinal submucosa (Bradding and Saito 2014, Wernersson and Pejler 2014, Gurish and Austen 2012, Galli et al. 2011, Beaven 2009, Matsue et al 2009). While, under physiological circumstances, MCs never enter the epithelium of human skin (incl. the HF ORS and the HB), they can migrate into human airway epithelium (Sugawara et al. 2013, Sugawara et al. 2012). CTMCs and MMCs show a distinct morphology, granule content and sensitivity to stimulation by various secretagogues, stabilizers and drugs **(Table 1.9)** (Bradding and Saito 2014, Gurish and Austen 2012, Galli et al. 2011, Beaven 2009, Metcalfe et al. 1997).

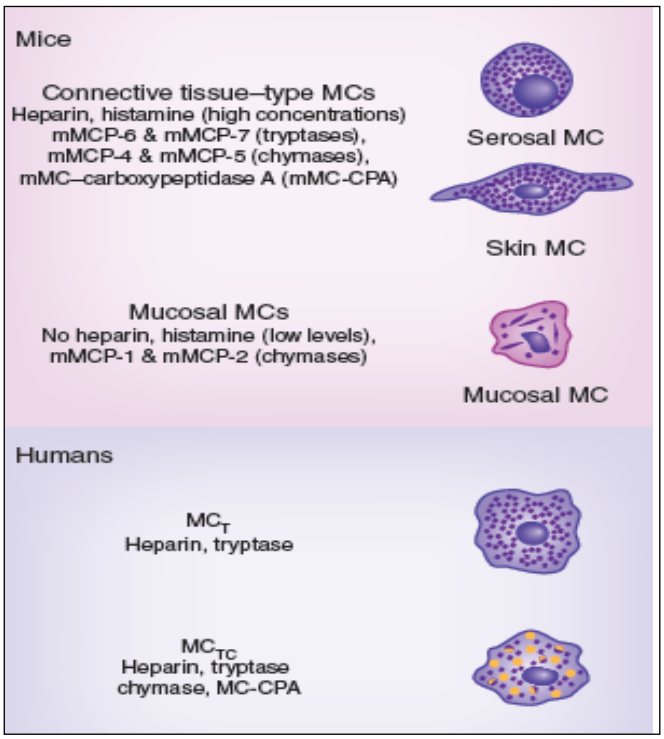

Figure 1.25: Mast cell subpopulations.
In human and rodents, MCs mature into MC_{TC} (CTMC) or serosal MCs containing both tryptase and chymase and MC_T (hMMC) or mucosal MCs containing mainly tryptase and depending on the anatomical location and/or mediator contents. Figure from Galli et al. 2011.

Human CTMCs (or MC_{TC}) which correspond to mucosal MCs in rodents, contain both tryptase and chymase, carboxypeptidase A and cathepsin G. Human MMCs (or MC_T) contain tryptase only and represent serosal MCs in rodents (Gri et al. 2012, Galli et al. 2011, Metcalfe et al. 1997, Yong 1997) **(Figure 1.25)**.

Characteristic	MC_T	MC_{TC}
Protease content	Tryptase	Tryptase Chymase
Proteoglycan content	Heparin	Heparin
Predominant granule patterning evident on electron microscopy	Scroll	Lattice
Common location	Epithelium	Lamina propria, connective tissue, skin, airway smooth muscle
Putative primary role	Host defense	Tissue repair
Relative LTC_4 release	High	Skin: low
Relative PGD_2 release	High	Skin: high
Cytokine profile	IL-4: low IL-5: high IL-6: high IL-13: low	IL-4: high IL-13: high
Activated by antigen	Yes	Yes
Activated by substance P	No	Yes
Responds to C5a	No	Yes
Responds to PAF Responds to opiates	Yes No	No Yes
Inhibited by sodium cromoglycate	Yes (weak effect)	No

Table 1.9 Human mast cell heterogeneity
Complement component 5a (C5a), connective tissue MC (MC_{TC}), leukotriene C4 (LTC_4), mucosal MC (MC_T), platelet-activating factor (PAF), prostaglandin D2 (PGD_2). Table modified after Bradding and Saito 2014.

MCs are activated by a multitude of different signalling pathways **(Figure 1.26)**, including numerous MC surface receptors, most prominently high-affinity receptors for IgE (FcεR) (Gri et al. 2012, Manikandan et al. 2012, Metz and Maurer 2007), corticotropin-releasing hormone receptor (CRH-R1 and CRH-R2) (Slominski et al. 2013, Ito et al. 2010), and SP (NK1) (Peters et al. 2007) **(Table 1.10)** (Gri et al. 2012, Migalovich-Sheikhet et al. 2012, Galli et al. 2011). MCs express also inhibitory receptors, such as CD200R (Bishoff 2007, Cherwinski et al. 2005), CD300A and C (Takahashi et al. 2013, Gri et al. 2012, Migalovich-Sheikhet et al. 2012, Karra et al. 2009, Bachelet et al. 2005), and TGFβ receptor type I (Gri et al. 2012, Bishoff 2007) **(Table 1.10)**. As ever-more MC surface receptors are being discovered (e.g. Migalovich-Sheikhet et al. 2012), the complexity of signalling pathways via which MC functions are regulated within their respective local microenvironment is continuously increasing.

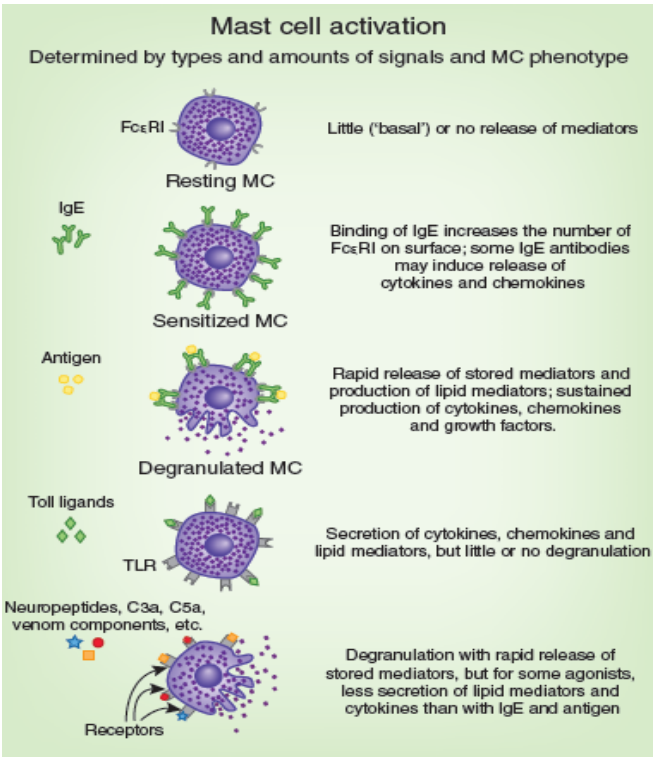

Figure 1.26: Mast cell activation
MCs constantly release little amount of mediators, also if not activated. In some cases, slight activation could result from the binding of IgE bound to high-affinity receptors for IgE (FceRI) on the cell surface. However, the classical activation of MCs, e.g. in allergic response, is mediated by the complex antigen:IgE to FceRI which causes the rapid exocytosis of the cytoplasmic granules (degranulation), the production of lipid mediators (such as leukotrienes and prostaglandins) and the release of cytokines, chemokines and growth factors. Toll-like receptors (TLRs) are expressed in a moltitudine of MCs and their stimulation promote the activation of MCs and the release of cytokines without degranulation. Finally, MCs present many surface receptors that if stimulated, cause the release of granule content and cytokines. Figure from Galli et al. 2011.

Receptor family	Members
FcR	
FcεR	FcεRI
FcγR	FcγRI[a], FcγRII, FcγRIII[b]
TLR	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10[a]
MHC	MHC class I, MHC class II
Complement receptor	CR1, CR2, CR3, CR4, CR5, C3aR, C5aR
Cytokine receptor	CD117, IL-1R, IL-3R, IL-10R, IL-12R, INFγR, TGFβR
Chemokine receptor	CCR1, CCR3, CCR4, CCR5,CCR7, CXCR1, CXCR2, CXCR3, CXCR4, CXCR6, CX3CR1
RECEPTOR FOR ENDOGENOUS MOLECULES	
Histamine receptor	H1/H2/H3/H4 receptor
Others	Endothelin-1, neurotensin, substance P, PGE2, adenosine
Adhesion molecules	ICAM-1, VCAM, VLA4, CD226 (DNAM-1), Siglec8, CD47, CD300a, CD72
CO-STIMULATORY MOLECULES	
TNF/TNFR family members	CD40L, OX40L, 4-1BB, GITR, CD153, Fas, TRAIL-R
B7 family member	CD28, ICOSL, PD-L1, PD-L2
TIM family members	TIM1, TIM3
Notch family members	Notch1, Notch2

Table 1.10: Summary of mast cell surface receptors.
Overview of surface receptors expressed either in CTMCs or MMCs or both. Some receptors have been only investigated in human[a] or murine[b] or not investigate[ni], if not indicated the molecules where studied in both species. Intercellular adhesion molecule (ICAM), prostaglandine (PG), T-cell immunoglobulin mucin domain (TIM), tumor necrosis factor (TNF), toll like-receptors (TRL), vascular cell adhesion molecule (VCAM). Table modified after Gri et al. 2012.

For example, it has recently surfaced that cannabinoid receptor 1-mediated signalling plays a crucial role in preventing excessive maturation of mucosal MCs from resident precursors in human skin and upper airway mucosa also counterbalances MC-activating stimuli (Sugawara et al 2013, Sugawara et al. 2012). Together with the well-recognized secretagogue activity of

CRH and SP this suggests that locally generated neuroendocrine signals are prominently involved in regulation MC activity. This notion is further supported by findings that CGRP, the α-MSH-derived peptide, K(D)PT all can inhibit human skin connective MC functions *in situ* (Kinori et al. 2012, Meyer et al. 2009)

Once activated MCs are able to immediately release an array of pre-formed mediators stored in cytoplasmic granules via exocytosis, such as histamine, proteases (tryptase, chymase), proteoglycans and many different cytokines (e.g. tumor necrosis factor, TNFα) and growth factors (e.g. TGFβ1). In addition, lipid mediators, cytokines, chemokines, growth and angiogenic factors are synthesized *de novo* upon MC activation (details and references, see **Table 1.11)**. The type and amount of mediators which are released depend on the nature, property and strength of the activation stimuli. Therefore, MCs are able to produce and release a vast array of molecules with either pro-inflammatory or immunoinhibitory functions, depending on the microenvironmental signalling context, systemic, neural, and environmental signals received, and on the cells they interact with (Hallgren and Gurish 2014, Wernersson and Pejler 2014, Frenzel and Hermine 2013, Nelissen et al. 2013, Voehringer 2013, Gri et al. 2012, Gurish and Austen 2012, Gilfillan and Beaven 2011, Beaven 2009, Galli 2005, Metcalfe et al 1997, Yong 1997).

Class	Mediators	Physiological effects
PREFORMED		
Biogenic amines	Histamine	Vasodilatation
	5-hydroxytryptamine	Leukocyte regulation, pain, vasoconstriction
Proteoglycans	Heparin, heparin sulfate	Angiogenesis, coagulation
	Chondroitin sulfate	Tissue remodeling
Proteases	Tryptase	Inflammation, pain, tissue damage, PAR activation
	Chymase	Inflammation, pain, tissue damage
	MC-CPA/Carboxypeptidase A	Enzyme degradation
	CathepsinB, C, D, E, G, L, S[b]	Pathogen killing, tissue remodeling
	MCP5/6	Pathogenesis of asthma and other allergic disorders
Lysosomial enzymes	β-hexosaminidase, β-glucuronidase, β-galactosidase, arylsulfataseA	ECM remodeling
Others	Nitric oxide synthase	NO production
	Endothelin	Sepsis
	Kinins	Inflammation, pain, vasodilatation
		Anti-inflammatory effects
NEWLY SYNTHESIZED		
Lipid-derived	LTB_4, LTC_4, PGD_2, PAF	Inflammation, leukocyte recruitment, endothelial adhesion, *smooth* muscle cells contraction, vascular permeability
Cytokines	IL-1α[a], IL-1β[a], IL-2[b], IL-3, IL-4, IL-5, IL-6, IL8[a], IL-9, IL-10, IL-11[a], IL-12, IL-13, IL-14[a], IL-15[a], IL-16, IL-17, IL-18[a], IL-22[b], IL-25[b], IL-33[b], MIF, TNFα, IFNα, IFNβ[b], IFNγ[b]	Inflammation, leukocyte proliferation and activation immunoregulation
Chemokines	CCL1, CCL2, CCL3[a,b], CCL4[a] CCL5[a], CCL7[a,b], CCL8[a], CCL11[a], CCL13[a], CCL16[a], CCL17, CCL19[a], CCL20[a], CCL22a,[b], CCL25[b] CXCL1[a], CXCL2, CXCL3[a], CXCL4, CXCL5, CXCL8[a], CXCL10[a], CX3CL	Leukocyte chemotaxis
Growth factors	TGFβ, SCF[a], G-CSF, M-CSF, GM-CSF, VEGF, NGFβ, LIF[a], bFGF	Growth of various cell types
Antimicrobic species	Antimicrobial peptides, NO, superoxide, ROS	Pathogen killing

Table 1.11: Summary of mast cell mediators.
Overview of mediators released either by CTMCs or MMCs or both. Some mediators have been only investigated in human[a] or murine[b] or not investigate[ni], if not indicated the markers where studied in both species. Colony-stimulating factor (CSF), fibroblast growth factor (FGF), leucocyte migration inhibitory factor (LIF), leukotriene (LT), nerve growth factor (NGF), nitric oxide (NO), platelet-activating factor (PAF), prostaglandine (PG), reactive oxygen species (ROS), stem cell factor (SCF), transforming growth factor (TGF). Table from Gri et al. 2012.

The ability of MCs to easily change their phenotype implicated them in numerous biological processes: host defence, innate and adaptive immunity, peripheral tolerance induction, immune suppression, autoimmunity, chronic inflammation, tissue remodelling/wound repair (collagen synthesis, angiogenesis), hair growth, tumour progression, and detoxification of venoms and other toxic substances **(Figure 1.27)** (Douaiher et al. 2014, Voehringer et al. 2013, Dyduch et al. 2012, Gilfillan and Beaven 2011, Della Rovere et al. 2009, de Vries et al. 2009a, Matsue et al. 2009, Bischoff 2007, Metz and Maurer 2007, Maurer et al 1995, Metcalfe et al. 1997, Paus et al. 1994).

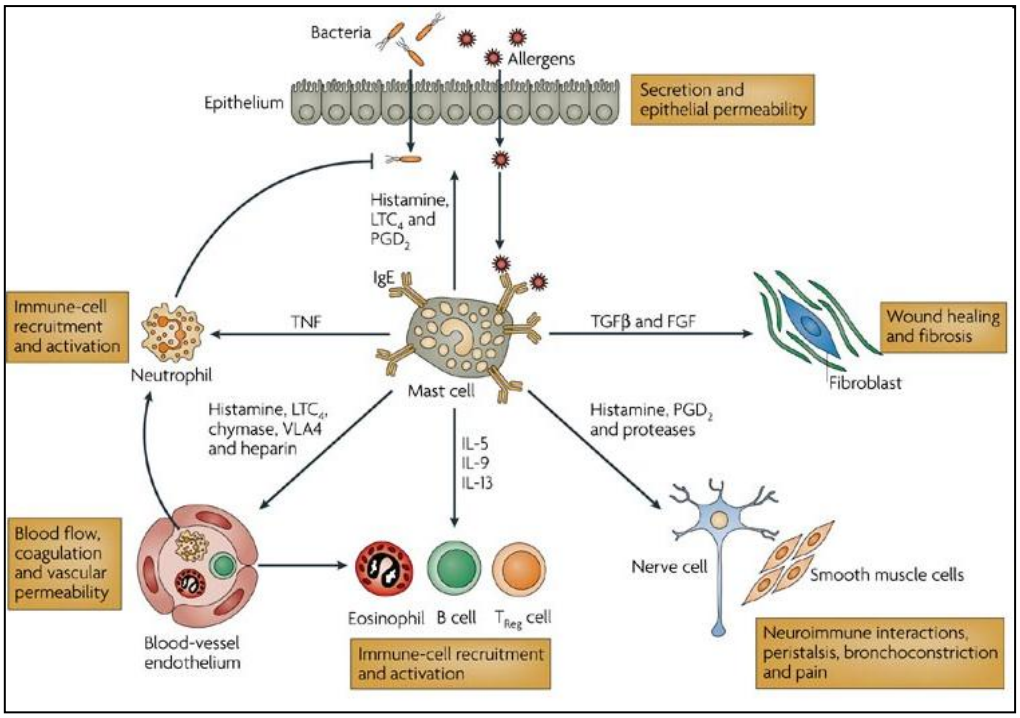

Figure 1.27: General function of human mast cells.
MCs are implicated in host defence, innate and adaptive immunity, peripheral tolerance induction, immune suppression, autoimmunity, chronic inflammation, tissue remodelling/wound repair, tumours progression, and detoxification. Fibroblast growth factor (FGF), interleukin (IL), leukotriene C4 (LTC4), prostaglandin D2 (PGD2), transforming growth factor (TGF), tumour-necrosis factor (TNF), CD4+CD25+ regulatory T cell (Treg cell), very late antigen 4 (VLA4). Picture from Bischoff 2007.

1.5.2 The immunomodulatory role of mast cells

MCs have long been viewed as the primary effector cells of innate immunity (Kurashima et al. 2014, St. John and Abraham 2013, Frenzel and Hermine 2013, Galli et al. 2011). However, they also play a key role in connecting innate and adaptive immune responses (Ebert et al. 2014, Frenzel and Hermine 2013, St John and Abraham 2013, Brown and Hatfield 2012, Gri et al. 2012, Tete et al. 2012, Harvima and Nilsson 2011, Tsai et al 2011, Frossi et al. 2010, Stelekati et al. 2009, Galli et al. 2008, Harvima et al. 2008, Sayed et al. 2008). In fact, MCs are able to attract and to regulate dendritic cells, B-cells, eosinophils, lymphocytes, NK cells, macrophages (Merluzzi et al. 2014, Chatterjee and Gashev 2014, Brown and Hatfield 2012, Gri et al. 2012, Migalovich-Sheikhet et al. 2012, Harvima and

Nilsson 2011, Sayed et al 2008, Christy and Brown 2007, Sayed and Brown 2007, Galli et al 2008) and can even control antigen-specific CD8+ T-cell responses (Stelekati et al. 2009) **(Figure 1.28)**.

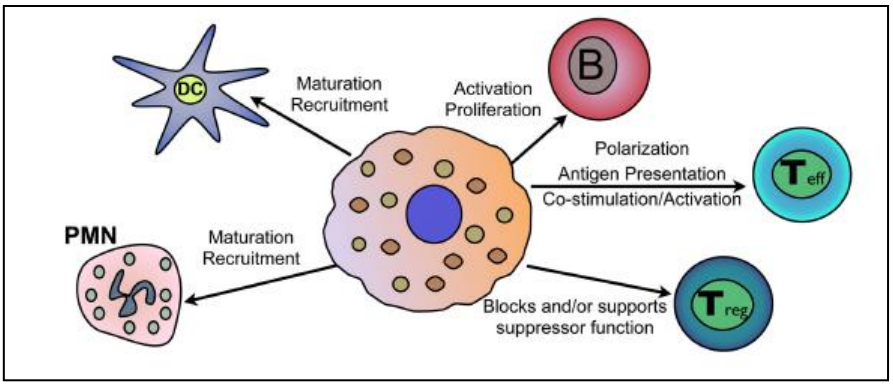

Figure 1.28: Immuno-modulatory functions of mast cells.
MCs are able to interact with many different immunocytes such as dendritic cells (DC), B cells (B), T effector cells (T eff), regulatory T-cells (T reg), neutrophils (PMN), macrophages, NK cell and γδ T-cells. Modified after Brown and Hatfield 2012.

Importantly, MCs exert a dual immunoregulatory role (Frenzel and Hermine 2013, Voehringer 2013, Brown and Hatfield 2012, Gri et al. 2012, Gilfillan and Beaven 2011, Harvima and Nilsson 2011, Tsai et al. 2011, Frossi et al. 2010, Galli et al. 2008, Sayed et al. 2008): Under physiological circumstances, they appear to exert largely immunoinhibitory functions, thus contributing to the maintenance of IP for example, and may function as intermediaries of peripheral tolerance and regulatory T-cell-dependent tolerance **(Figure 1.28** and **1.29)** (Chan et al. 2013, Voehringer 2013, Gan et al. 2012, Kalesnikoff and Galli 2011, Tsai et al. 2011, Frossi et al. 2010, de Vries et al. 2009a, Sayed et al. 2008, Lu et al. 2006, Waldmann 2006).

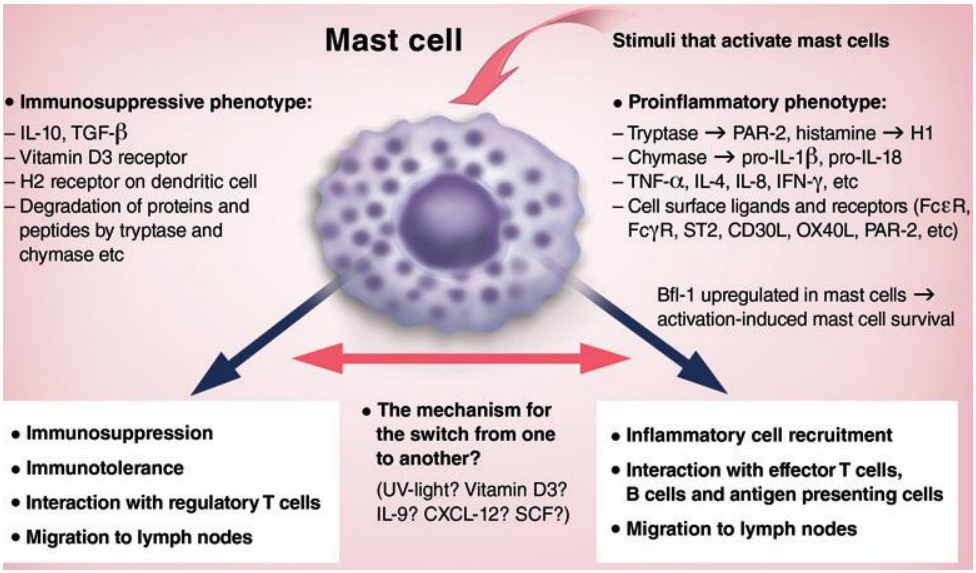

Figure 1.29: Mast cell phenotype switch.
MCs are able to switch from an immunosuppressive, important for peripheral tolerance and IP maintenance to a pro-inflammatory phenotype which promotes pathogenic environment. Interferon (IFN), interleukin (IL), protease activated receptor (PAR2), stem cell factor (SCF), tumor necrosis factor (TNF), transforming growth factor (TGF), Picture from Harvima and Nilsson 2011.

For example, in the bladder, MCs are the major source of IL-10 which promotes a tolerogenic microenviroment and is important to suppress immunocytes during infection in order to permit long-term bacterial persistence (Chan et al. 2013). Similarly, MCs promote the release of IL-9 from T-regulatory cells which contributes to the peripheral tolerance in a mouse model of skin allografts (Lu et al. 2006). However, if MCs are strongly activated by MC secretagogue (compound 48/80) the allogenic graft is rejected due to a T-cell dependent immuno-response (de Vries et al. 2009b).

Therefore, as cells primed to rapidly secrete significant, proinflammatory 'danger' signals upon activation/degranulation, this role can quickly switch into a tolerance-breaking and potentially autoimmunity-promoting role, for example during allograft rejection and experimental autoimmune encephalomyelitis, rheumatoid arthritis, psoriasis, atopic dermatitis **(Figure 1.30** and **Table 1.12)** (Frenzel and Hermine 2013, Voehringer 2013, Brown and Hatfield 2012, Walker et al. 2012, Gilfillan and Beaven 2011, de Vries et al. 2009b, Sayed et al. 2008, Lu et al. 2006, Waldmann 2006).

Etiologies	Diseases
Autoimmune diseases	Rheumatoid arthritis, Sjögren's syndrome, lupus, scleroderma, dermatopolymyositis and polymyositis, pemphigus and pemphigoid, psoriasis
Chronic inflammatory diseases	Ankylosing spondylitis, Crohn's disease and ulcerative colitis, ANCA vasculitis, sarcoidosis
Neurological diseases	Multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, anxiety-depression syndrome, autism
Allergic diseases	Atopic dermatitis and eczema, urticarial, asthma
Malignancies	Systemic mast cell disease, Hodgkin and non-Hodgkin lymphomas, solid malignancies (lung, breast, colon and rectum, pancreas, skin)

Table 1.12: List of diseases in which mast cells are considerate to play a role. Table from Frenzel and Hermine 2013

1.5.3 Mast cells in autoimmune diseases

Recently, strong evidence has supported MCs make pathobiologically important contributions to the development of selected to autoimmune disorders such as multiple sclerosis, rheumatoid arthritis, type 1 diabetes and bullous penphigoid **(Figure 1.30)** (Christy et al. 2013, Frenzel and Hermine 2013, Anand et al. 2012, Brown and Hatfield 2012, Gan et al. 2012, Walker et al. 2012, Gilfillan and Beaven. 2011, Sayed et al. 2008).

In multiple sclerosis for example, MCs not only release preformed mediators such as tryptase, TNFα which contribute to target tissue destruction, but also are fundamental for maximal autoreactive T-cell responses and the recruitment of neutrophils which in turn recruit additional autoreactive T-cells and cause additive tissue damage, at least in the mouse model (Christy et al. 2013, Walker et al. 2012, Sayed et al. 2011, Sayed et al. 2010, Gregory et al. 2005).

In rheumatoid arthritis, the MC number is strongly up-regulated in the joint and the release of tryptase and cytokines (TNF, IL-1β, IL-17) is indirectly responsible of neutrophils chemotaxis and cartilage and bone destruction by activating synovial fibroblasts. Moreover, MC tryptase increases vascular permeability and inflammation in the joint (Christy et al. 2013, Kritas et al. 2013, Frenzel and Hermine 2013, Brown and Hatfield 2012, Shin et al. 2009).

The inhibition of MCs by cromolyn sodium in a rat model for type 1 diabetes delay the onset the disease (Geoffrey et al. 2006) and the specific inhibition of c-Kit only slightly limit the disease progression in NOD mice (Brown and Hatfield 2012, Shi et al. 2012, Louvet et al. 2008).

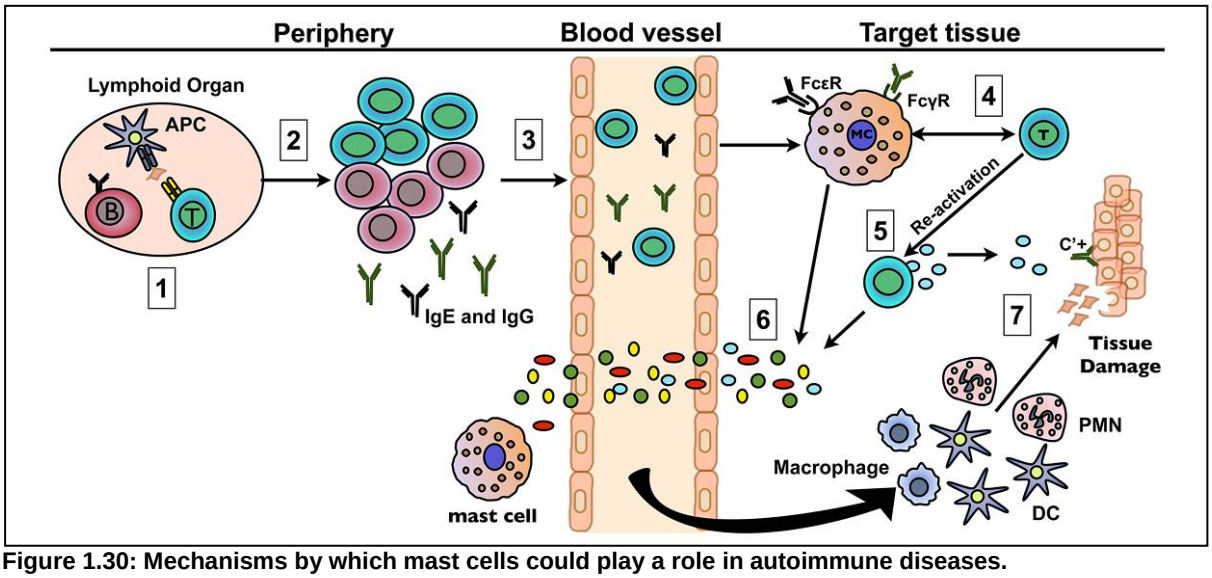

Figure 1.30: Mechanisms by which mast cells could play a role in autoimmune diseases.
MCs are able to cross-talk with autoreactive T and B cells, present the antigens, release mediators that increase vessels permeability and direct damage the tissue and recruit inflammatory cells thus amplifying the autoimmune response. Antigen-presenting cells (APC), dendritic cell (DC), polymorphonuclear leukocytes (PMN). Figure from Brown and Hatfield 2012.

However if MCs play a role in autoimmune disease is still controversy since in some animal experiments MCs revealed to be protective for the disease development (Gutierrez et al. 2014, Brown and Hatfield 2012).

1.5.4 Mast cells, hair follicles and alopecia areata

In mammalian skin, MCs are located in the dermal and subcutaneous tissue and are particularly prominent in the CTS of the HF **(Figure 1.5)** (Metz and Maurer 2009, Christoph et al. 2000), where they play a role in the regulation of HF cycling (Liu et al. 2013, Paus et al. 1998, Botchkarev et al. 1997, Maurer et al. 1997, Botchkarev et al. 1995, Maurer et al. 1995, Paus et al. 1994c, Moretti et al. 1967). Namely, after stimulation with the endogenous MC secretagogues, SP or corticotrophin-releasing hormone (Ito et al. 2010, Ito et al. 2005a, Maurer et al. 1997), anagen HFs can be induced to prematurely enter into the regression phase of HF cycling, i.e. catagen (Peters et al. 2007, Siebenhaar et al. 2007, Ito et al. 2005a, Maurer et al. 1997). This is also suggested by substantial fluctuations in the number, activity and histochemical staining characteristics of skin MCs during synchronized HF cycling (Paus et al. 1998, Botchkarev et al. 1997, Botchkarev et al. 1995, Moretti et al. 1967).

Moreover, the HF mesenchyme in human and murine skin harbours resident MC progenitor cells, from which fully functional, mature skin MCs can differentiate *in loco* (Sugawara et al. 2012, Ito et al. 2010, Kumamoto et al. 2003). In the HF CTS, MCs may collaborate to maintain the IP (Gilhar et al. 2012, Waldmann 2006, Maurer et al. 2003). Their action is considered to be connected to the release of immunosuppressive cytokines, in particularly, IL-10 and TGFβ, which may decrease the immune response indirectly by interacting with T-regulatory cells or CD8+ T lymphocytes (Stelekati et al. 2009, Sayed et al. 2008, Waldmann 2006, Maurer et al. 2003).

Several studies have reported an increase of the number of peribulbar and perivascular MCs in AA lesions (Cetin et al. 2009, D'Ovidio et al. 1988, Finzi and Landi 1964, Baccaredda-Boy and Giacometti 1959). However, other authors did not find any differences in AA skin compared to controls with respect to MC histochemistry (Spath and Steigleder 1970). Given this conflicting literature, recent published results generated during the Master´s thesis project of the current PhD candidate revealed that the number of immature and mature MCs is indeed strongly increased in AA, as well as the degranulation and the proliferation *in situ* around lesional HFs **(Figure 1.31)** (Bertolini et al. 2014, Bertolini et al. 2010a,b).

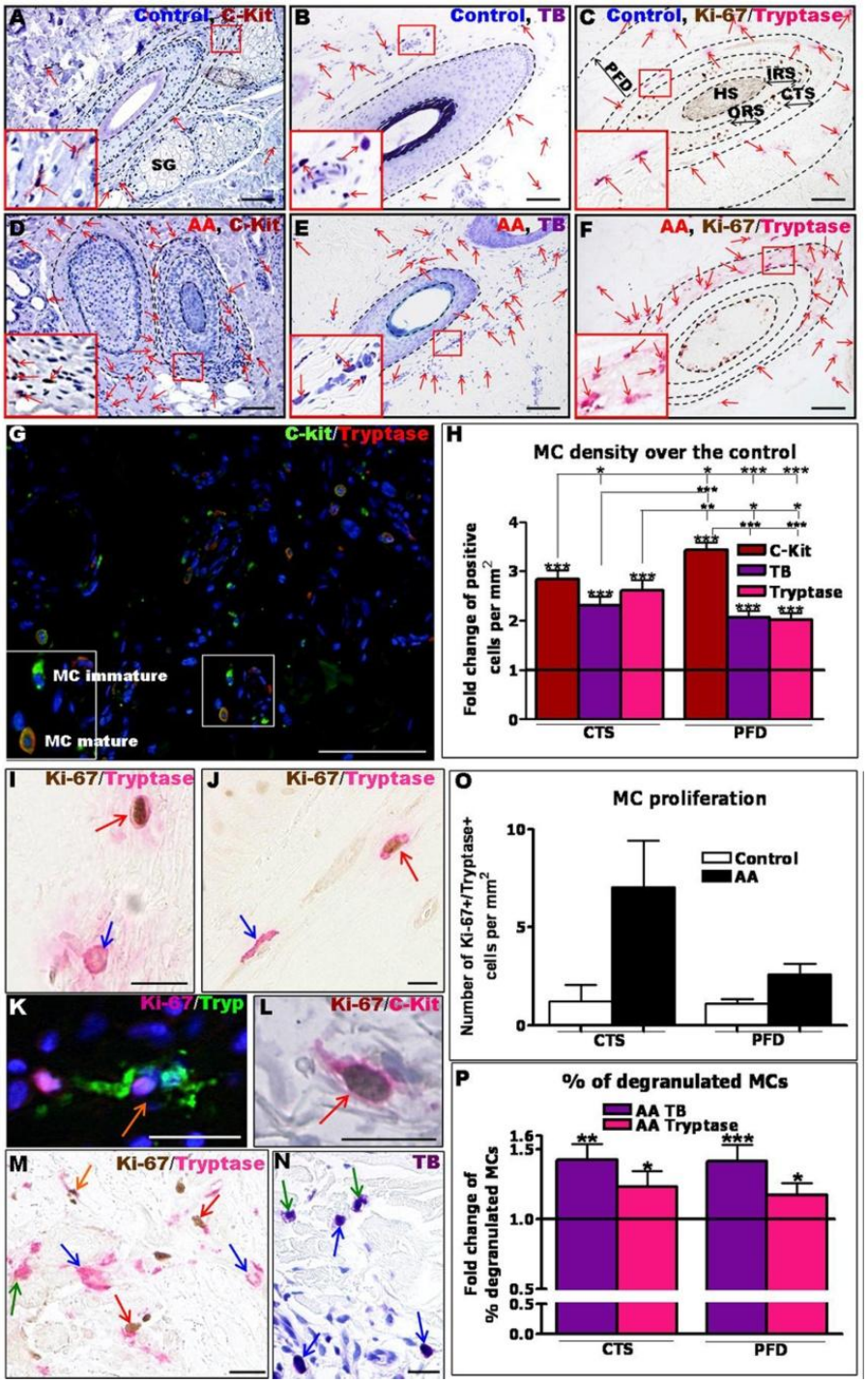

Figure 1.31: **Human alopecia areata lesions show increased density, proliferation and degranulation of perifollicular mast cells.** The immunohistochemical identification and evaluation of MCs by c-Kit (A,D), TB (B,E) or Ki-67/tryptase (C,F) revealed a strong increase of MC numbers in AA (D-F) compared to control healthy (A-C) skin. Red arrows indicate MCs. C-Kit/tryptase double-IF showing immature c-Kit+ MCs (stained in green) and mature c-Kit+/tryptase+ MCs in AA skin (stained in green and red) (G). See inserted panels in the bottom left of each Figure for higher magnification views of the area highlighted in the small boxes. Reference area for the quantitative analysis using (immuno-)histomorphometry for the cell counting in the connective tissue sheath (CTS) and perifollicular dermis (PFD). CTS+PFD represents the total area including the space demarcated up to 200µm from the HF basement membrane (C,F). Fold change of MC density detected by c-Kit, TB and tryptase (H). Black line indicates the control. Analysis derived from 69-81areas (HFs) of 11-17 AA patients and from 50-69 areas (HFs) of 5-7 healthy controls, ±SEM, *p≤0.05, **p≤0.01, ***p≤0.001, Mann-Whitney-U-Test or Student t-test (for c-Kit, TB and tryptase compared to respective controls and for comparing bars between CTS and PFD), Kruskal-Wallis test (p<0.0001) followed by Dunn's test (for comparing c-Kit, TB and tryptase within CTS and PFD). Immunohistochemical identification of MCs by Ki-67/tryptase IHC (I,J,M), Ki-67/tryptase IF (K), Ki-67/c-Kit (L) and TB (N) showing non-degranulating, non proliferating MCs (blue arrows), degranulating, non proliferating MCs (green arrows), non-degranulating MCs in proliferation (red arrows) and degranulating MCs in proliferation (orange arrows). Quantitative analysis of MC proliferation by Ki-67/Tryptase IHC (O). Analysis derived from 81 areas (HFs) of 17 AA patients and 50areas (HFs) of 7 healthy controls, ±SEM, Mann-Whitney-U-Test (ns).Quantitative analysis of MC degranulation by TB histochemisty and Ki-67/tryptase IHC (P). Black line indicates the control. Analysis derived from 69-81 areas (HFs) of 11-17 AA patients and 50-69 areas (HFs) of 5-7 healthy controls, ±SEM, *p≤0.05, **p≤0.05, ***p≤0.001 Mann-Whitney-U-Test (compare to control), Mann-Whitney test (TB compare to Tryp) (ns). Scale bars: 100µm (A-G) and 20 µm (I-N). Connective tissue sheath (CTS), hair shaft (HS), inner root sheath (IRS), outer root sheath (ORS), perifollicular dermis (PFD), toluidine blue (TB), sebaceous gland (SG). (Figure and legend derived from data generated during M. B.'s M.Sc. thesis project, included in Bertolini et al. 2014).

1.6 Mast cells and CD8+ T-cell interactions in autoimmune diseases

A co-localization of MCs with T-cells, including CD8+ T-cells, has been observed in several T-mediated inflammatory processes (Harries et al. 2013, Brown and Hatfield 2012, Gri et al. 2012). Moreover, MCs are able to activate an antigen specific MHC class I dependent CD8+ T-cells immune response **(Figure 1.32)** (Stelekati et al. 2009, Malaviya et al. 1996). MCs can co-stimulate CD8+ T-cells via molecular mediators and adhesion pathways **(Figure 1.33)** (Christy et al. 2013, Frenzel and Hermine 2013, Brown and Hatfield 2012, Gan et al. 2012, Gri et al. 2012, Walker et al. 2012, Gilfillan and Beaven 2011, Stelekati et al. 2009, Sayed et al. 2008).

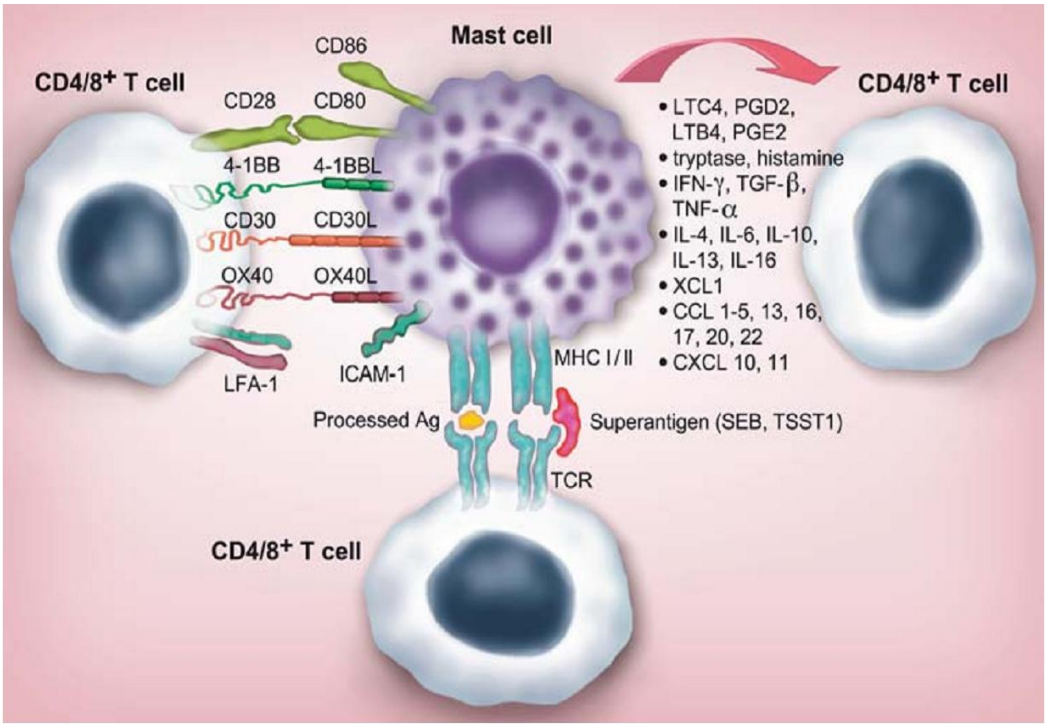

Figure 1.32: A selection of pro-inflammatory mediators and surface molecules involved in mast cell-CD8+ T-cell interactions.
MCs are able to present antigen via MHC class I to CD8+ T-cells and to costimulate them via OX40L, 4-1BBL, CD30L, ICAM-1, CD80 and granules-associated molecules such as histamines, tryptase and chymase. Moreover MCs release potent mediators which influence CD8+ T-cell activities. Interferon (IFN), interleukin (IL), intercellular adhesion molecule (ICAM), leukocyte functional associated antigen (LFA), leukotriene (LT), prostaglandine (PG), transforming growth factor (TGF), tumor necrosis factor (TNF). Picture from Harvima et al. 2008.

1.6.1 Mast cell mediators and CD8+ T-cells: A selection

Most MC mediators play a role in the recruitment, activation of CD8+ T-cells during inflammatory processes, however some of them are important to maintain homeostasis and to inhibit immuno cells such as CD8+ T-cells (Wernersson and Pejler 2014, Christy et al. 2013, Frenzel and Hermine 2013, Brown and Hatfield 2012, Gan et al. 2012, Gri et al. 2012, Walker et al. 2012, Gilfillan and Beaven 2011, Harvima et al. 2008, Sayed et al. 2008). Here below, a list of MC mediators able to modulate CD8+ T-cell functions:

- Histamine is an organic nitrogenous molecule that exerts its function by binding to four different receptors: H1, H2, H3 and H4, with both pro-inflammatory and immuno-inhibitory capacity **(Table 1.10)**. Among its complex functions, histamine contributes to the polarization of T-cell responses (Galli et al. 2005) and can stimulate the production of INFγ by some CD8+ T-cells **(Figure 1.32)** (Hanzlikova et al. 2012).

- Tryptase is a trypsin-like protease stored together with heparin within MCs, whose functions are mostly mediated mostly by signalling via the protease-activated receptor (PAR)-2 receptor (Wernersson and Pejler 2014, Gri et al. 2012, Harvima & Nilsson 2011, Sayed et al. 2008, Shin et al. 2009) and by activating other proteases such as collagenases (Magarinos et al. 2013, Sayed et al. 2008). Since PAR-2 is also expressed by MCs, it is also possibly a paracrine activator of MCs (Wernersson and Pejler 2014, Hernandez-Hernandez et al. 2012, Harvima and Nilsson 2011, Dvorak 2005). Apart from providing protection against several pathogens (St. John and Abraham 2013, Gri et al. 2012, Pejler et al. 2010), controlled "MC-directed collagenolysis" (Hammel et al. 2010, Krejci-Papa and Paus 1998), i.e. activation of procollagenase, tryptase activates keratinocytes (Kempkes et al. 2014, Harvima and Nilsson 2011, Steinhoff et al. 1999), elicits action potentials in sensory skin nerves (Harvima and Nilsson 2011, Lohi et al. 1992) **(Table 1.10)**, and activates and recruits T-cells **(Figure 1.28)** (Harvima and Nilsson 2011, Spinnler et al. 2010, Li and He 2006).

- Chymase, a chymotryptic serine proteases, is fundamental in the degradation of eotaxin and neuropeptides **(Table 1.10)** but it is also able to recruit eosinophils and neutrophils *in vivo* and to stimulate inflammatory cells including T-cells *in vitro* **(Figure 1.28)** (Harvima and Nilsson 2011).

- The release of leukotriene B_4 by MCs **(Table 1.10)** contributes to the chemotaxis of effector CD8+ T-cells in target tissues affected by allergic disease, acting on high-affinity BLT1 leukotriene receptor (Ott et al. 2003). This receptor on CD8+ T-cells seems to be fundamental for the migration of CD8+ T-cells in tumour (Sharma et al. 2013) and CD8+ T-cell mediated colitis **(Figure 1.31)** (Nancey et al. 2011).

- IL-10 is a pleiotropic cytokine which is secreted from MCs during cross-talk with other cells **(Table 1.10)** (Soyer et al. 2013, Mosser and Zhang 2008, Groux et al. 1998). It exerts mostly immunosuppressive functions, such as the inhibition of T-cells (Rowbottom et al. 1999) or the maintenance of peripheral tolerance, supporting regulatory T-cells (Chan et al. 2013, Gan et al. 2012, Gri et al. 2012, Chacon-Salinas et al. 2011, Harvima and Nilsson 2011, de Vries et

al. 2009b, Lu et al. 2006). However, under certain circumstances and in defined microenvironments, IL-10 can also activate natural killer and cytotoxic CD8+ T-cells **(Figure 1.28)** (Hofmann et al. 2012).

- TGFβ-1 **(Table 1.10)**, is a potent IP guardian which is also able to inhibit CD8+ T-cells and to reduce their proliferation, cytolitic activity, differentiation, and acquisition of effector molecules. Moreover, TGFβ-1 is a fundamental factor for the differentiation of regulatory T-cells **(Figure 1.28)** (Oh and Li 2013, Wahl et al. 2006).

1.6.2 Co-stimulatory molecules for CD8+ T-cells expressed on mast cells

MCs on their surface may express after appropriate stimuli, an array of co-stimulatory molecules, including OX40L, CD30L, 4-1BBL, ICAM-1 which have been shown to modulate MC-CD8+ T-cell interaction (Frenzel and Hermine 2013, Brown and Hatfield 2012, Gri et al. 2012, Harvima and Nilsson 2011, Harvima et al. 2008, Sayed et al. 2008, Galli et al. 2008, Stelekati et al. 2009). Here below, a list of MC co-stimulatory molecules able to influence CD8+ T-cell functions:

- OX40L (syn CD252, TNFSF4, gp34) **(Table 1.11)** is a type II transmembrane and soluble glycoprotein express after activation, which binds OX40 during cell contact. The receptor OX40 it is not expressed on naïve or memory T-cells but it is transiently expressed after antigen presentation (Ilves and Harvima 2013, Zhang et al. 2013b, Weinberg et al. 2011, Croft 2010, Ishii et al. 2010, Kober et al. 2008, Nakae et al. 2006, Kashiwakura et al. 2004). The expression of OX40L on MCs seems to be induced by GM-CSF, IL-1 and TNF and to promote the expansion, survival and cytokine production in CD8+ T-cells (Croft 2014, Croft 2010, Nakae et al. 2006, Kashiwakura et al. 2004). It was shown that the binding of OX40L+ MCs with OX40 regulatory T-cells, which could be also CD8+, is fundamental for regulatory T-cell-dependent peripheral tolerance in skin allografting (Frossi et al. 2010).

- CD30L (syn: CD153, TNFSF8) **(Table 1.11)** depending on the environment exerts several pleiotropic biological effect on T-cells, including proliferation, activation, differentiation and cell death. Recently, it was shown that not only CD4+ T-cells but also CD8+ T-cells can express CD30 (Croft 2014, Cabrera et al. 2013, Zhang et al. 2013a, Kober et al. 2008, Horie and Watanabe 1998, Gruss et al. 1996).

- 4-1BBL (syn.: CD137L, TNFSF9) **(Table 1.10)** modulates the antigen presentation by MCs to CD8+ T-cells (Stelekati et al. 2009). In fact, 4-1BB can be express on T-cells (apart resting T-cells) but is induced more on CD8+ T-cells compared to other T-cells. The binding 4-1BBL/4-1BB mediates the survival, activation, expansion, up-regulation of effector molecules

(perforin, granzyme) in CD8+ T-cells and promote Th1 response (Croft 2014, Chacon et al. 2013, Vinay and Kwon 2012, Shao and Schwarz 2011, Wu et al. 2011, Wang et al. 2009, Kober et al. 2008, Watts 2005).

- The interaction between ICAM-1 **(Table 1.11)** and LFA-1 is well known to mediate the immunological synapse during antigen presentation from APCs to T-cells (Springer and Dustin 2012), and it is also the first signaling pathway which was found to mediate MC-T-cell interactions (Gri et al. 2012). However, the role of ICAM-1/LFA-1 signaling in MC-CD8+ T-cell interactions is as yet insufficiently characterized. In fact, most of the studies consider only the effect of activated T-cells to MCs (Nagai et al. 2009, Brill et al. 2004, Inamura et al. 1998). Only in rare case, like in Skokos et al. (2001), the scenario is seen the other way around, in fact exosomes containing also ICAM-1 and derived from mouse bone marrow-derived MCs promote T-cell proliferation and cytokine production (Skokos et al. 2001, Galli et al. 2005). Since ICAM-1/LFA-1 interactions are likely to play an important role in the HF immune system under physiological and pathological circumstances (Joachim et al. 2008, Müller-Röver et al. 2000, Eichmüller et al. 1998, Limat et al. 1994) and have also been implicated in the context of AA pathobiology (Ghersetich et al. 1996, Zhang and Oliver 1994, Mcdonagh et al. 1993), the role of ICAM-1/LFA-1 signaling in MC-CD8+ T-cell interactions during AA may be of particular relevance.

1.6.3 Inhibitory molecules for CD8+ T-cells expressed on mast cells

Vice versa, the MC surface also expresses immuno-inhibitory receptors important for the maintenance of peripheral tolerance and the inhibition of CD8+ T-cells (Chan et al. 2013, Voehringer 2013, Gan et al. 2012, Kalesnikoff and Galli 2011, Tsai et al. 2011, Frossi et al. 2010, de Vries et al. 2009a, Sayed et al. 2008, Lu et al. 2006, Waldmann 2006). The most important is PD-L1 (syn: B7-H1, CD274) **(Table 1.11)**, which is a type I transmembrane protein. PD-L1 seems to be implicated also in HF-IP maintenance (Wang et al. 2014). It delivers an inhibitory signal through its receptor on T-cells (PD-1), inhibiting cytokine production and proliferation while stimulating T-cell death (Podojil and Miller 2013, Saresella et al. 2012, Wu et al. 2012, Keir et al. 2008). However, the expression of PD-L1 has so far only been investigated in mouse bone marrow-derived MCs (Nakae et al. 2006), therefore it would be interesting to study this receptor in human MCs *in situ* and in the context of AA pathogenesis.

1.7. Selected open questions in the field

If one accepts the concept that AA is an organ-restricted CD8+ T-cell dependent autoimmune disease during which a collapse of the HF's IP plays a critical role (Paus et al. 1993, Gilhar et al. 2012, McElwee et al. 2013), many important questions remain unsolved. These include, for example: 1) What exactly stimulates the HF-IP collapse in AA? 2) Which T-cells are the autoreactive in AA? 3) What is (are) the auto-antigen(s) in AA? 4) What is the role of autoantibodies in AA? 5) What is the role of the innate immune system in AA, namely the role of MCs, NK and NKT-cells and the endogenous agonists of NKG2D such as MICA, ULBP3? 6) What is the role of regulatory T-cells?

Among these multiple open questions, the current thesis project focuses on the potential role of MCs in AA, given that a) MCs are up-regulated in AA lesion (Bertolini et al. 2014, Bertolini et al. 2010a,b, Cetin et al. 2009, D'Ovidio et al. 1988, Finzi and Landi 1964, Baccaredda-Boy and Giacometti 1959), b) that they are able to co-stimulate CD8+ T-cells (Christy et al. 2013, Frenzel and Hermine 2013, Brown and Hatfield 2012, Gan et al. 2012, Gri et al. 2012, Walker et al. 2012, Gilfillan and Beaven. 2011, Harvima et al. 2008, Sayed et al. 2008), and c) can even present specific antigens to CD8+ T-lymphocytes (Stelekati et al. 2009), the accepted key effector lymphocytes in AA pathogenesis (McElwee et al. 2013, Gilhar et al. 2012, McElwee et al. 2005, Gilhar et al. 1998, McElwee et al. 1996). Therefore the main research challenge in this context is to systematically explore the role of MC-CD8+ T-cell interactions in human AA pathobiology *in situ.*

1.8. Working hypothesis

The following working hypothesis regarding MC and CD8+ T-cell interactions was formulated so as to guide the experimental design chosen for this thesis project: in physiological state, MCs around the HFs interact with CD8+ T-cells in order to preserve the HF-IP and peripheral tolerance; instead, under pathological conditions, such as in AA, MCs switch to pro-inflammatory phenotype and activate pathogenic CD8+ T-cells as well as autoimmunity-promoting MC-CD8+ T-cell interactions. Taken together, this causes HF-IP collapse and breaks the peripheral tolerance to HF-associated autoantigens, which in turn results in the development of AA **(Figure 1.34)**.

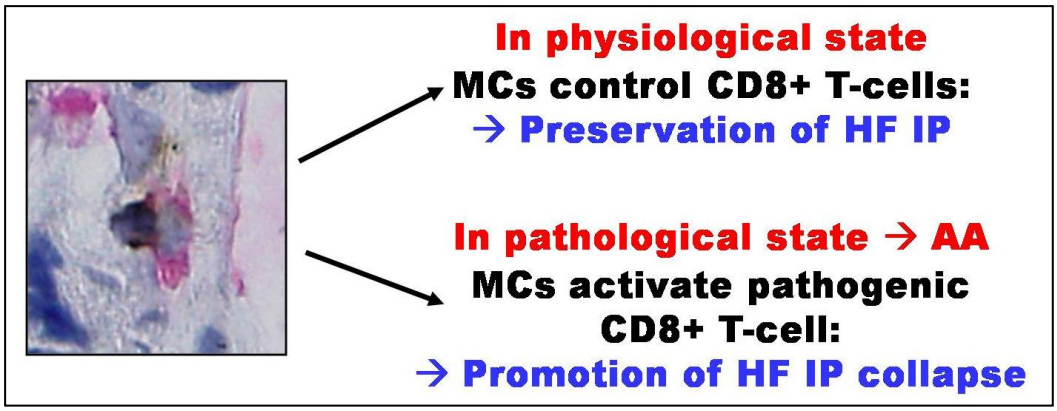

Figure 1.34: Overall hypothesis: Mast cell-CD8+ T-cell interactions in physiological hair follicle-immune privilege versus alopecia areata.
In the picture tryptase+ MC (pink) interacting with CD8+ T-cell (brown).

1.9. Overall aim and specific questions addressed

The overall aim of the current project was: **to dissect the role of MC and CD8+ T-cell interactions in AA pathogenesis.**

With this overarching aim in mind, the following specific questions were addressed:

1) Do MCs switch to a pro-inflammatory phenotype in AA?

2) Do MCs interact with CD8+ T-cells in AA?

3) Are the observed interactions between MCs and CD8+ T-cells likely to be pro-inflammatory or immuno-inhibitory?

4) Is it possible to investigate and modulate MC-CD8+ T-cell interactions *ex-vivo*?

5) Do abnormal MCs interact with CD8+ T-cells *in vivo* in AA lesions from animal models for AA?

The specific questions addressed in each set of experiments in this thesis project are summarized in **Table 1.13**.

Model	*Question addressed*	*Investigated read-out parameters*
Lesional and non-lesional skin from AA patients versus healthy skin	**Do MCs switch to a pro-inflammatory phenotype in AA?**	Evaluation of TGFβ1 and tryptase contents within MCs using TGFβ1/C-Kit and Ki-67/Tryptase stainings. Evaluation of MC number positive for OX40L, CD30L, 4-1BBL, ICAM-1, IL-10, PD-L1, CD200 using corresponding triple-staining.
	Do MCs interact with CD8+ T-cells in AA?	Evaluation of MC number in close contact with CD8+ T-cells using CD8/Tryptase and CD8/C-Kit
	Are the observed interactions between MCs and CD8+ T-cells likely to be pro-inflammatory or immuno-inhibitory?	Evaluation of MC degranulation and expression MHC class I, OX40L, CD30L, 4-1BBL, ICAM-1, IL-10, PD-L1 CD200 when in close contact with CD8+ T-cells using corresponding double- and triple-staining.
Hair follicle organ culture	**Is the HF organ culture suitable for investigating MC-CD8+ T-cell interactions after MC secretagogue treatment?**	Evaluation of MC number in close contact with CD8+ T-cells using CD8/Tryptase and C-Kit/CD8 immunostainings in SP and compound 48/80 treated HFs
Full-thickness healthy skin organ culture	**Do perifollicular MCs and their activities increase in full-thickness healthy skin organ-culture after MC secretagogue treatment?**	Evaluation of MC number using C-Kit/Ki-67 and Ki-67/Tryptase stainings. Evaluation of MC degranulation using Ki-67/Tryptase stainings. Evaluation of MC proliferation using Ki-67/Tryptase, C-Kit/tryptase and Ki-67/C-Kit and HF IP collapse using MHC class I and TGFβ1 stainings in SP and compound 48/80 treated healthy skin
	Is the full-thickness healthy skin organ-culture suitable to investigate MC-CD8+ T-cell interactions after MC secretagogue treatment?	Evaluation of MC number in close contact with CD8+ T-cells using CD8/Tryptase in SP and compound 48/80 treated healthy skin
Full-thickness AA skin organ culture	**Do perifollicular MCs and their activities increase in full-thickness AA skin organ-culture after MC secretagogue and stabilizer treatment?**	Evaluation of MC number using C-Kit and Ki-67/Tryptase stainings. Evaluation of MC degranulation using Ki-67/Tryptase stainings. Evaluation of MC proliferation using Ki-67/Tryptase, C-Kit/tryptase and Ki-67/C-Kit stainings in SP, compound 48/80, cromoglycate treated AA skin
	Is the full-thickness AA skin organ-culture suitable to investigate MC-CD8+ T-cell interactions after MC secretagogue and stabilizer treatment?	Evaluation of MC number in close contact with CD8+ T-cells using CD8/Tryptase in SP, compound 48/80, cromoglycate treated AA skin
C3H/HeJ mice	**Does the subcutaneous injection with IFNγ accelerate the spontaneous development of AA in C3H/HeJ mice?**	Determinate the first episode of AA in vehicle and INFγ treated C3H/HeJ mice
	Are C3H/HeJ mice subcutaneously injected with IFNγ suitable for investigating MC-CD8+ T-cell interactions?	Evaluation of MC number in close contact with CD8+ T-cells using CD8/C-Kit in AA affected skin?
Grafted C3H/HeJ mice	**Do perifollicular MCs and their activities increase in AA affected mice?**	Evaluation of MC number using C-Kit/CD8 and mMCP6/CD8, stainings. Evaluation of MC degranulation using mMCP6/CD8 staining.
	Are MCs and MC-CD8+ T-cell interactions also abnormal in the best-established AA mouse model?	Evaluation of MC number in close contact with CD8+ T-cells using mMCP6/CD8 and C-Kit/CD8 stainings.
Humanized-mouse model of AA	**Can key findings made in the skin of AA patients with respect to excessive MC number/activities and MC-CD8+ T-cell interactions be reproduced in experimentally induced AA-like lesions in previously healthy human skin?**	Evaluation of MC number using c-Kit and Tryptase/CD8, stainings. Evaluation of MC number degranulation using tryptase/CD8 staining. Evaluation of MC number in close contact with CD8+ T-cells using tryptase/CD8 staining.

Table 1.13: List of models used, question addressed formulated and investigated read-out parameters evaluated in the current project.
Underline in light blue the *in situ* analyses performed on lesional and non-lesional skin section from AA patients and control skin sections from healthy individuals, in pink *ex-vivo* analyses performed in HF or full thickness skin organ culture and in green *in-vivo* analyses performed in C3H/HeJ mice and human skin transplanted on SCID mice. Intercellular adhesion molecule (ICAM), programmed death-ligand (PD), transforming growth factor (TGF).

1.10. Experimental Design

To dissect the role of MC-CD8+ T-cell interactions in AA pathogenesis, in the current project, several complementary models were employed **(Table 1.2)** to overcome the frustrating fact the MC cannot be strictly selectively depleted or modulated neither in human or in mouse skin (see Discussion for details).

MC and CD8+ T-cell interactions were first investigated in AA lesional skin from AA patients compared to non-lesional skin and to control skin from healthy human subjects. Here, the MC phenotype and number of MC-CD8+ T-cell interactions were evaluated by employing single-, double-, and triple- immunostainings for several MC markers known to take part in MC-CD8+ T-cell interactions (tryptase, TGFβ1, OX40L, CD30L, 4-1BBL, ICAM-1, IL-10, PD-L1 and CD200) **(Table 1.12)**. These were evaluated by quantitative (immuno-)histomorphometry (Bertolini et al. 2013, Kloepper et al. 2013, Harries et al. 2013, Meyer et al. 2008).

As a first attempt towards establishing an *ex-vivo* model in which MC-CD8+ T-cell interactions can be studied and experimentally manipulated, human scalp HF organ culture was performed in which HFs were treated with SP and compound 48/80. The number of MCs and CD8+ T-cell interactions were evaluated using immunofluorescent double-stainings **(Table 1.12)**. In addition, organ cultures of full-thickness human healthy skin were carried out treated with endogenous or exogenous MC secretagogues (SP or compound 48/80). The number of MCs and CD8+ T-cell interactions were evaluated using double-immunohistochemistry techniques and HF-IP was assessed by quantitative immuno-histomorphometry **(Table 1.12)**. This was complemented by full-thickness organ culture from a patient with long-standing AA who underwent cosmetic facelift surgery (4mm punches were treated with SP, compound 48/80, or the MC stabilizer, cromoglycate). The number of MCs and CD8+ T-cell interactions were evaluated using double-immunohistochemistry techniques **(Table 1.12)**.

Finally, MC-CD8+ T-cell interactions were also evaluated *in vivo*, namely in C3H/HeJ mice injected subcutaneously with INFγ, in grafted C3H/HeJ mice, and in a newly established humanized-mouse model for AA **(Table 1.12)**.

2 Material and Methods

2.1 Human tissue collection

All experiments on human tissue were performed according to Helsinki guidelines, and after written patient consent (where applicable) and ethics committee approval.

2.1.1 Human healthy tissue collection from plastic surgery clinics

Clinically “healthy” frontotemporal human scalp skin samples were obtained from 23 women without a record of AA (mean age: 55 years) undergoing cosmetic facelift surgery after ethics committee approval (University of Lübeck, n. 06-109, 18-07-06) and written patient consent (Vidali et al. 2014, Ernst et al. 2013, Harries et al. 2013, Ramot et al. 2013, Kinori et al. 2012). The skin samples, stored in William's E medium (Biochrom, Cambridge, United Kingdom), were shipped by collaborating plastic surgeons overnight at 4°C to the laboratory and was used within 24 hours after extraction **(Figure 2.1)**.

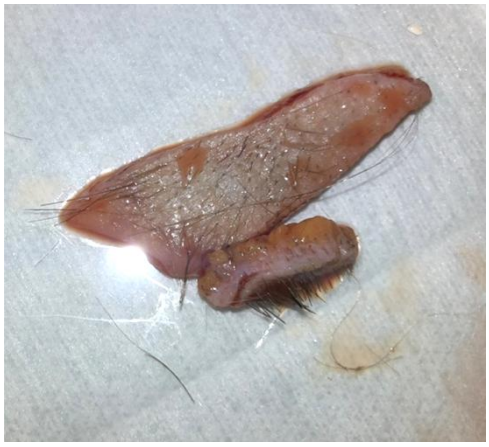

Figure 2.1: Human scalp skin samples received from collaborating plastic surgeons.

If the skin was used as control for the (immuno-)histomorphometry analyses on AA patients, small skin pieces were cut out from the skin samples and fixed in 10% formalin (Merck, Darmstadt, Germany) at least for 24 hours. After proper fixation steps in alcohol solutions done at the histology laboratory at the department of dermatology, University of Lübeck, the skin pieces were embedded in paraffin (Leica Microsystems, Wetzlar, Germany) **(Table 2.1)**.

Patient number	Sex	Age	Scalp skin region
HS 173	Female	59	Temporal-Frontal
HS 340	Female	56	Temporal-Frontal
HS 343	Female	40	Temporal
HS 507	Female	35	Frontal
HS 08-022	Female	60	Occipital
HS 08-186	Female	67	Occipital-Temporal
HS 09-020	Female	54	Occipital
HS 09-024	Female	61	Temporal
HS 09-033	Female	53	Temporal
HS10-037	Female	56	Temporal
HS 10-097	Female	59	Frontal
HS10-100	Female	70	Temporal
HS11-070	Female	66	Temporal
HS 11-116	Female	66	Occipital-Temporal
HS 12-022	Female	19	Temporal
HS 12-031	Female	59	Temporal
HS 12-078	Female	50	Temporal
HS 12-089	Female	52	Occipital-Temporal
HS 12-094	Female	40	Temporal
HS 12-098	Female	58	Temporal

Table 2.1: Details of the human skin samples used as control for (immuno-)histomorphometry analysis on alopecia areata patients. Human skin (HS).

If the skin was used for HF or full-thickness human healthy skin organ culture, it was processed as explain below **(Table 2.2)**.

Patient number	Organ culture number	Sex	Age	Scalp skin region
HS 10-100	HS 10-089	Female	70	Temporal
HS 11-015	HF 11-020	Female	52	Temporal
HS 11-095	HS 11-123	Female	41	Temporal
HS 12-129	HS 12-136	Female	57	Temporal

Table 2.2: Details of the human skin samples used either for hair follicles or full-thickness human healthy skin organ culture. Hair follicle (HF), human skin (HS).

2.1.2 Alopecia areata samples, University of Lübeck, Lübeck

25 human lesional scalp skin biopsies from AA patients were obtained from archival paraffin blocks (up to 10 years old) from biopsies that had been taken exclusively for diagnostic purposes, filed in the Dermatopathology Paraffin Block Collection, Dept. of Dermatology University of Lübeck after ethics committee approval (University of Lübeck, n. 13-007, 13-03-13) **(Table 2.3)**. In this case, it was not possible to obtain the written consent forms from all patients because only several of them were still traceable after such a long period (pseudoanonymous use of these tissue blocks without formal written consent was, therefore, approved by the ethics committee). These human skin biopsies had been fixed in formalin and had been processed for paraffin embedding as describe above.

Patient number	Sex	Age	Clinical evaluation and histological features
05-14975	Female	36	Many HFs, all in catagen with few lymphicytic infiltrate, no mucous, maybe pharmaceutical induced (remicad/infliximab)
94-1781	Female		
98-10264	Female	57	Perifollicular lymphocytic infiltrate with some eosinophils, reduced number of HFs + scalp eczema (slight parakeratosis, irregular acanthosis of the epidermis, focal spongiosis)
97-4642	Male	57	Follicle in corium massive reduced, catagen or atrophic, rare deeper HFs with dense lymphocytic infiltrates, no distinct scarring
96-9417	Male	21	A lot of mucous, lymphohistocytic infiltrate also around the hair bulbs, more catagen HF, collagenous strands around the hair bulbs
96-2473	Female	59	Predominantly orthokeratosis, edematous, interstitial cell infiltrate with mucous, dilatated bloodvessels, a little granulomatous infiltrate around the hair bulbs in the subcutis, partially exocytosis in follicular epithelium, partially fibrotic strands in subcutis (=destroyed HFs). Early, inflammatory AA
96-11655	Female	36	Small number of HFs in the dermis, follicle with lymphohistiocytic infiltrate around the bulb, in upper part of the subcutis only follicle residues with a dense infiltrate
93-9377	Female	57	Only slight lymphohistiocytic infiltrate, non inflammatory active AA. Mild AA?
95-16885	Female	38	Lymphocytic inflammatory infiltrates, many catagen/telogen HF, atrophic HF. Strong/definite AA
97-6245	Male	35	Early disease, many HFs visible, perifollicular infiltrates. Early mild AA
96-10392	Male	34	Lymphocytic infiltrates around the bulbs, which partially invade into the follicle epithelium
96-3492	Male	15	In the corium only small HFs, in the deep corium very small anagen HFs with a lymphocytic infiltrate around the hair bulb
96-14982	Male	28	Perivasculary lymphohistiocytic infiltrate, partially many eosinophils, Lymphocytic infiltrates around the bulbs, which partially invade into the follicle epithelium
04-10186	Female	72	Lymphocytic infiltrate around anagen HFs, infundibula inconspicuous, no scarring alopecia, interfollicular epptihelium inconspicuous
07-15881	Female	48	Massive lymphocytic infiltrate around hair bulb + eosinophils, hyperparakeratosis. Active, inflammatory AA
08-5728	Female	51	Many HFs, lymphocytic infiltrate around hair bulb, no scarring
97-12930	Female	35	In lower and deeper parts + subcutis many HFs, surrounded by perifollicular lymphocytic infiltrates. Probably early AA
98-14080	Female	70	Kerathosis in the epidermis, in the dermis the collagen fibers increasing the content of mucin. HF number is not reduced and the HB are in the subcutis. Lymphocytic infiltrate around hair bulb and the HF structure has clear degeneration (hair matrix, hair shaft)
04-20338	Male	59	Perifollicularis lymphocytic infiltrate and next to it atrophy. HF epithelium is unaffected.
08-5312	Female	49	Kerathosis and atrophy in the epidermis, HF with rudimental hair shaft and moderate hyperkeratosis. Hair bulb is found in the reticular dermis and you can find some fibrotic rest of HF in the subcutis. Perifollicularis lymphocytic infiltrate, also inside the epithelium. Angiogenesis. No degeneration of basement membrane.
07-14504	Female	41	Hair bulbs only in dermis, peribulbar lymphocytic infiltrate, unaffected epidermis
07-12896	Female	47	The HFs are at the same level and with perifollicular lymphocyte infiltrate. Melanin clumping
05-16335	Female	20	No change in the HF density. The epidermis appears normal. In the upper dermis and adipose tissue reveals a lymphocytic inflammatory infiltrate which is going to the hair bulb. No scarring
03-669	Male	13	Inconspicuous epi. The HFs are small, high standing and surrounded in the depth of a sparse lymphocytic infiltrate. They do not contain hair shafts. Collagen downright largely, no evidence of scarring. PAS: the basement membrane appears normal, no evidence of the suspected fungal infection elsewhere (Candida).The finding corresponds to the late stage of AA, no significant acute inflammation, no scarring alopecia.
07-6996	Female	44	Many HB in the dermis + lymphocyte infiltrate. No fibrosis.

Table 2.3: Details of archival human lesional alopecia areata skin samples obtained from the Dept. of Dermatology, University of Lübeck, Germany. Alopecia areata (AA), Hair follicle (HF).

2.1.3 Alopecia areata samples, University “La Sapienza”, Rome, Italy

Human lesional and non-lesional scalp skin biopsies (4-6mm) were obtained from 7 AA patients (n= 7, lesional skin, n= 4, non lesional skin) by Prof. Alfredo Rossi at the University "La Sapienza", Rome, Italy, after written patient consent and approval of the ethical committee (n. 2973,28-11-13), University “La Sapienza” of Rome.

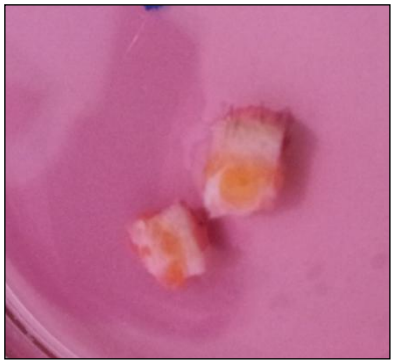

Figure 2.2: Human alopecia areata lesional skin punch biopsy cut into two pieces, one for embedding in cryomatrix and one for embedding in paraffin.

The skin pieces were delivered to the laboratory overnight by courier in William's E medium at 4 °C. After arrival, the skin punches were cut into pieces **(Figure 2.2)**, and one fragment was fixed in Shandon Cryomatrix (Pittsburgh, USA) and frozen in N_2 while the second fragment was fixed in formalin and embedded in paraffin. Only paraffin embedded skin was used for the (immuno-)histomorphometry analyses reported in this thesis **(Table 2.4)**.

Patient number	Sex	Age	Clinical evaluation and histological features
1	Female	26	>50% affected scalp, bad prognosis
2#	Male	36	<50% affected scalp, reticolar, bad progosis
3#	Male	71	<50% affected scalp, multiple patches, bad prognosis
5	Male	23	>50% affected scalp, universalis, bad prognosis
7#	Male	45	<50% affected scalp, universalis, bad prognosis
8#	Female	59	<50% affected scalp, 1 patch, bad prognosis
10	Female	37	<50% affected scalp, ophiasis, bad prognosis

Table 2.4: Details of the human lesional alopecia areata skin biopsies obtained from Prof. Alfredo Rossi, University "La Sapienza", Rome, Italy. # indicates that from this patient lesional and non-lesional skin punches were obtained.

2.1.4 Alopecia areata sample, Klinik Dr. Koslowski, Munich

One of the collaborating surgeons (Dr. Wolfgang Funk, Klinik Dr. Koslowiski, Munich) contributed a unique cosmetic facelift surgery skin sample from a female patient (age 67) with long-standing AA (duration: 43 years). After receiving the fresh skin samples (in William’s E medium, 4 °C, transported over night), 4mm punches were taken with a standard 4 mm skin punch (pfm medical, Cologne, Germany). These AA skin fragments were

processed for full-thickness serum-free skin organ culture, following our basic protocol (Langan et al. 2013, Lu et al. 2007) (see below).

2.1.5 Tissue samples taken as positive control for immunohistology, University of Lübeck, Lübeck

As positive control tissues for different immunostaining protocols, human tonsil and placenta tissue samples fixed in 10% of formalin and embedded in paraffin were obtained from the Dept. of Pathology, University of Lübeck, with ethics approval (n. 06-109, 18.07.06).

2.2 Organ cultures

2.2.1 Hair follicle organ culture

Human scalp skin received by plastic surgeons **(Table 2.2** and **Figure 2.3A,B)**, was cut into 0.5 cm x 1 cm thin strips **(Figure 2.3C)**. The epidermis and part of the dermis were cut off with a scalpel blade **(Figure 2.3D)**. The HF bulge region and SGs were cut off as well. After that, intact HFs were dissected from the skin under a binocular microscope **(Figure 2.3E,F)**. A careful check of the HFs followed this procedure, to ensure that the HFs were not in catagen and that they were surrounded by their full corresponding CTS (Kinori et al. 2011, Kloepper et al. 2010).

36 HFs were then placed in groups of three in a 24-well plate (Greiner Bio One, Wemmel, Belgium) with 500µl of supplemented William's E medium, containing 2mmol/L L-glutamine (Invitrogen, Paisley, UK), 10µg/ml insulin (Sigma-Aldrich, Taufkirchen, Germany), 10ng/ml hydrocortisone (Sigma Aldrich) and 1% antibiotic/antimycotic mixture (100x Gibco, Karlsruhe, Germany) **(Figure 2.3G,H)**. The HFs were incubated in Hera cell incubator (Heraeus, Kendro, Artisan Scientific Corporation, Champaign, United States) at 37°C with an atmosphere of 5% CO_2 and 95% air. After one day of preincubation in order to allow the stabilization of the system after the trauma of surgery and HF microdissection, the medium was changed, and treatment with the test substances was initiated (Vidali et al. 2014, Kinori et al. 2012, Kinori et al. 2011, Kloepper et al. 2010, Peters et al. 2007). In this assay, HFs were incubated for 24 hours either with only supplemented William's E medium (vehicle) or supplemented William's E medium added of 10^{-8}M or 10^{-10}M SP (Sigma-Aldrich) or 5µg/ml compound 48/80 (Sigma-Aldrich) (Sugawara et al. 2013, Peters et al. 2007). The concentrations of SP were chosen following previous obtained results in our laboratory, in fact 10^{-8}M and 10^{-10}M SP treatments were shown to stimulate MC degranulation in HF culture (Sugawara et al. 2013, Peters et al. 2007). Regarding the concentration for compound 48/80, it was shown by Kambe and colleagues that skin-derived MCs respond and degranulate in

culture in the same way after the treatment from 3 to 100 µg/ml of compound 48/80 (Kambe et al. 2008). Therefore, in order to keep the concentration of coumpound 48/80 as low as possible but considering that in this experiment the HFs were treated and not isolated cells, the concentration of 5µg/ml was chosen. Finally, the HFs were fixed in cryomatrix and frozen in liquid nitrogen and stored at -80°C (Kinori et al. 2012, Kinori et al. 2011, Kloepper et al. 2010, Peters et al. 2007).

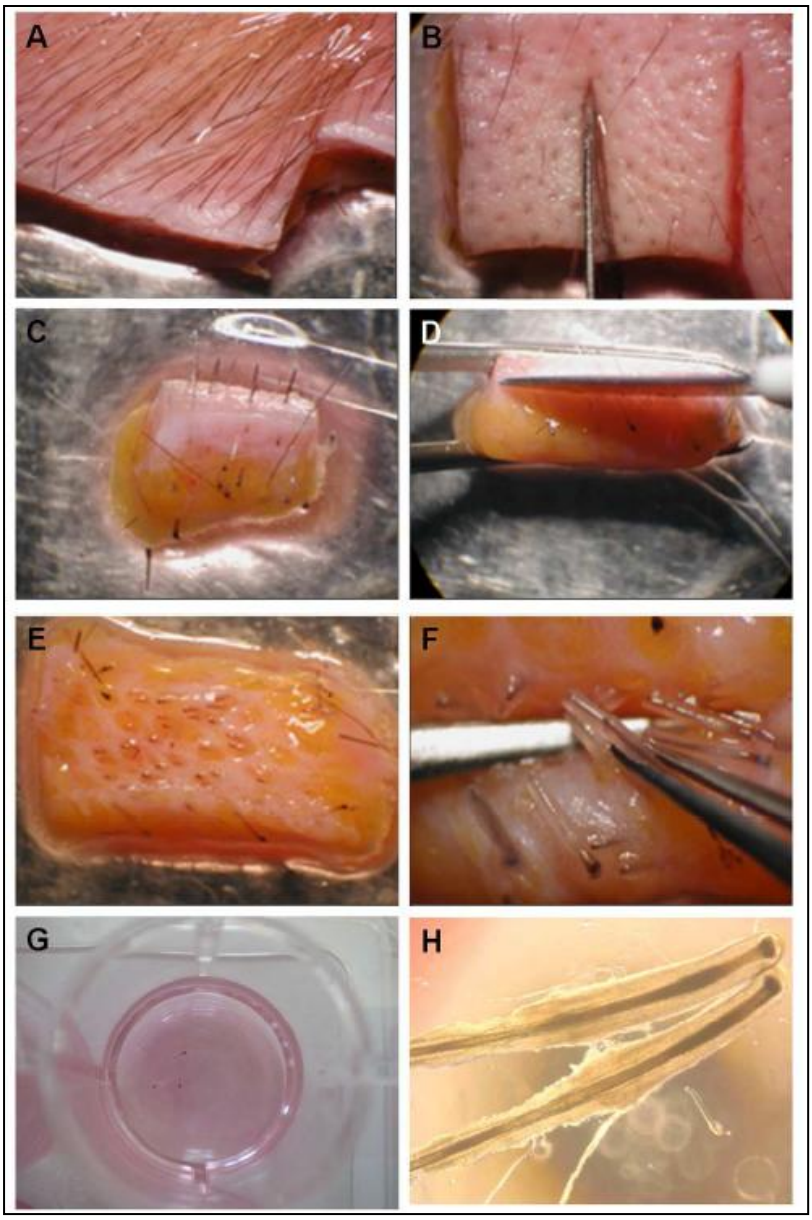

Figure 2.3: Hair follicle isolation and culture procedure.
Human scalp skin (A) is shaved and cut in thin strips with a scalpel blade (B), following the growing orientation of the HFs (C). The skin is then deprived of the epidermis and part of the dermis (D). If a net of white dermal collagen fibers spread all over the subcutaneous fat tissue is visualized, the cut was successful (E) and the HFs could be dissected (F). The HFs were culture in threesome (G) in a 24-wells plate and observed under a microscope (H). Modified after Kinori et al. 2011.

2.2.2 Full-thickness human skin organ culture

4 mm punches were obtained from human scalp skin **(Figure 2.4A,B)**, placed in a 6-wells plate in with 5 ml of supplemented William's E medium **(Figure 2.4C)** and incubated at 37°C with an atmosphere of 5% CO_2 and 95% air (Ramot et al. 2013, Langan et al 2012,

Sugawara et al. 2012, Lu et al. 2007). The treatment stared after 24 hours, when the medium was changed.

For full-thickness human healthy skin organ cultures, punches were treated for 24 hours or 72 hours with vehicle (only medium), 10^{-8}M or 10^{-10}M of SP and 5µg/ml compound 48/80 (Sugawara et al. 2012). In the case of 72 hours of incubation the medium was changed every 24 hours. However, after the first experiment it was clear that the concentration of compound 48/80 was too low to obtain significant results. Therefore, for following experiments a higher concentration (50µg/ml) was also used.

For the organ culture of full-thickness human AA skin, punches were treated for 24 hours with vehicle (only supplemented medium), 10^{-8}M or 10^{-10}M of SP, 5µg/ml of compound 48/80 and 10^{-4}M or 10^{-7}M of cromoglycate (Sigma-Aldrich) (Leung et al. 1988).

At the end of the experimentation, the skin fragments were fixed in 10% buffered formalin for at least 48 hours and embedded in paraffin as described above.

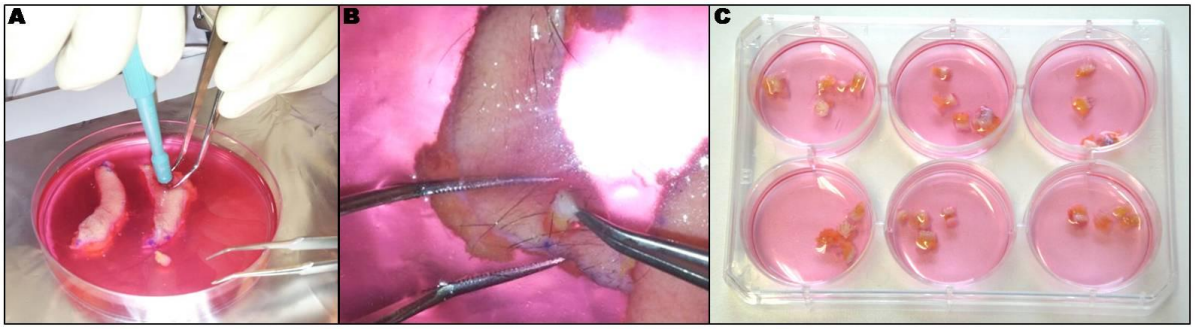

Figure 2.4: Full-thickness skin organ culture.
4mm punches were performed (A), extracted (B) from human scalp skin and placed in 6-wells plate (C).

2.3 Animal experiments

2.3.1 C3H/HeJ mice, University of Lübeck, Lübeck

42 female C3H/HeJ mice **(Figure 2.5A,B)** were purchased from Charles Rivers Laboratories (Sulzfeld, Germany) (JAX 000569), 14-15 weeks older (Wang et al. 2013, Ohyama et al. 2010, King et al. 2008). The mice were housed at the animal facility of the University of Lübeck **(Figure 2.5A)** and were subcutaneously injected once a week near the neck on the back either with 100 µl of 10mM $NaPO_4$ + 0,1% bovine serum albumin (BSA) (vehicle) (Sigma Aldrich) or 10mM $NaPO_4$ + 0,1% BSA + 10000UI INFγ (treated) (Peprotech, Hamburg, Germany). Every week the mice were observed to search of AA sign. Once that a mouse revealed AA lesion were immediately killed by cervical dislocation together with a

vehicle treated healthy mouse. After that, the mice were processed for tissue sampling **(Figure 2.5C,D)**.

AA lesional and peri-lesional skin fragments together with back hairy skin as control were harvested from AA mice. Back skin and skin from the same site in which alopecic mouse presented AA lesion were harvested from non-alopecic vehicle injected mice. For all mice also lymph nodes, spleen and nails were obtained, however these samples were not used for this thesis project. All tissue samples were harvested in duplicate or cut into two pieces so that cryo and paraffin blocks could be prepared. This experiment was conducted following ethics approval by the governmental authorities: Ministerium für Landwirtschaft, Umwelt und ländliche Räume, V 312-72241.122-5 (66-6/10).

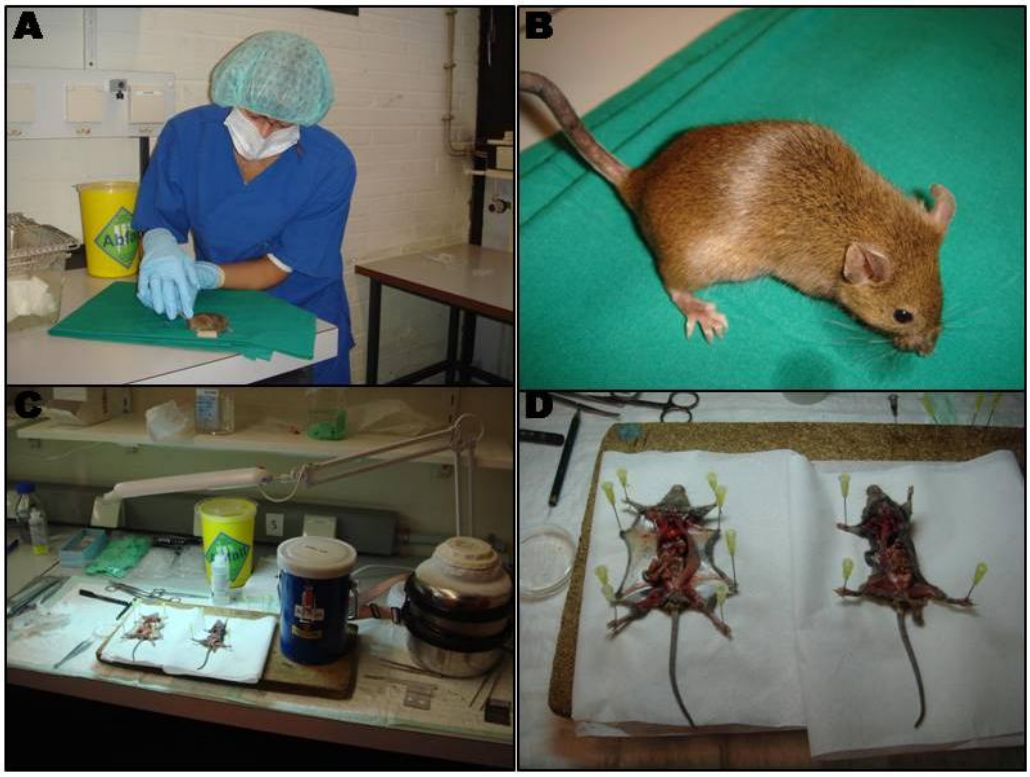

Figure 2.5: C3H/HeJ mice.
Management of C3H/HeJ mice (A). Picture of a C3H/HeJ mouse (B). Mouse tissue sampling (C,D).

2.3.2 Grafted C3H/HeJ mice, University of British Columbia, Vancouver, Canada

13 weeks-old female C3H/HeJ mice were purchased from The Jackson Laboratory, Bar Harbor, Maine USA and housed in the British Columbia University facility. The experiment was conducted by Eddy Wang and Prof. Kevin McElwee. The mice were transplanted with healthy hairy or alopecic skin isolated from older C3H/HeJ donors as previously described (Silva and Sundberg 2013, Wang et al. 2013, King et al. 2008). Most of the mice transplanted with alopecic lesions developed AA on the belly skin which developed fast in all body, here

called AA mice (mAA), while few mice failed to develop AA, here called failed-grafted mice (fAA). Mice transplanted with hairy skin did not develop AA, here called sham-grafted mice (mSH). For comparison also normal not-transplanted mice were used, here called normal mice (NM). After about one year the mice were killed and the tissue samples were collected from the mid to lower back close to the midline, fixed in cryo matrix and frozen in liquid nitrogen. The skin samples were then shipped to the University of Lübeck for (immuno-)histomorphometry analyses. This experiment was performed following ethics approval by the University of British Columbia Animal Care Committee (n. A10-0166, 16-08-13).

2.3.3 Humanized-mouse model for alopecia areata, Technion-Israel Institute, Haifa, Israel

6 C.B-17/IcrHsd-Prkdc^{SCID}Lyst^{bg-J} mice (Shultz et al. 1995) (derived from 2 independent experiments) were purchased from The Jackson Laboratory and housed at the facility of the Technion-Israel Institute. Immunologically, this mouse strains is characterized by the lack of T-cells and B-cells as well as severe lymphopenia (Mosier et al. 1993, Carroll et al. 1989). In addition to other SCID models, this strain reveals also diminished NK cell activity (Shultz et al. 1995). For this reasons, this mouse model is very much in use and widely accepted in the field in xenograft transplantation experiments (Lai et al. 2014, Zhang et al. 2013). The mice were transplanted with healthy human scalp skin which was subsequently injected intradermally with allogeneic, IL-2 or PHA-treated PBMCs from healthy donors enriched for NKG2D+/CD56+ cells (for details, see: Gilhar et al. 2013a,b).

Mice that received an injection of IL-2-treated NKG2D+/CD56+ cell-enriched PBMCs clinically and histologically developed characteristic AA lesions in the transplanted, previously healthy and hair-bearing human skin, while control mice receiving NKG2D+/CD56+-enriched cells cultured with PHA instead of IL-2 failed to develop AA in the human skin transplants. This xenotransplant model has been advocated as the best currently available animal model for AA since it imitates human AA more closely than any other animal models. In addition, it permits one to transform previously healthy human skin *in vivo* into one that phenotypically copies this autoimmune disease and thus allows one to dissect all stages of disease development (Gilhar et al. 2013a,b).

After euthanasia, the human skin was harvested from the mice and frozen in liquid nitrogen or fixed in 10% formalin and embedded in paraffin. The cryoblocks were used in the lab in Haifa during my visit while paraffin skin samples were shipped to the University of Lübeck for (immuno-)histomorphometry analyses. Human skin samples were obtained after informed patient consent and ethics approval (n. 919970072, 13-05-97) from the Flieman Medical Center and the Ministry of Health, Israel, and the study was performed in adherence with the

Declaration of Helsinki Principles. Animal care and research protocols were in accordance with institutional guidelines and were approved by the Technion Institute Committee on Animal Use (n. IL-087-08-2011, 08-11). These experiments were conducted by Dr. Aviad Keren and Prof. Amos Gilhar, who hosted the PhD candidate for two weeks in his lab in Haifa so that the candidate could handle these mice and could witness how the xenotransplantation was done.

2.4 Stainings

Before each staining, paraffin sections from blocks of human skin from healthy individuals and AA patients, of human cultured skin and of human skin transplanted on SCID mice (Humanized-mouse model) were cut of 4.5 µm thickness using a Leica RM 2255 microtome (Leica Microsystems) and devolved on superfrost plus slides (Menzel GmbH & Co KG, Braunschweig, Germany), were deparaffinized in xylene for 20 min and were immersed 4-5 times into different alcohol-content bath sequentially (100%, 96%, 70%, 50%) (Merck, Darmstadt, Germany) and subsequently re-hydrated in distillate water. The deparaffinization was followed by an antigen retrieval step either with sodium-citrate (pH 6.1) or TRIS-EDTA (pH 9) buffers for 20 min in the microwave (Europastyle) respectively at 650 and 800 watt **(Table 2.5)**.

Cryosections from blocks of C3H/HeJ mouse skin and of cultured HFs were cut of 7µm thickness using a Leica 3500 cryostat (Leica) and devolved on superfrost plus slides were dried for 10 min at room temperature (RT) and fixed either in acetone at -20°C or in 1% paraformaldeyde in phosphate buffered saline (PBS) at RT for 10 min; in some cases, cryosections were alternatively post-fixed with ethanol and acetic acid (2:1) for 5 min at -20°C **(Table 2.5)**.

Paraffin- and cryo-sections were then rinsed in Tris-buffered saline (TBS) or Tris-NaCl-Tween buffer (TNT) or distilled water.

2.4.1 Haematoxylin-eosin histochemistry

The haematoxylin-eosin (H&E) staining is a dichromatic staining, based on the basic dye haematoxylin and the alcohol-based acidic dye eosin. The nucleus is stained in blue-purple while intracellularly or extracellularly structures are stained in a brilliant pink colour by the eosin (Bancrof and Gamble 2008).

Paraffin sections were stained in Mayer's haematoxylin (Merck, Darmstadt, Germany) for 10 min and rinsed under tap water approximately for 15 min. The counterstaining was performed with 0.1% eosin (Sigma Aldrich) for approximately 1 min. The final water elimination was done by incubating the slides in ascending ethanol series: 70%, 96%, 100% ethanol and immersing two times into xylene, for 10 min each. Finally, the slides were mounted with Eukitt, a synthetic resin (Eukitt, O.Kindler &Co, Freiburg, Germany).

Antigen(s)	Specimen	Antigen retrievel/ fixation	1st detection system	2nd detection system	3rd detection system	Counter staining
TGFβ1/c-Kit	Human	Sodium Citrate	IF, FITC or Rho	IF, Rho or FITC		DAPI
Ki-67/Tryptase	Human	Sodium Citrate	ABC-HRP, DAB	ABC-AP, SIGMAFAST™		
CD8	Human	TRIS-EDTA	IF, Alexa 488			DAPI
CD8/Tryptase	Human, Hu-mouse	Sodium Citrate	ABC-HRP, DAB	ABC-AP, SIGMAFAST™		Haematoxylin
CD8/MHCI/Tryptase	Human	TRIS-EDTA	IF, Alexa 488	IF, Rho	IF, Dy Light 350	
OX40L/CD8/Tryptase	Human	TRIS-EDTA	ABC-HRP, AEC	ABC-AP, Vector Blue®	ABC-AP, SIGMAFAST™	
CD30L/c-Kit/CD8	Human	Sodium Citrate	ABC-HRP, AEC	ABC-AP, Vector Blue®	ABC-HRP, DAB	
4-1BBL/c-Kit/CD8	Human	Sodium Citrate	ABC-HRP, AEC	ABC-AP, Vector Blue®	ABC-HRP, DAB	
ICAM-1/CD8/Tryptase	Human	Sodium Citrate	ABC-HRP, AEC	ABC-AP, Vector Blue®	ABC-AP, SIGMAFAST™	
IL-10/c-Kit/CD8	Human	TRIS-EDTA	ABC-HRP, AEC	ABC-AP, Vector Blue®	ABC-HRP, DAB	Methyl-green
PD-L1/c-Kit/CD8	Human	Sodium Citrate	ABC-HRP, AEC	ABC-AP, Vector Blue®	ABC-HRP, DAB	
CD200/CD8/Tryptase	Human	TRIS-EDTA	DAB	ABC-AP, Vector Blue®	ABC-AP, SIGMAFAST™	
CD8/c-Kit	Human, Hu-mice	1%PFA, EtOH + Ac. acid 2:1	IF, Rho	TSA, FITC		DAPI
Tryptase/CD8	Human	1%PFA, EtOH + Ac. acid 2:1	TSA, FITC	IF, Rho		DAPI
Ki-67/Tryptase	Human	Sodium Citrate	ABC-HRP, DAB	ABC-AP, SIGMAFAST™		Haematoxylin
Ki-67/c-Kit	Human	Sodium Citrate	ABC-HRP, DAB	ABC-AP, SIGMAFAST™		
MHCI	Human	TRIS-EDTA	IF, Alexa 488			DAPI
TGFβ1	Human	Sodium Citrate	ABC-HRP, DAB			
CD8/c-Kit	Mouse	Acetone	ABC-HRP, AEC	ABC-AP, SIGMAFAST™		Haematoxylin
mMCP6/CD8	Mouse	Acetone	Envision®-HRP, AEC	ABC-HRP, DAB		Haematoxylin
C-kit	Hu-mouse	Sodium Citrate	ABC-AP, SIGMAFAST™			Haematoxylin

Table 2.5: List of all immunostainings which were performed and relevant details.
Avidin-biotin complex, alkaline phosphatase (ABC-AP), Avidin-biotin complex, horseradish peroxidase (ABC-HRP), 3-amino-9-ethylcarbazole (AEC), 3,3'-diaminobenzidine (DAB), diamidino-2-phenylindole (DAPI), Envision-horseradish peroxidise (Envision®-HRP), Fluorescein isothiocyanate (FITC), immunofluorescence (IF), Fast Red TR/Naphthol AS-MX tablets (SIGMAFAST™), Rhodamine (Rho).

2.4.2 Immunohistochemistry

For detection of single antigen, the skin sections were immunostained following previously established protocols (Vidali et al. 2014, Bertolini et al. 2013; Harries et al. 2013, Sugawara et al. 2013, Sugawara et al. 2012, Meyer et al. 2008) by using either the avidin-biotin complex method (Vector Laboratories, Burlingame, CA, USA) or Envision™ method (DAKO, Hamburg, Germany) and the corresponding chromogen **(Table 2.5)** (Buchwalow and Böcker 2010, Hsu et al. 2006). Similar protocols were used for each protein antigen assessed by double or triple-immunostainings in which the sections were serially stained for each antigen **(Table 2.5).**

Briefly, in the avidin-biotin complex method after antigen retrieval or fixation the sections **(Table 2.5)** were washed three times for 5 min in TBS. At this point, either the endogenous peroxides (Emprove®) or the endogenous expression of avidin and biotin (Vector Laboratories) of the tissue were blocked depending on which enzyme was used in the detection system. After pre-incubation with either normal serum, normal serum plus bovin serum albumine (BSA), BSA or normal serum plus BSA and X-triton 0,5% or 1% casein (Sigma Aldrich) to block reactive sites of the tissue and therefore unspecific antibody binding, the sections were incubated with the correct primary antibody **(Table 2.6)** dissolved either in TBS, TBS and normal serum, antibody diluent (DAKO, Glostrup, Denmark) or DCS LabLine Antikörper-Verdünnungspuffer (DCS, Innovative Diagnostik-Systeme, Hamburg, Germany) for 60 min at 37°C or ON at +4°C.

After washing, the sections were incubated with goat biotinylated antibodies against rabbit or mouse IgG (Jackson Immunoresearch Laboratories (JIR), West Grove, United States or Beckman Coulter, Brea, CA, USA) **(Table 2.7)** at 1:200 either in TBS, TBS and normal serum, antibody diluent or DCS LabLine Antikörper-Verdünnungspuffer for 45 min at RT. Slides were washed three times for 5 min in TBS again and treated with the detection system alkaline phosphatase- (AP) or horseradish peroxidase-based (HRP) avidin biotin complex (ABC) **(Table 2.5)** for 30 min at RT. The sections were labeled with the AP-chromogen Fast Red TR/Naphthol AS-MX tablets (SIGMAFAST™) (Sigma Aldrich, Saint Louis, USA) or Vector blue® (Vector Laboratories) for AP-ABC and 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories) or 3,3'-diaminobenzidine (DAB) (Vector Laboratories) for HRP-ABC for 3-15 min **(Table 2.5)**.

Primary antibody	Origin	Clone	Vendor	Dilution	Sections	References
Anti-human antibodies						
C-Kit	Rabbit		DAKO	1:100 1:400	Paraffin,IHC	Sugawara et al. 2013
C-Kit	Rabbit	YR145	Cell Marque	1:1000	Cryo, TSA	Sugawara et al. 2012
Tryptase	Mouse	AA1	Abcam	1:500 1:1000 1:5000	Paraffin, ICH, IF Cryo, TSA	Sugawara et al. 2013 Sugawara et al. 2012
Ki-67	Mouse	Tec-3	DAKO	1:10	Paraffin, IHC	Kinori et al. 2011
TGFβ1	Rabbit		Santa Cruz	1:100	Paraffin, IHC, IF	Bertolini et al. 2013
MHCI	Mouse	EMR8-5	Abcam	1:50	Paraffin, IF	Lee et al. 2012
CD8	Mouse	C8/144B	DAKO	1:100 1:500	Paraffin, IHC, IF	Harries et al. 2013
OX40L	Mouse	159403	R&D Sytem	1:25	Paraffin, IHC	Kashiwakura et al. 2004
CD30L	Mouse	116614	R&D Sytem	1:50	Paraffin, IHC	Diaconu et al. 2007
4-1BBL	Rabbit		Abcam	1:200	Paraffin, IHC	Zhao et al. 2013
ICAM-1	Rabbit	EP1442Y	Abcam	1:100	Paraffin, IHC	Delfortrie et al. 2004
IL-10	Mouse	23738	R&D Sytem	1:25	Paraffin, IHC	Poindexter et al. 2004
PD-L1	Mouse	29E2A3	BioLegend	1:100	Paraffin, IHC	Shi et al. 2011
CD200	Goat		R&D System	1:200	Paraffin, IHC	Garza et al. 2011
Anti-mouse antibodies						
C-Kit	Rat	2B8	BD Pharmigen	1:100	Cryo, IHC	Paus et al. 1998
CD8a	Rat	53-6.7	BD Pharmigen	1:10	Cryo, IHC	Bertolini et al. 2013
mMCP6	Rabbit		M. Gurish	1:500	Cryo, Envision	Shin et al. 2008

Table 2.6: Primary antibodies employed.
Antibodies used for immunohistochemical and immunofluorescence stainings are listed and described in detail. Immunohistochemistry (IHC), Immunofluorescence (IF).

As explained, for double or triple-immunostaining, skin sections were serially stained for each antigen. Skin sections were incubated with the correct dilution of the first primary antibody (**Table 2.6**), followed by a biotinylated secondary goat anti-mouse or goat anti-rabbit antibody (1:200) **(Table 2.7)** and the colour reaction was developed using either the ABC-HRP and DAB or AEC as substrate **(Table 2.5)**. After proper blocking (see above), this was followed by immunostaining for the second primary antibody **(Table 2.6)**. The skin sections were then incubated with an appropriate secondary antibody **(Table 2.5)** and the AP-ABC detection system and developed either using SIGMAFAST™ or Vector Blue® as substrate **(Table 2.5)**. In order to detect the third antigen, after proper blocking, the third primary antibody **(Table 2.6)** was applied, followed by the incubation of the secondary antibody **(Table 2.7)**, which

were visualized either with HRP-ABC or AP-ABC and DAB or SIGMAFAST™ colour reaction **(Table 2.5)**.

For mMCP6/CD8 double-immunostaining, the mMCP6 protein **(Table 2.6)** was detected by using the Envision™-HRP kit (DAKO) (Buchwalow and Böcker 2010), following the manufacturer´s protocol **(Table 2.5)**. In order to block endogenous peroxidise, the sections were pre-treated for 5 min at RT with a peroxidase block. The incubation of the primary antibody **(Table 2.6)** was followed by incubation of a labelled polymer-HRP anti-rabbit and the staining was developed using AEC as substrate chromogen. All these substances were contained in the kit. Since in this method the secondary antibody molecules are carried on an enzyme-conjugated polymer backbone, it is a high sensitive technique that allows one to detect also very low concentrated antigens in the tissue.

For CD200 triple staining, a HRP conjugated donkey anti-goat (JIR) as a secondary antibody **(Table 2.7)** was used for goat anti-human CD200, therefore it was not necessary to use ABC-HRP **(Table 2.5)**.

In some cases **(Table 2.5)**, skin sections were finally incubated with a counterstain, either Mayer's haematoxylin (Merck) for 30 sec or Methyl green (DAKO) for 1 min and rinsed in tap water for 5 min (Bancrof and Gamble 2008) **(Table 2.5)**. Finally the slides were mounted with Faramount (DAKO).

Secondary antibody	Vendor	Dilution
Goat anti-mouse biotinylated	Beckman Coulter	1:200
Goat anti rabbit	Jackson Immunoresearch Laboratories	1:200
Goat anti-rabbit FITC	Jackson Immunoresearch Laboratories	1:400
Goat anti-rabbit Rhodamine	Jackson Immunoresearch Laboratories	1:400
Goat anti-mouse Alexa fluor 488	Invitrogen	1:400
Goat anti-mouse Rhodamine	Jackson Immunoresearch Laboratories	1:400
Goat anti-mouse Dy Light 350	Thermo Scientific	1:50
Goat anti-mouse FITC	Jackson Immunoresearch Laboratories	1:400

Table 2.7: List of secondary antibodies.
Fluorescein isothiocyanate (FITC), Rhodamine (Rho).

2.4.3 Immunofluorescence

For the detection of single antigen, the skin sections were immunostained following established protocols by using either the classical indirect immunofluorescence (IF) (Kloepper et al. 2014, Vidali et al. 2014, Ernst et sl. 2013, Harries et al. 2013, Samuelov et al. 2012, Meyer et al. 2008) or the tyramide amplification method (TSA) (Perkin-Elmer, Boston, United States) (Sugawara et al. 2013, Buchwalow and Böcker 2010, Bobrow et al. 1989) **(Table 2.6)**. Similar protocols were then used for each protein stained in double or triple-immunostainings in which the sections were serially stained for each protein **(Table 2.5).**

Sections stained with IF, after antigen retrieval or fixation, were washed in TBS for three times for 5 min each. After pre-incubation with 10% goat serum in TBS or 1% BSA the slides were incubated with the corresponding primary antibody **(Table 2.6)** for 60 min at 37°C or ON at +4°C. After three washing steps in TBS, 45 or 60 min incubation at RT with the correct secondary antibody **(Table 2.5** and **2.7)** was arranged.
To perform double- (Kloepper et al. 2014, Ito et al. 2005a) and triple-immunofluorescence (IF), we used the appropriate primary antibody **(Table 2.6)** and secondary antibodies conjugated to the correct fluorophore, goat anti-mouse/goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (1:400, JIR), Alexa Fluor 488 (1:400, Invitrogen, Xuhui, Shanghai, China), rhodamine (1:400, JIR) or Dy Light 350 (1:50, Thermo Scientific, Marietta, OH, United States) were used **(Table 2.5** and **2.7)**.

For the TSA protocol, sections were pre-treated to block the endogenous peroxidase and the endogenous expression of avidin and biotin. The sections were then incubated with the correct primary antibody **(Table 2.6)** ON at +4°C. After washing, the correct biotinylated secondary **(Table 2.7)** antibody was applied**.** Next, streptavidin HRP (TSA kit) was administrated (1:100 in TNT) for 30 min at RT. The reaction was amplified by FITC tyramide amplification reagent at RT for 5 min (1:50 in amplification diluent, TSA kit). This method is highly sensitive compared to the indirect conventional technique. In fact, the detection of the antigens is improved up to 100 fold. This is due to the oxidation by HRP of the phenol moiety of FITC-labelled tyramide that once activated, suddenly bind convalently the protein immediately close to the immunoreaction (Buchwalow and Böcker 2010, van Gijlswijk et al. 1997).

Finally, only in some stainings **(Table 2.5)**, the samples were washed three times for 5 min in TBS and counterstained with 4’,6-diamidin-2’-phenylindoldihydrochlorid (DAPI, Boehringer Mannheim, Germany) for 1 min and mounted with Fluoromount-G (Southern Biotechnologies, Birmingham, USA).

2.4.4 Problems overcome during the establishment of the stainings

The establishment of all these immunostainings was not easy and most of the time frustrating. One has to consider which is the most difficult antigen to stain, what is the correct order of antibodies and what are the correct blocking steps. Here, will follow some examples of how it was possible to overcome methodological problems.

The skin is one of the tissues with many autofluorescent structures, e.g. blood vessels, nerve endings and with structures that stick the antibodies, e.g. collagen, AMP, SGs. Therefore, this become a great problem when one want to establish a double- or triple-IF, most when already labelled antibodies are not available. One attempt was to pre-labelled the primary antibody using a labelling kit, Zenon® labelling technology, which uses a new method to labelled primary antibody without covalent binding. Following the manufactory procedure, 1µg of the primary antibody (e.g. mouse anti-human CD8) was incubated with 5µl of Alexa Fluor labelled Fab mixture for 5 min at RT and then 5µl of blocking reagent was added and the mixture incubated for other 5 min at RT. Once the labelled primary antibody was prepared, it could be use directly on the sections. However, since this technology, did not consider the removal of free Fab the result obtained was not satisfactory enough.

Therefore, another method was used to decrease skin structures autofluorescence, the incubation with 0,25% ammonia in 70% ethanol for 60 min at RT after deparafinization of the sections and the incubation with 0,1% Sudan Black B (Sigma Aldrich) in 70% ethanol after counterstaining. However, after observation it was denoted that this procedure has to be carefully used when the antigen should be stained with a chromosphore excited in the red spectrum.

The establishment of OX40L/CD8/Tryptase triple staining was rather extreme complicate because after reaching a beautiful and specific staining for OX40L, the treatment of the sections with the blocking buffers and mostly for the ones that contained BSA or goat serum caused the lost of OX40L IR. Therefore, in this case, the normal three days protocol for a triple-staining had to be tremendously shortened (1 day and half) and the casein was used instead of BSA. However, the blocking ability of casein it was lowered compared to BSA, so the success of this staining was dependent on the ability of controlling the development time of the substrates.

In some cases, the primary antibody already in the single ABC-based staining revealed unspecific background, as for a monoclonal rabbit anti-human 4-1BBL (Abcam, ab68185). Therefore, alternative methods were employed, e.g. Envision®-base IHC. However, also with

these methods the result was unchanged. Therefore, to overcome this problem another primary antibody was purchased.

Some antigens were not possible to stain, even in a single IHC (e.g.IL-15), or to combine with other antigens in an IF (e.g. F-actin) because of the unspecific staining.

Finally, critical was the establishment and the performance of double-staining in cryosections of mouse skin. Given that the skin of mouse is so thin and breckable, a particular attention has to be made on applying the solutions to the sections if one wants to perform a long staining.

Once that the staining is established, the success of the staining on valuable tissue samples is depending on many factors, room temperature, humidity which varies the time of substrate incubation. So, to perform triple-immunohistochemistry protocols one has to know perfectly the antibodies, the substrate development time and has to play, each time in a different way, to avoid cross-reaction between substrates.

2.4.5 Positive and negative controls

Each immunostaining protocol was also conducted with the appropriate positive and negative controls (see **Figure 2.6** legend), which confirmed both the sensitivity and specificity of the IR patterns reported here in order to avoid false positive results **(Figure 2.6)**.

MHC class I is expressed in all nucleated cells, apart those which reside in IP sites (Murphy 2012). Therefore, the anagen HF, which harbours two IP sites, is an optimal positive and negative control for MHCI class I staining. In fact, the HF infundibulum is characterized by a strong MHC class I IR, which is then down-regulated in the bulge and almost absent in the bulb (Meyer et al. 2008). The same pattern of MHC class I expression was detected in the corresponding IF staining **(Figure 2.6A)**. Tonsil sections were used to confirm the specificity of the triple CD8/MHCI/Tryptase IF staining, since in this tissue samples many CD8+ T-cells, MCs and MHC class I+ cells could be detected **(Figure 2.6B)**.

Tonsil tissue samples were employed as positive and negative control for OX40L IR, since tonsillar MCs, lymphoid tissue inducer (LTi), activated macrophages and dendritic cells express this glycoprotein on their surface (Kim et al. 2011, Kashiwakura et al. 2004, Croft 2010). As shown in **Figure 2.6C,D**, OX40L IR could be found in the marginal zone of tonsil follicle where mostly OX40L+ cells may be LTi or macrophages and in some MCs localized in the sinuses (Shikotra et al. 2012).

For confirming the CD30L IR, placenta tissue sections were stained since CD30L is expressed in placental villous endothelial cells but not in placenta syncytiotrophoblasts **(Figure 2.6E)** (Phillips et al. 2003). In tonsil, some activated MCs in the sinuses expressed CD30L, as confirmed in the established staining **(Figure 2.6F)** (Molin et al. 2001).

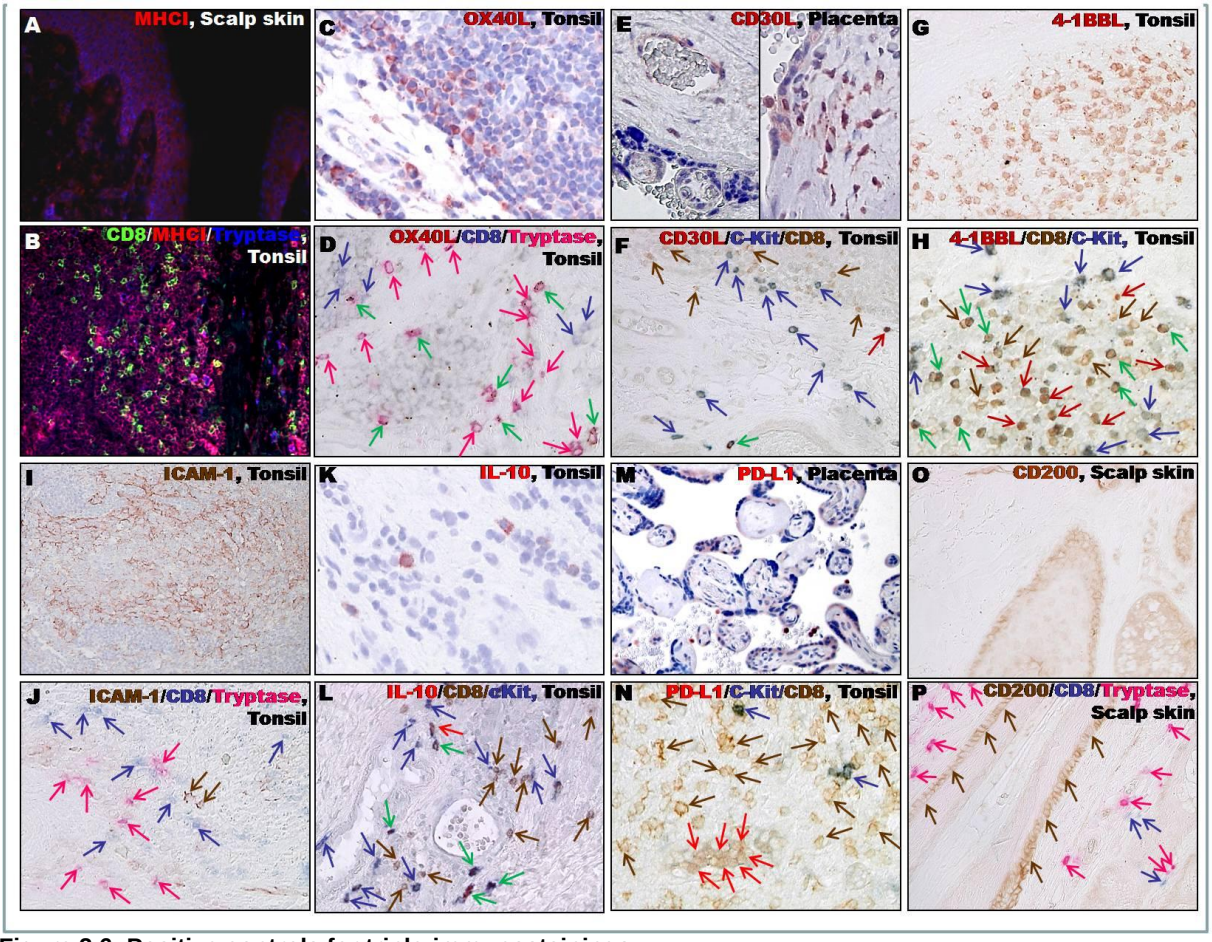

Figure 2.6: Positive controls for triple-immunostainings.
Specific MHC class I in the HF infundibulum (A) (Meyer et al. 2008) and triple CD8/MHCI/Tryptase IF in the tonsil (B). Green cells are CD8+ T-cells, red cells are MHC class I+ cells and blue cells are tryptase+ cells. Specific single OX40L staining (C) showing OX40L+ activated T lymphocyte in the marginal zone of tonsil follicle (Shikotra et al. 2012) and triple OX40L/CD8/Tryptase staining (D). Blue arrows indicate CD8+ T-cells, pink arrows indicate tryptase+ MCs and green arrows indicate OX40L+/tryptase+ MCs. CD30L is expressed in placental villous endothelial cells (Phillips et al. 2003) (E), our positive control together with tonsil (F) for the triple staining CD30L/C-kit/CD8. Red arrows indicate CD30L+ cells, blue arrows indicate c-Kit+ MCs, brown arrows indicate CD8+ T-cells and green arrows indicate CD30L+/c-Kit+ MCs. 4-1BBL+ cells are found in the marginal zone of tonsil follicles (Zhao et al., 2013) (G) as shown also in the triple 4-1BBL/c-Kit/CD8 staining (H). Red arrows indicate 4-1BBL+ cells, blue arrows indicate c-Kit+ MCs, brown arrows indicate CD8+ T-cells and green arrows indicate 4-1BBL+/c-Kit+ MCs. Specific single staining for ICAM-1 (I) showing ICAM-1 IR in the germinal centre (Goval et al. 2006) and sparse positive cells of tonsils (J), our positive control for ICAM-1/CD8/Tryptase. Brown arrows indicate ICAM-1+ cells, blue arrows indicate CD8+ T-cells, pink arrows indicate tryptase+ MCs. Many IL-10+ cells are found in tonsil (Poindexter et al. 2004) as shown in our single IL-10 (K) but also triple IL-10/c-Kit/CD8 (L) stainings. Red arrows indicate IL-10+ cells, blue arrows indicate c-Kit+ MCs, brown arrows indicate CD8+ T-cells and green arrows indicate IL-10+/c-Kit+ MCs. PD-L1 IR in chorionic villi of placenta (Kshirsagar et al. 2012) detected by single PD-L1 (M) and triple PD-L1/c-Kit/CD8 (N) stainings. Red arrows indicate PD-L1+ cells, blue arrows indicate c-Kit+ MCs, brown arrows indicate CD8+ T-cells and green arrows indicate PD-L1+/c-Kit+ MCs. Positive CD200+ cells are found in the HF bulge (Harries et al. 2013) (O) also for the triple staining CD200/CD8/Tryptase (N). Brown arrows indicate CD200+ cells, pink arrows indicate tryptase+ MCs, blue arrows indicate CD8+ cells.

In healthy tonsil, the mantle zone of secondary lymphoid follicles is populated by 4-1BBL+ cells. No 4-1BBL+ cells are found in tonsil sinuses (Zhao et al. 2013). This 4-1BBL expression pattern is revealed also in the corresponding established stainings **(Figure 2.6G,H).**

Specific single staining for ICAM-1 is reported in the germinal centre of tonsil follicles where mostly intrafollicular dendritic cells are positive (Goval et al. 2006) and in sparse positive cells of tonsils but not in tonsil mantle zone **(Figure 2.6I,J)**.

Many IL-10+ cells are found in tonsil sinuses **(Figure 2.6K)** (Poindexter et al. 2004) and some of them are MCs, as shown in the triple IL-10/c-Kit/CD8 **(Figure 2.6L)** staining. However, no IL-10+ cells were found in germinal center or mantle zone **(Figure 2.6L).**

PD-L1 IR is expressed in chorionic villi of placenta but not in their mesenchyme, (Kshirsagar et al. 2012) as detected by the single PD-L1 staining **(Figure 2.6M)**, and in few immunocytes in tonsil tissue samples **(Figure 2.6N)** (Latchman et al. 2001).

Since CD200 is a hallmark for bulge IP (Harries et al. 2013, Meyer et al. 2008), skin tissue was used as internal negative and positive control. In fact, CD200+ cells are found only in the HF bulge (Harries et al. 2013) **(Figure 2.6O,P)**.

2.5 Microscopy

For fluorescence and light microscopy, Keyence Biozero-8000 and Keyence Biozero-8100 microscopes (Keyence Corporation, Biozero-8000, Higashi-Nakajima, Osaka, Japan) were used, in combination with Nikon lenses (Japan). The software used was Biozero Image Analyzer, Version 2.5.

For fluorescence microscopy, a Zeiss LSM 700 confocal microscope (Carl-Zeiss-Strasse, Oberkochen, Germany) were used, in combination with ZEN imaging software.

2.6 Quantitative (immuno-)histomorphometry

In human skin, the cell density of MCs, CD8+ T-cells and MC-CD8+ T-cell interactions was evaluated by quantitative (immuno-)histomorphometry in the HF CTS and the PFD, using previously established general histomorphometry principles (Bertolini et al. 2013, Harries et al. 2013, Kloepper et al. 2013, Sugawara et al. 2012). The total reference area (CTS+PFD),

demarcated using Biozero Image Analyzer software, included all human skin tissue within a distance of up to 200µm from the HF basement membrane in human skin **(Figure 2.7A)**.

In murine skin, positive cells were counted in a perifollicular tissue area at a distance of up to 50µm **(Figure 2.7B)** (Bertolini et al. 2013, Kloepper et al. 2013).

For the current study, the HF CTS and PFD (defined as indicated in **Figure 2.7A**) were considered to be the most important reference areas for many evident reasons: 1) MCs are prominent in the HF CTS in human and mice (Metz and Maurer 2009, Christoph et al. 2000, Paus et al. 1998, Botchkarev et al. 1995, Paus et al. 1994c) where they seems to play a role also in HF cycle (Liu et al. 2013, Paus et al. 1998, Botchkarev et al. 1997, Maurer et al. 1997, Botchkarev et al. 1995, Maurer et al. 1995, Paus et al. 1994c, Moretti et al. 1967) and HF-IP (Gilhar et al. 2012, Waldmann 2006); 2) CD8+ T-cells in the HF mesenchyme are mostly found in the infondibulum and isthmus CTS (Christoph et al. 2000, Paus et al. 1998); 3) CTS+PFD represents the perifollicular mesenchyme tissue in which MCs and CD8+ T-cells can interact and have an effect on the proximal HF and not on other neighbour HFs. In fact, in human skin 300-400µm separates one HF from another HF nearby, while in mouse skin 80-100µm (personal observation). So, evaluating an area of 200µm in human and 50µm in mouse skin around the HF, it was possible to exclude the skin mesenchyme in which MC-CD8+T-cell interactions might have an effect on a second HF.

MCs with more than five granules located outside of the cell membrane were regarded as “degranulated” (Bertolini et al. 2013, Sugawara et al. 2012, Paus et al. 1994c).

The staining intensity of TGFβ1 and tryptase of individual MCs **(Figure 2.7C)**, of TGFβ1 in the HF ORS **(Figure 2.7D)** and MHC class I in the hair bulb **(Figure 2.7E)** was evaluated by quantitative analysis, using NIH image J software (National Institute of Health, Bethesda, Maryland) (Bertolini et al. 2013, Harries et al. 2013, Meyer et al. 2008).

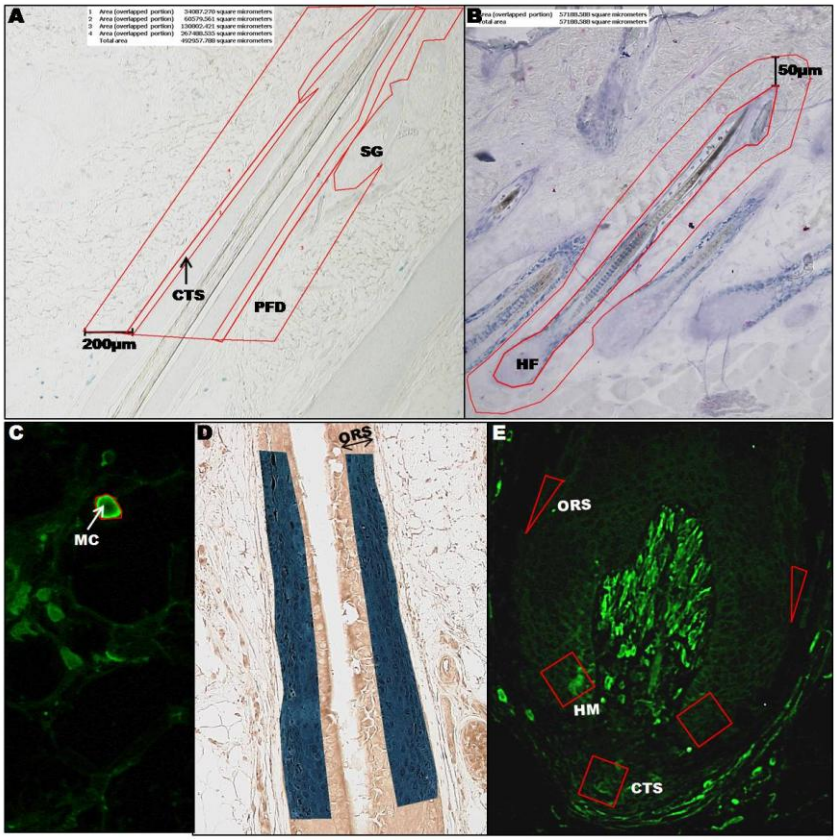

Figure 2.7: Reference areas for quantitative (immuno)-histomophometry.
In human skin the cell density was counted in the HF CTS and PFD. The total reference area (CTS+PFD) included the human skin tissue within a distance of up to 200µm from the HF basement membrane (A). In murine skin, positive cells were counted in a perifollicular tissue area around the HFs at a distance of up to 50µm from the basement membrane (B). The staining intensity of TGFβ1 and tryptase of individual MCs was evaluated drawing the area around MC (C). TGFβ1 IR in the HF ORS was evaluated drawing 2 areas in the ORS (D). MHC class I in the hair bulb was evaluated in CTS, HM and ORS (E). Connective tissue sheath (CTS), hair matrix (HM), outer root sheath (ORS), perifollicular dermis (PFD).

2.7 Statistical analysis

All data were analyzed by Student´s *t*- or Mann-Whitney-U- tests when only two groups were compared, or by One Way-ANOVA or Kruskal-Wallis tests followed by Bonferroni´s or Dunn´s multiple comparison tests, respectively, when more than two groups were analyzed, using GraphPad (GraphPad Prism version 4.00 for Windows; GraphPad Software, San Diego, CA, USA) (Dytham 2011, Bremer and Doerge 2010). Data are expressed as mean ±SEM; p values of <0.05 were regarded as significant.

3 Results

3.1 Mast cell and CD8+ T-cell interactions are enhanced and abnormal *in situ* in the perifollicular mesenchyme of human alopecia areata lesional skin

3.1.1 Mast cells in alopecia areata lesional skin are skewed towards a pro-inflammatory phenotype

MCs are able to both inhibit and promote inflammation by releasing either immuno-inhibitory or pro-inflammatory mediators (see Introduction, chapters 1.5 and 1.6). Specifically, MCs may help to maintain the HF-IP (Gilhar et al. 2012a) by releasing potent immunoinhibitors such as IL-10 and TGFβ1 (Gri et al. 2012, Harvima and Nilsson 2011, Hugle et al. 2011, Kalesnikoff and Galli 2011, Tsai et al. 2011, Aceves et al. 2010, Gordon and Galli 1994), which may decrease the immune response indirectly by interacting with regulatory T-cells (Sayed et al. 2008, Waldmann 2006, Maurer et al. 2003). Therefore, the immune-phenotype of lesional MCs in AA skin was investigated.

3.1.1.1 Lesional AA mast cells contain less TGFβ1 compared to control mast cells

TGFβ1 is one of the most important "guardians of HF-IP" (Gilhar et al. 2012, Kang et al. 2010, Kinori et al. 2011, Meyer et al. 2008, Wahl et al. 2006, Paus et al. 2005, Ito et al. 2004) and was found to be down-regulated in the ORS of lesional AA-HFs (Bertolini et al. 2014), suggesting compromised HF-IP (Harries et al. 2013, McElwee et al. 2013, Gilhar et al. 2012, Wahl et al. 2006). This observation raised the question whether the TGFβ1 content of perifollicular MCs was also abnormal in AA lesional skin.

To explore this, double-immunostainings for c-Kit and TGFβ1 were established in which the antigens were detected either by using a FITC-labelled secondary antibody (green signal) for c-Kit and a rhodamine-labelled secondary antibody (red signal) for TGFβ1 **(Figure 3.1A)** or by using a FITC-labelled secondary antibody for TGFβ1 and a rhodamine-labelled secondary antibody for c-Kit. The latter combination led to higher-quality immunostaining result for TGFβ1 and therefore was used for the evaluation of TGFβ1 IR within MCs **(Figure 3.1B,C)**. Indeed, perifollicular MCs in lesional AA skin **(Figure 3.1C)** showed a reduced TGFβ1 content compared to control MCs from healthy scalp skin **(Figure 3.1B-D)**.

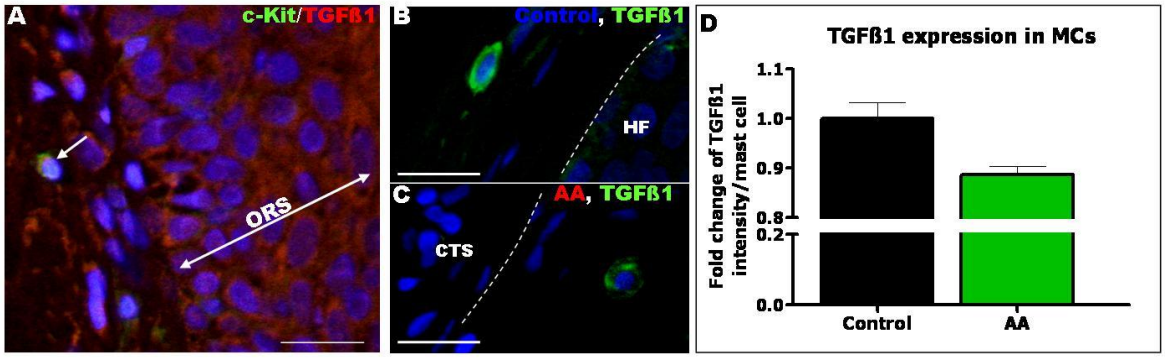

Figure 3.1: Lesional alopecia areata mast cells contain less TGFβ1 compared to control MCs from healthy scalp skin.
Representative picture of TGFβ1(red)/C-Kit(green) double-staining in control healthy skin showing a double stained perifollicular MC (white arrow) and TGFβ1 IR in the ORS of the HF (A). Representative pictures of the spectral emission channel for green fluorescence showing TGFβ1+ MCs in control skin from healthy subjects (B) and lesional skin from AA patients (C) stained by TGFβ1(green)/C-Kit(red) double-staining and used for the evaluation of TGFβ1 IR within MCs. Scale bars: 20µm. Quantitative analysis revealed a down-regulation of TGFβ1 IR within perifollicular MCs in AA lesional skin compared to the control skin (D). Analysis derived from 272 MCs around 29 HFs of 10 AA patients and 175 MCs around 19 HFs of 2 healthy controls, ±SEM, Mann-Whitney-U-Test (ns). Connective tissue sheath (CTS), hair follicle (HF), outer root sheath (ORS). This evaluation was performed together with other colleagues.

This phenomenon suggests that the TGFβ-based immuno-inhibitory phenotype of perilesional MCs is attenuated in AA.

3.1.1.2 Lesional AA mast cells contain more tryptase than control mast cells

Additionally, MCs can release numerous pro-inflammatory mediators (Frenzel and Hermine 2013, Voehringer 2013, Gri et al. 2012, Gurish and Austen 2012, Gilfillan and Beaven 2011, Beaven 2009, Galli 2005, Metcalfe et al 1997, Yong 1997). One of the most important ones among these, is tryptase, a trypsin-like protease stored together with heparin within MCs and released upon MC degranulation (Li et al. 2013, Magarinos et al. 2013, Voehringer 2013, Gri et al. 2012, Hernandez-Hernandez et al. 2012, Sugawara et al. 2012, Harvima and Nilsson 2011, Pejler et al. 2010, Shin et al. 2009).

To investigate any fluctuations in tryptase content within perifollicular MCs of AA lesions, tryptase IR (pink colour) was evaluated by re-analysing previously immunostained sections for Ki-67/Tryptase IHC of AA lesional and control skin **(Figure 1.31C,F,I,J,M)**. This analysis revealed a significant up-regulation of tryptase expression in perifollicular MCs of lesional AA skin compared to control MCs from healthy scalp skin **(Figure 3.2A-E)**.

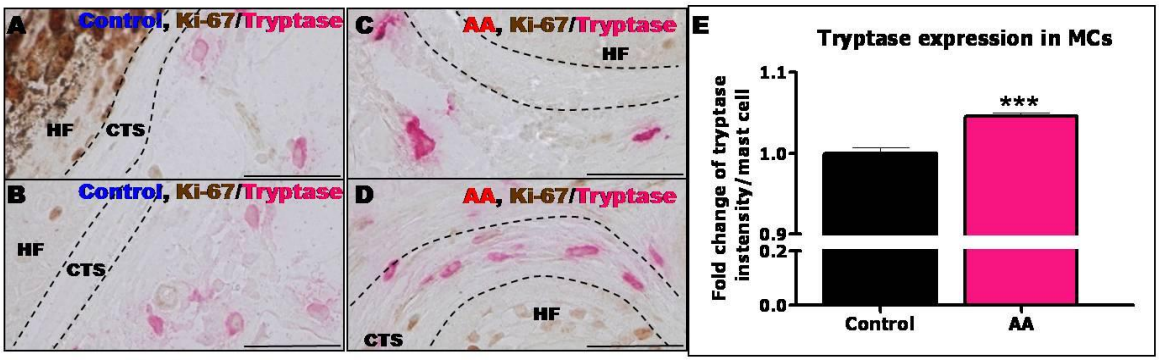

Figure 3.2: Lesional alopecia areata mast cells contain more tryptase compared to control MCs from healthy scalp skin.
Representative pictures of tryptase+ MCs in human scalp skin of control (A,B) and lesional skin from AA patients (C,D) stained by Ki-67/Tryptase double-staining used for the evaluation of tryptase IR within MCs (pink cells). Quantitative analysis of tryptase IR in perifollicular MCs revealed an increased expression of tryptase within MCs in AA lesional skin compared to the control (E). Analysis derived from 272 MCs around 41 HFs of 14 AA patients and 182 MCs around 19 HFs of 2 healthy controls, ***p≤0.001, ±SEM, Mann-Whitney-U-Test. Scale bars: 50µm. Connective tissue sheath (CTS), hair follicle (HF). This evaluation was performed together with other colleagues.

This suggests that the production of pro-inflammatory mediators (such as tryptase) in lesional MCs is strongly increased in AA.

Taken together, compared to control MCs of non-affected skin from healthy individuals, lesional AA MCs revealed less TGFβ1, an immuno-inhibitory cytokine, along with more tryptase, a pro-inflammatory mediator. This supports the concept that perifollicular MCs in AA switch from an immuno-inhibitory to a pro-inflammatory phenotype at some stage during AA pathogenesis.

3.1.2 Mast cell-CD8+ T-cell contacts are significantly increased in alopecia areata lesional skin

Considering that CD8+ T-cells have a key role in AA pathogenesis (McElwee et al. 2013, Gilhar et al. 2012, McElwee et al. 2005, Gilhar et al. 1998, McElwee et al. 1996, Paus et al. 1993) and that MCs can activate CD8+ T-cells (Ebert et al. 2014, Frenzel and Hermine 2013, Brown and Hatfield 2012, Gri et al. 2012, Harvima and Nilsson 2011, Stelekati et al. 2009, Harvima et al. 2008, Nakae et al. 2006, Kashiwakura et al. 2004), it was hypothesized that interactions between CD8+ T-cells and MCs in AA may be abnormal. Namely, perifollicular MCs in AA may not control CD8+ T-cells as efficiently as it may be the case under physiological conditions.

This hypothesis was supported by the observation that MCs in AA revealed a pro-inflammatory phenotype (see above). In order to further probe this hypothetical concept, it was investigated whether the number of MC-CD8+ T-cell contacts is altered in human lesional AA compared to control healthy scalp skin since direct cell-cell contact is a fundamental prerequisite for their interaction (Stelekati et al. 2009, Nakae et al. 2006). This

was possible by analysing the number of MC-CD8+ T-cell interactions in sections previously double-stained for CD8 and tryptase antigens during the candidate's Master´s thesis project, in which CD8+ T-cells were labelled in brown while tryptase+ MCs in pink **(Figure 3.4C,D)**.

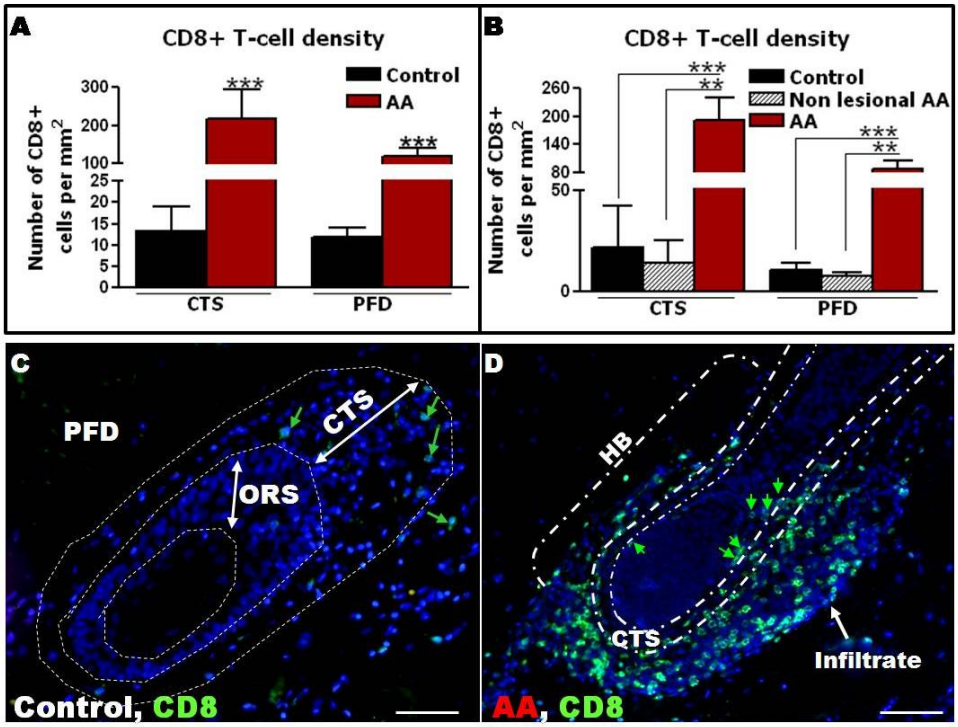

Figure 3.3: The number of perifollicular CD8+ T-cells is increased in alopecia areata lesional skin. Quantitative analysis of CD8+ T-cells revealed more perifollicular CD8+ T-cells in AA lesional skin compared to control skin from healthy individuals (A). This evaluation was performed in sections stained with CD8/Tryptase double-IHC. Analysis derived from 56 areas (HFs) from 13 AA patients and 44 areas (HFs) of 7 healthy controls, ***p≤0.001, ±SEM, p value was calculated by Mann-Whitney-U-Test. Quantitative analysis of CD8+ T-cells revealed more perifollicular CD8+ T-cells in AA lesional skin compared to non-lesional skin from AA patients and to control skin from healthy individuals (B). This evaluation was performed in sections stained with CD30L/C-Kit/CD8 triple-IHC. Analysis deriving from 12-18 areas of 4-16 HFs of 6 healthy controls and 7 AA patients (non lesional skin; only from 3 AA patients), ***p≤0.001, **p≤0.01, ±SEM, One-Way ANOVA or Kruskal-Wallis test followed respectively by Bonferroni´s or Dunn´s multiple comparison tests. Representative pictures showing CD8+ T-cells, stained by CD8 IF (green cells, green arrows), around healthy (C) and AA lesional (D) HFs. The latter picture (D) was provided by M. Bertolini for incorporation into Gilhar et al. 2012. Scale bars: 50µm. Connective tissue sheath (CTS), hair bulb (HB), outer root sheath (ORS), perifollicular dermis (PFD).

The number of CD8+ T-cells in the perifollicular mesenchyme was evaluated first. As expected from the literature (Ito et al. 2013c, Alli et al. 2012, Cetin et al. 2009, Nagai et al. 2006, Yano et al. 2002, Tsuboi et al. 1999), lesional AA skin revealed significant higher CD8+ T-cell numbers compared to healthy anagen HFs **(Figure 3.3A,C,D)**. This was later confirmed also in comparison to non-lesional skin from AA patients when CD30L/C-Kit/CD8 triple immunostains were evaluated in lesional and non-lesional skin from AA patients as well as in healthy control skin **(Figure 3.3B)**.

Importantly, the analysis of MC-CD8+ T-cells interactions provided the first evidence that tryptase+ MCs co-localize with CD8+ T-cells around the HFs in lesional AA skin **(Figure 3.4A,D)** significantly more frequently than in healthy control skin **(Figure 3.4A,C,D).** Later on,

this was confirmed also for c-Kit+ MC-CD8+ T-cell interactions and compared to non-lesional skin **(Figure 3.4B)**. Calculation of the percentage of MC-CD8+ T-cell interactions among CD8+ T-cells and MCs revealed that this was independent of the numeric CD8+ T-cell increase, but reflected the number increase of MCs in lesional AA skin (data not shown).

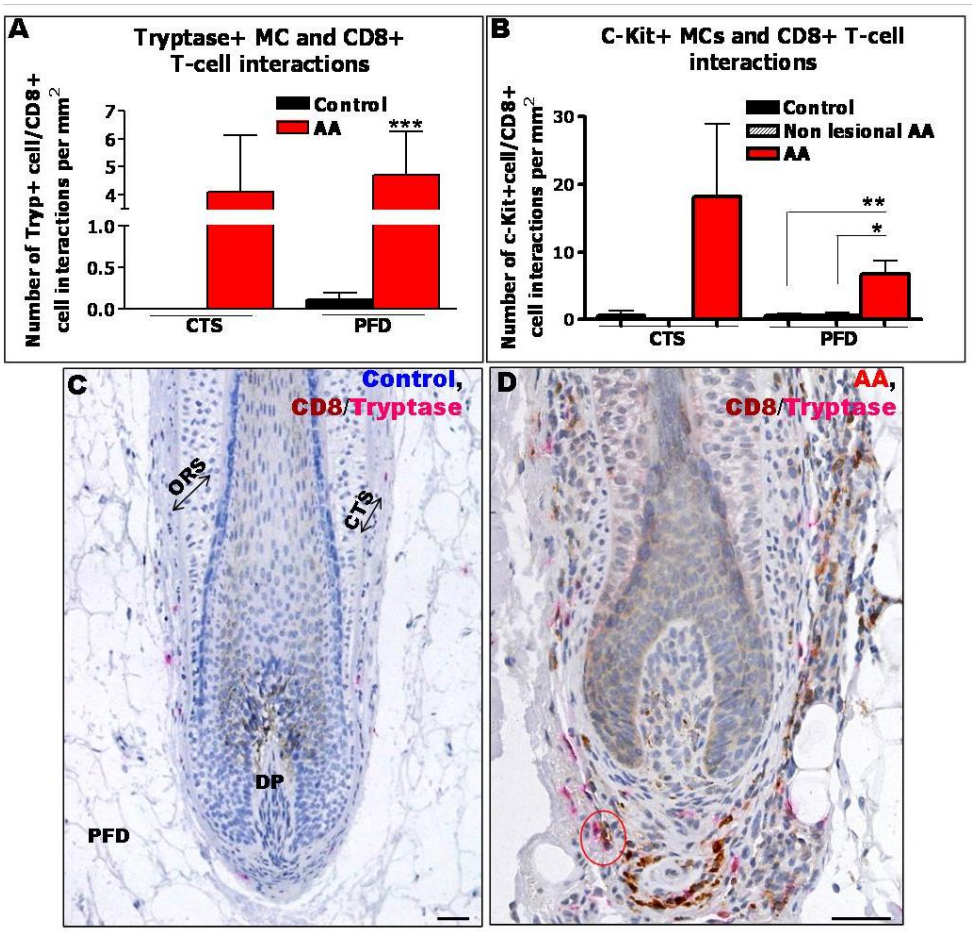

Figure 3.4: The number of perifollicular mast cell-CD8+ T-cell interactions is increased in alopecia areata lesional skin.
Quantitative analysis of tryptase+ MC-CD8+ T-cell interactions showing that their number is up-regulated in lesional compared to control skin (A). Analysis derived from 56 areas (HFs) from 13 AA patients and 44 areas (HFs) of 7 healthy controls, ***p≤0.001, ±SEM, p value was calculated by Mann-Whitney-U-Test. Quantitative analysis of c-Kit+ MC-CD8+ T-cell interactions showing that their number is up-regulated in AA lesional skin compared to AA non-lesional and control skin (B). This evaluation was performed in sections stained with CD30L/C-Kit/CD8 triple-IHC. Analysis deriving from 12-18 areas of 4-16 HFs of 6 healthy controls and 7 AA patients (non lesional skin only from 3 AA patient), **p≤0.01, *p≤0.05, ±SEM, Kruskal-Wallis test followed by Dunn´s multiple comparison test. Representative pictures showing tryptase+ MCs and CD8+ T-cells around HFs from control healthy (C) and AA lesional skin (D) stained by CD8/Tryptase double-IHC. Red circles indicate MC-CD8+ T-cell interaction. Pink cells are tryptase+ MCs and brown cells are CD8+ T-cells. Scale bars: 50µm. Connective tissue sheath (CTS), dermal papilla (DP), outer root sheath (ORS), perifollicular dermis (PFD).

The above results demonstrate for the first time that human skin MCs are able to directly interact with CD8+ T-cells in both healthy and AA affected human skin *in situ*. Moreover, that the number of MC-CD8+ T-cell interactions is increased and that MCs have pro-inflammatory phenotype, provides phenomenological support for the working hypothesis that MCs in AA lesional skin may promote pathogenic CD8+ T-cell responses.

3.1.2.1 Alopecia areata mast cells show prominent MHC class I immunoreactivity during their interaction with CD8+ T-cells

MCs are able to present autoantigens to CD8+ T-cells via MHC class I and to thus drive and control CD8+ T-cell-dependent immune responses (Stelekati et al. 2009). Therefore, after establishing a triple immunofluorescent staining for MHC class I (red signal), CD8 (green signal) and tryptase (blue signal) **(Figure 3.5A-B)**, we investigated whether MCs express MHC class I during the interaction with CD8+ T-cells in lesional AA skin.

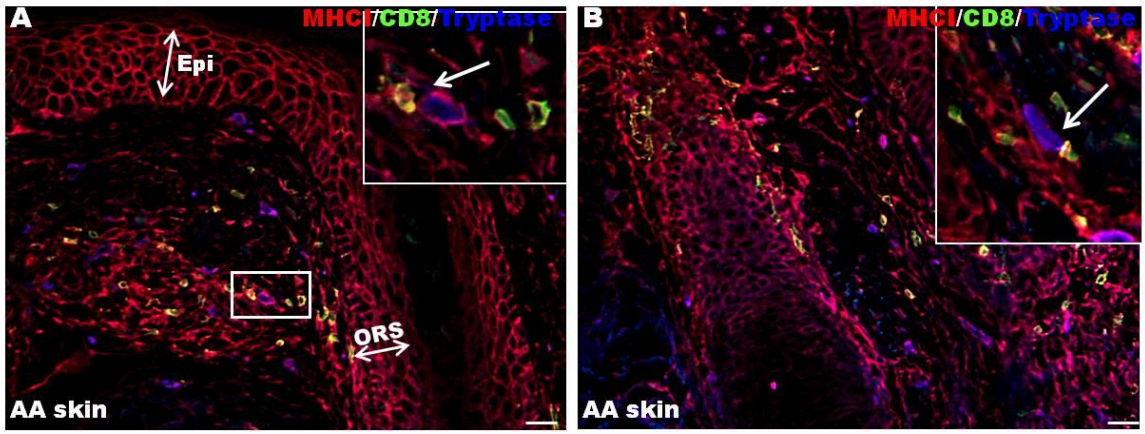

Figure 3.5: Alopecia areata mast cells show prominent MHC class I immunoreactivity during their interaction with CD8+ T-cells.
Representative pictures of MHCI(red)/CD8(green)/Tryptase(blue) showing the expression of MHC class I within MCs during the interaction with CD8+ T-cells in human AA lesional skin from AA patients (A-B). In small panels, higher magnifications of MHC class I+ MCs in close contact with CD8+ T-cells. Scale bars: 50µm. Epidermis (Epi), outer root sheath (ORS).

This showed that perifollicular human MCs strongly express MHC class I in lesional human AA skin, also when they physically interact with CD8+ T-cells **(Figure 3.5A,B**). Therefore, it is conceivable in principle that MCs are capable of presenting autoantigens to cognate CD8+ T-cells at some stage during AA pathogenesis.

3.1.2.2 Mast cells degranulate during their interaction with CD8+ T-cells in alopecia areata lesional skin

Upon activation, MCs can immediately release mediators stored in cytoplasmatic pre-formed granules, such as neutral proteases (tryptase, chymase), histamine, proteoglycans (heparin and chondroitin sulphate E) and cytokines like TNF-alpha (Christy et al. 2013, Frenzel and Hermine 2013, St John and Abraham 2013, Brown and Hatfield 2012, Gri et al. 2012, Harvima and Nilsson 2011, Frossi et al. 2010, Pejler et al. 2010, Shin et al. 2009, Galli et al. 2008) and therefore create a strong pro-inflammatory environment. Since most of these molecules have direct impact on CD8+ T-cells activation (Frenzel and Hermine 2013, Brown and Hatfield 2012, Gri et al. 2012, Tete et al. 2012, Harvima and Nilsson 2011, Tsai et al. 2011, Frossi et al. 2010, Galli et al. 2008, Sayed et al. 2008), it was next investigated

whether MCs degranulate during the interaction with CD8+ T-cells in AA lesional skin. This was done by assessing the degranulation status using CD8/Tryptase IHC.

Indeed, during MC-CD8+ T-cell interactions, almost 50% of MCs in AA lesional skin were found to be degranulated (when more than five granules located outside of the cell membrane, see chapter 2.6) **(Figure 3.6A-F)** while in control healthy skin most of them were non degranulated **(Figure 3.6G,H)**. Hence, in AA lesional skin, pre-formed MC mediators may activate CD8+ T-cells by binding the corresponding receptors of these lymphocytes (Frenzel and Hermine 2013, Brown and Hatfield 2012, Gri et al. 2012, Tete et al. 2012, Harvima and Nilsson 2011, Tsai et al. 2011, Frossi et al. 2010, Galli et al. 2008, Sayed et al. 2008).

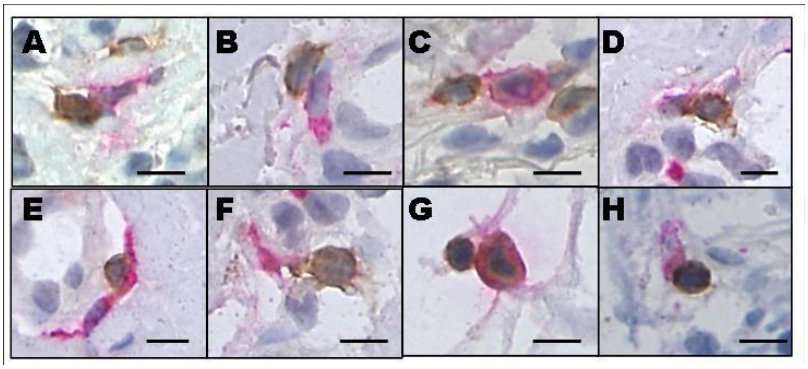

Figure 3.6: Half of the mast cells interacting with CD8+ T-cells are degranulated in alopecia areata lesional skin.
Immunohistochemical identification of tryptase+ MCs (pink cells) interacting with CD8+ T-cells (brown cells) in lesional skin from AA patients (A-F) and in control skin from healthy individuals (G-H). Non-degranulated MCs (A-C, G-H) and degranulated MCs (D-F). Scale bars: 10µm.

Taken together, the above results show that perifollicular MHC class I+ MCs interact with CD8+ T-cells much more frequently in AA lesional compared to control healthy skin.

3.1.3 Mast cells in lesional alopecia areata skin up-regulate co-stimulatory molecules for CD8+ T-cells

Given that MHC class I+ MC-CD8+ T-cell interactions are increased in AA lesional skin but that only half of MCs are degranulated during these contacts, this raised the question how MCs and CD8+ T-cells may interact in AA. It is known that the release of MC mediators is not the only mechanism by which MCs and CD8+ T-cells may talk to each other during their interaction, since MCs can express many cell surface molecules that are either co-stimulatory or inhibitory for CD8+ T-cells (see Introduction, chapter 1.6) (Frenzel and Hermine 2013, Brown and Hatfield 2012, Gri et al. 2012, Harvima and Nilsson 2011, Kalesnikoff and Galli 2011, Tsai et al. 2011, Galli et al. 2008, Harvima et al. 2008, Sayed et al. 2008). Therefore, as a first attempt towards dissecting these interactions *in situ*, triple-immunostainings were established for MCs, CD8+ T-cells and some of the best-

characterized co-stimulatory molecules known to modulate MC-CD8+ T-cells interactions: OX40L, CD30L, 4-1BBL and ICAM-1 (Frenzel and Hermine 2013, Brown and Hatfield 2012, Gri et al. 2012, Harvima and Nilsson 2011, Galli et al. 2008, Sayed et al. 2008). In order to be precise with the evaluation, for each established immunostaining the total number of MCs, CD8+ T-cells and MC-CD8+ T-cell interactions was first evaluated in order to confirm the pattern of expression for these antigens. Once these analyses had revealed similar results to the ones obtained with the double-staining of CD8 and tryptase by IHC, the expression of the corresponding co-stimulatory molecules by MCs was investigated. Moreover, for each triple-staining the correct positive control tissue was immunostained together with sample sections (see Material and methods, **Figure 2.6**).

3.1.3.1 The total number and the percentage of OX40L+ mast cells are significantly increased in alopecia areata lesional skin, at least compared to non-lesional skin.

OX40L+ MCs can stimulate CD8+ T-cell proliferation, survival, and/or cytokine production (Ilves and Harvima 2013, Zhang et al. 2013, Weinberg et al. 2011, Croft 2010, Ishii et al. 2010, Kober et al. 2008, Nakae et al. 2006, Kashiwakura et al. 2004). This raised the questions if MCs are expressing OX40L in healthy human skin *in situ* (unknown so far), whether OX40L expression is up-regulated in AA lesional MCs, and whether MCs interacting with CD8+ T-cells in AA lesional skin are expressing OX40L. To answer these questions lesional and non-lesional AA skin as well as healthy control skin sections were triple-stained for OX40L/CD8/Tryptase. This demarcated OX40L+/tryptase+ MCs as brown-pink cells (**Figure 3.7A,B**), OX40L-/tryptase+ MCs as pink cells and CD8+ T-cells as blue cells (**Figure 3.7C-F**).

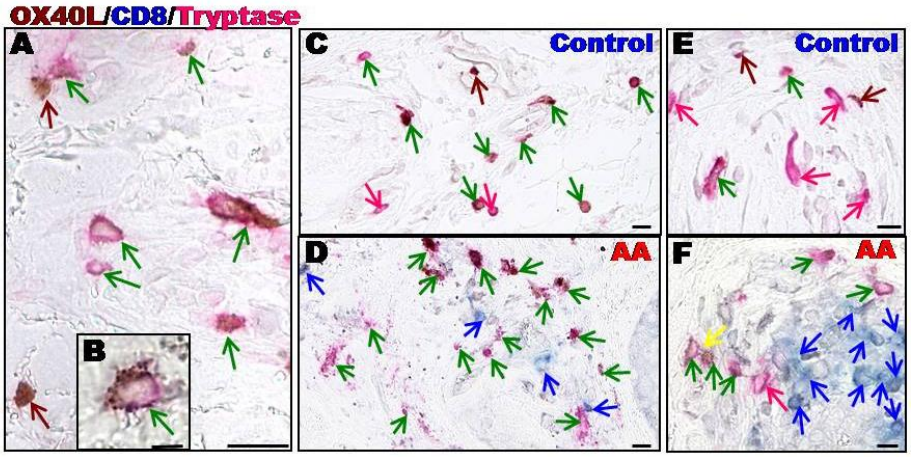

Figure 3.7: OX40L expression of mast cells in healthy control versus lesional alopecia areata skin. Immunohistochemical identification of OX40L/tryptase+ MCs, detected using OX40L/CD8/Tryptase staining, showing the expression pattern of OX40L within MCs (A). Higher magnification of an OX40L+/tryptase+ MC (B). Representative pictures of OX40L/CD8/Tryptase triple staining in human scalp skin of controls (C,E) and lesional skin from AA patients (D,F). Brown cells are OX40L+ (brown arrows), blue cells are CD8+ (blue arrows), pink cells are tryptase+ cells (pink arrows), pink-brown cells are OX40L+/tryptase+ cells (green arrows). Of note, that the yellow arrow indicate exchange of small molecules via pseudopodial extensions (Carroll-Portillo et al. 2012, Della Rovere et al. 2009). Scale bars: 20µm (A,C,D), 5µm (B) and 10µm (E,F).

In line with the literature (Croft 2010, Nakae et al. 2006), OX40L was found in the nuclei, cytoplasm or in the cell membrane of human skin MCs. Typically, the expression of OX40L within MCs was mostly localized at one side of the cells at the cell membrane **(Figure 3.7A-F)**. This feature could be related to the receptor binding, since three copies of OX40 interact with three ligand molecules creating an accumulation of OX40L molecules at one side of MC surface (Croft 2010).

Quantitative (immuno-)histomorphometry showed that the total number of OX40L+ MCs was increased in lesional skin of AA patients **(Figure 3.8A-C)**. However, the percentage of OX40L+ MCs among all tryptase+ MCs was significantly increased only compared to non-lesional AA skin but not compared to healthy skin **(Figure 3.8A,B,D)**. This intriguing result raises the possibility of a constitutive genetic abnormality in MCs of AA patients, which might be involved in AA pathogenesis by predisposing individuals prone to develop AA to OX40L-dependent activating interactions with CD8+ T cells.

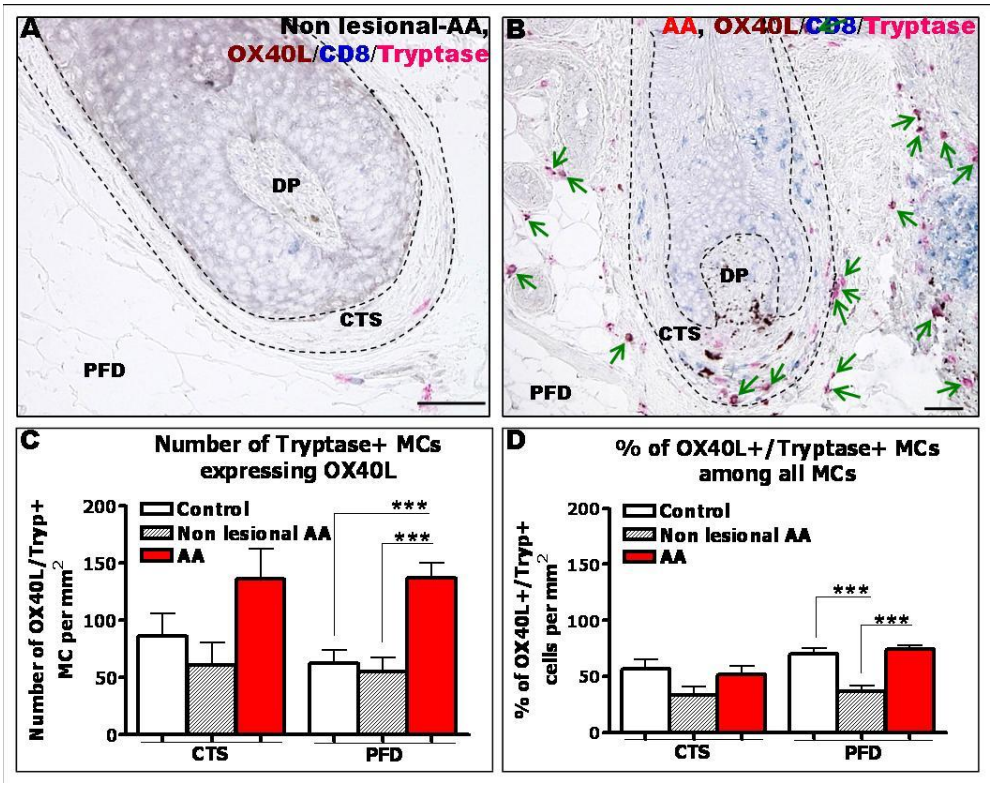

Figure 3.8: The total number and percentage of OX40L+ mast cells are increased in lesional alopecia areata skin compared to non-lesional skin.
Immunohistochemical identification of OX40L/Tryptase+ MCs and CD8+ T-cells in human hair bulb from scalp skin from non-lesional (A) and lesional (B) AA skin showing an increase of OX40L+ MC numbers around AA lesional HFs. Green arrows indicate OX40L+/tryptase+ MCs. Scale bars: 50µm (A,B). Quantitative analysis of OX40L+/tryptase+ MCs number (C) and % of OX40L+/tryptase+ MCs among all MCs (D) in AA lesional skin compared to controls which confirmed the qualitative observation. Analysis deriving from 20-23 areas of 12-14 HFs of 7 healthy controls and 8 AA patients (non lesional skin only from 4 AA patients), ***p≤0.001, ±SEM, One-Way ANOVA or Kruskal-Wallis test followed respectively by Bonferroni´s or Dunn´s multiple comparison tests. Connective tissue sheath (CTS), dermal papilla (DP), perifollicular dermis (PFD).

Most of the MCs interacting with CD8+ T-cells in healthy and non-lesional skin were expressing OX40L **(Figure 3.9A)**, however a significant up-regulation of OX40L+ MCs that were in direct contact with CD8+ T-cells was found in AA lesional skin **(Figure 3.9A-C)**. Unfortunately, it was not possible calculate the ratio for the small number of interactions in healthy and AA non-lesional skin.

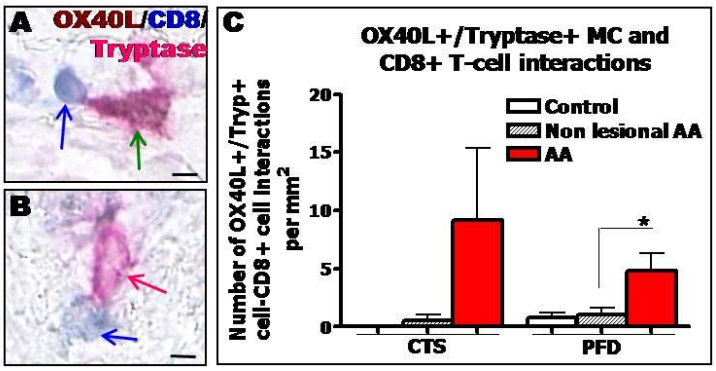

Figure 3.9: The number of OX40L+ mast cells interacting with CD8+ T-cells is increased in alopecia areata lesional skin.
Representative pictures of OX40L+ (A) and OX40L- (B) MCs (detected by tryptase) interacting with CD8+ T-cells. Blue cells are CD8+ T-cells (blue arrows), pink cells are tryptase+ MCs (pink arrows), pink-brown cells are OX40L+/tryptase+ MCs (green arrows). Scale bars: 5µm (A-B). Quantitative analysis reports an up-regulation of OX40L+/tryptase+ MCs interacting with CD8+ T-cells in AA lesional skin compared to controls (C). Analysis deriving from 20-23 areas of 12-14 HFs of 7 healthy controls and 8 AA patients (non lesional skin only from 4 AA patients), *p≤0.05, ±SEM, One-Way ANOVA or Kruskal-Wallis test followed respectively by Bonferroni´s or Dunn´s multiple comparison tests. Connective tissue sheath (CTS), perifollicular dermis (PFD).

These findings further corroborate the concept that MCs are phenotypically abnormal in lesional skin of AA patients and suggest that OX40L might be involved in the excessive interaction between MCs and CD8+ T-cells seen in AA skin.

3.1.3.2 The total number and percentage of CD30L+ mast cells is significantly up-regulated in alopecia areata lesional skin

The expression of CD30L by MCs is up-regulated under pro-inflammatory conditions (Diaconu et al. 2007, Fischer et al. 2006, Molin et al. 2001). Moreover, activated CD8+ T-cells can express CD30, which is implicated in the control of CD8+ T-cell proliferation and cytokine production (Croft et al. 2014, Cabrera et al. 2013, Zhang et al. 2013, Kober et al. 2008, Horie and Watanabe 1998, Gruss et al. 1996). In order to investigate whether CD30L+ MCs were up-regulated and if MCs during the interaction with CD8+ T-cells were expressing CD30L in AA lesional skin, it was attempted to establish a triple immunostaining technique for CD30L/C-Kit/CD8. However, this initially exposed major cross-reaction problems between the substrates AEC and DAB (not shown). Therefore, first, a double staining for CD30L/C-Kit was established and used for the evaluation of CD30L+ MC numbers in lesional and non-lesional AA skin from AA patients and control skin from healthy individuals. CD30L+/c-Kit+ MCs were revealed as red-blue cells and CD30L-/c-Kit+ MCs as blue cells (**Figure 3.10A-C**).

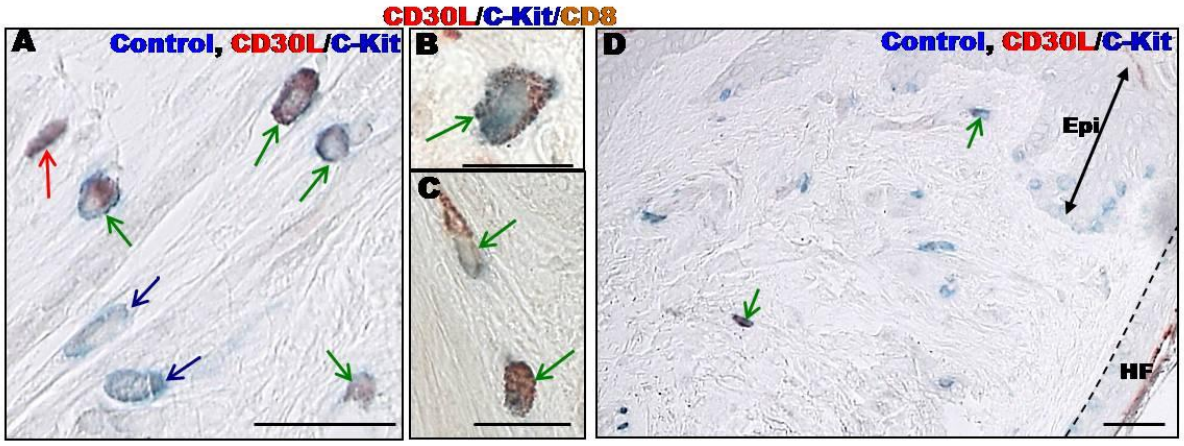

Figure 3.10: Immunohistochemical identification of CD30L expression by mast cells.
Representative pictures showing the expression pattern of CD30L within MCs in healthy control skin (A-D), detected using CD30L/C-Kit double staining (A,D) and CD30L/C-Kit/CD8 triple staining (B-C). Red cells are CD30L+ cells (red arrows), blue cells are c-Kit+ MCs (blue arrows), red-blue cells are CD30L+/c-Kit+ MCs (green arrows). Scale bars: 50µm (A,D) and 20µm (B,C). Epidermis (Epi), hair follicle (HF).

Only very few MCs positive for CD30L were detectable in non-lesional skin from AA patients and in control skin from healthy individuals **(Figure 3.10A-D** and **3.11A)**. Instead, in lesional AA skin, the number of CD30L+ MCs was significantly up-regulated (**Figure 3.11B**). In addition, the percentage of CD30L+ MCs among all MCs in the PFD was also significantly higher than in non-lesional skin from AA patients and healthy control skin (**Figure 3.11A-C**).

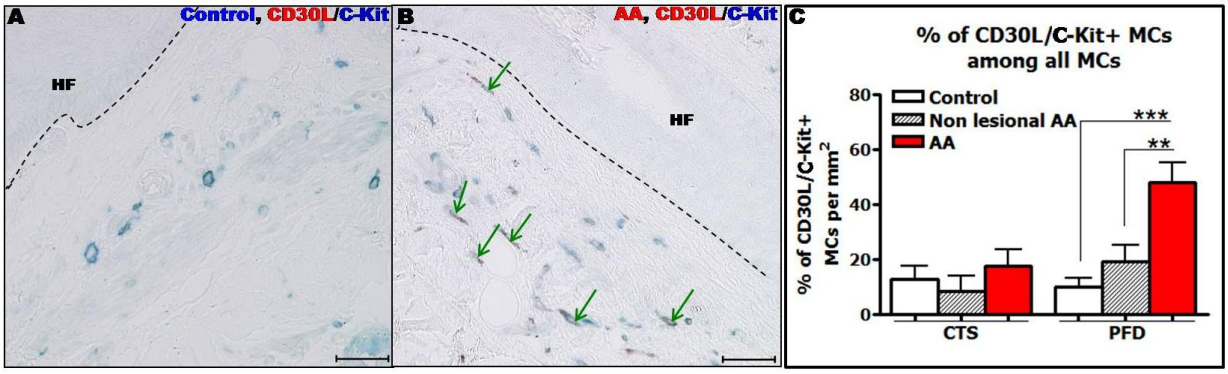

Figure 3.11: The total number and percentage of CD30L+ mast cells are increased in lesional alopecia areata skin.
Immunohistochemical identification of CD30L/c-Kit+ MCs in human HFs from healthy scalp skin (A) and lesional AA skin (B) showing an increase of CD30L+ MC numbers around AA lesional HFs. Green arrows indicate CD30L+/c-Kit+ MCs. Scale bars: 50µm. Quantitative analysis of the percentage of CD30L/c-Kit+ MCs among all MCs (C) in AA lesional skin compared to controls which confirmed the qualitative observation. Analysis deriving from 12-17 areas of 4-17 HFs of 6 healthy controls and 7 AA patients (non lesional skin only from 4 AA patients), ***p≤0.001, **p≤0.01, ±SEM, One-Way ANOVA or Kruskal-Wallis test followed respectively by Bonferroni´s or Dunn´s multiple comparison tests. Connective tissue sheath (CTS), hair follicle (HF), perifollicular dermis (PFD).

Later on, it became possible to establish the desired CD30L/C-Kit/CD8 staining avoiding the cross-reaction problems reducing the temperature and the time of incubation for the substrates. This led the possibility to investigate if CD30L+ MCs that were in close contact with CD8+ T-cells. This triple immunostaining was repeated once to confirm the pattern of expression. CD30L+ cells were detected as red cells, CD30L+ MCs as red-blue cells,

CD30L- MCs as blue cells and CD8+ T-cells as brown cells (**Figure 3.12A-D**). However, hardly any CD30L+ MCs were found to be in contact with CD8+ T-cells, neither in AA lesional skin, nor in non-lesional AA skin, nor in healthy control skin (**Figure 3.12A-C**). Only in one area out a total of 17 examined, it was possible to detect a CD30L+ MCs that was in close contact with CD8+ T-cells (interestingly, this skin section was from an AA patient) **(Figure 3.12D)**.

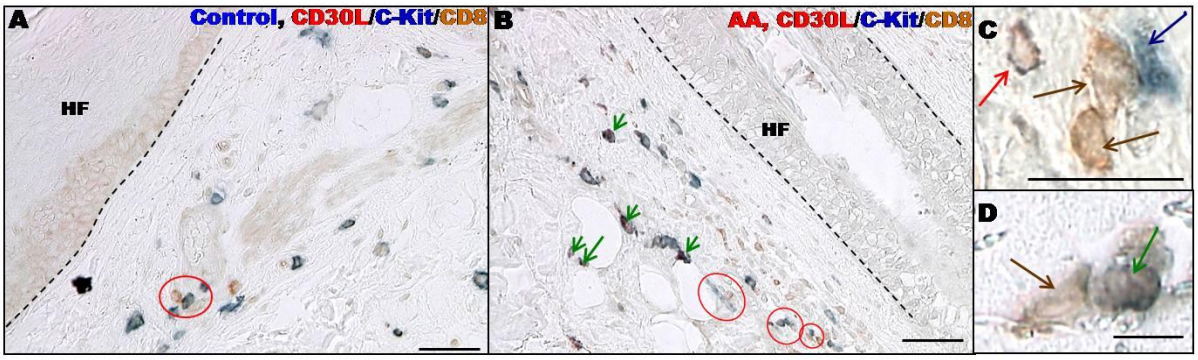

Figure 3.12: The number of CD30L+ mast cells interacting with CD8+ T-cells does not change in alopecia areata lesional skin compared to non-lesional alopecia areata and healthy controls skin.
Representative pictures of CD30L/C-Kit/CD8 triple staining in control (A) and AA lesional (B) skin showing an increased CD30L+ MC numbers in AA lesional skin and MC-CD8+ T-cell interactions. Representative pictures of CD30L- (C) and CD30L+ (D) (detected by c-Kit) MCs interacting with CD8+ T-cells. Brown cells CD8+ cells (brown arrows), blue cells are c-Kit+ MCs (blue arrows), red cells are CD30L+ cells (red arrows), blue-red cells are CD30L+/c-Kit+ MCs (green arrows). Red circles underline MC-CD8+ T-cell interactions. Of note, the brown cells in the HF bulge are mostly cytokeratin 15+ cells, not CD8+ T-cells. Scale bars: 50µm (A,B) and 20µm (C,D).

In sum, MCs in AA lesional skin up-regulate CD30L. However, since CD30L+ MCs do not appear to interact with CD8+ T-cells in AA lesional skin, CD30L-CD30 pathways might mediate MC interaction with other intracutaneous immunocytes (e.g. CD4+ T-cells), or with the soluble sCD30 (Velasquez et al. 2013), which is produced by the cleavage of membrane-bound CD30 by metalloproteinase (Hansen et al. 1995).

3.1.3.3 4-1BBL+ mast cells are slightly increased in alopecia areata lesional skin

4-1BBL is expressed by activated MCs (Stelekati et al. 2009, Kashiwakura et al. 2004, Sayama et al. 2002) and supports CD8+ T-cell survival/expansion after binding its receptor (4-1BB) on activated T-cells (Croft et al. 2014, Chacon et al. 2013, Vinay and Kwon 2012, Shao and Schwarz 2011, Wu et al. 2011, Kober et al. 2008, Wang et al. 2009, Watts 2005). In order to investigate whether the number of 4-1BBL+ MCs is up-regulated in lesional compared to non-lesional AA and control healthy skin, a 4-1BBL/C-Kit/CD8 triple-immunostaining was established, performed and analysed in which 4-1BBL+/c-Kit+ MCs were detected as blue-red cells, 4-1BBL-/c-Kit+ MCs as blue cells and CD8+ T-cells as brown cells **(Figure 3.13A)**.

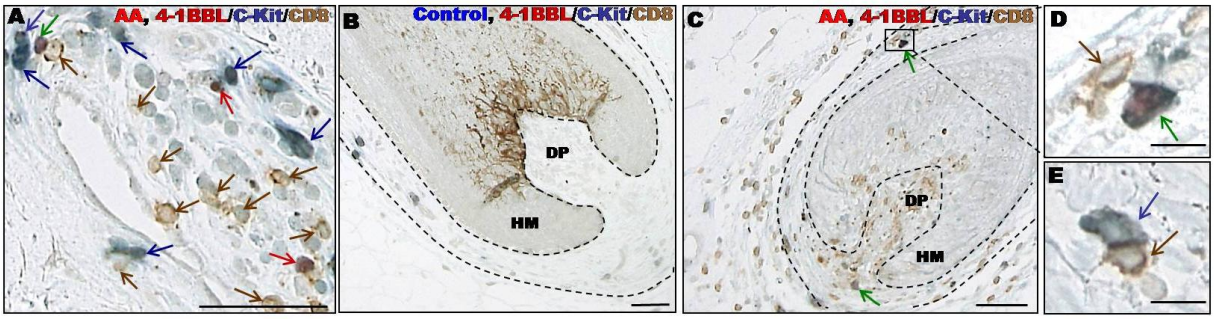

Figure 3.13: 4-1BBL+ cells are exceptionally rare in healthy and non-lesional alopecia areata skin, while more 4-1BBL+ mast cells are detectable in alopecia areata lesional skin.
Representative pictures showing the expression pattern of 4-1BBL within MCs in AA lesional skin detected by using 4-1BBL/C-Kit/CD8 triple staining (A). Immunohistochemical identification of 4-1BBL+/c-Kit+ and 4-1BBL-/c-Kit+ MCs, detected using 4-1BBL/C-Kit/CD8 staining around the HF bulb of control (B) and lesional skin of an AA patient (C) showing higher number of 4-1BBL+/c-Kit+ MCs in AA lesional skin. Representative pictures of 4-1BBL+/c-Kit+ (D), 4-1BBL-/c-Kit+ (E) MCs interacting with CD8+ T-cells. Brown cells are CD8+ T-cells (brown arrows), blue cells are c-Kit+ MCs, red cells are 4-1BBL+ cells (red arrows), blue-red cells are 4-1BBL+/c-Kit+ MCs (green arrows). Of note, the brown colour in panel B, in the HM is the melanin content of melanocytes, not CD8+ T-cells.This staining was observed in one section/subject of 8 healthy individuals and non-lesional skin from 4 AA patients and lesional skin from 11 AA patients. Scale bars: 50µm (A,B,C) and 10µm (D,E). Dermal papilla (DP), hair matrix (HM).

However, in lesional skin from AA patients the number of 4-1BBL+ cells was slightly increased and also more 4-1BBL+ MCs were detectable, notably in a peribulbar location **(Figure 3.13C)** (fitting to the typical peribulbar inflammatory infiltrate in AA) (McElwee et al. 2013, Gilhar et al. 2012, Cetin et al. 2009, Whiting 2003). 4-1BBL+ MCs were seen occasionally in close proximity to CD8+ T-cells in lesional AA skin **(Figure 3.13D)**.

The above results indicate that 4-1BBL+ MCs are up-regulated in AA lesional skin and raise the possibility that the 4-1BBL-4-1BB signalling pathway may be involved in the regulation of MCs-CD8+ T-cell interactions in AA lesional skin.

3.1.3.4 ICAM-1+ mast cells are slightly increased in alopecia areata lesional skin

Considering that MCs are able to present antigens to CD8+ T-cells (Stelekati et al. 2009) and that they can express ICAM-1 (Nagai et al. 2009, Galli et al. 2005), it is possible that ICAM-1 expressed by MCs play a role in MC-CD8+ T-cell interaction in AA. Moreover, ICAM-1 stored in exosomes derived from mouse bone marrow-derived MCs promotes T-cell proliferation and cytokine production (Skokos et al. 2001).

Therefore, the corresponding triple-immunostaining ICAM-1/CD8/Tryptase was established in order to analyse any changes on ICAM-1+ MCs number in AA lesional skin and any involvement of ICAM-1 in mediating MCs and CD8+ T-cell interactions in AA pathogenesis. In this staining, ICAM-1+ cells were detected as brown cells, CD8+ T-cells as blue cells, tryptase+ MCs as pink cells and ICAM-1+/tryptase+ MCs as brown-pink cells **(Figure 3.14A).**

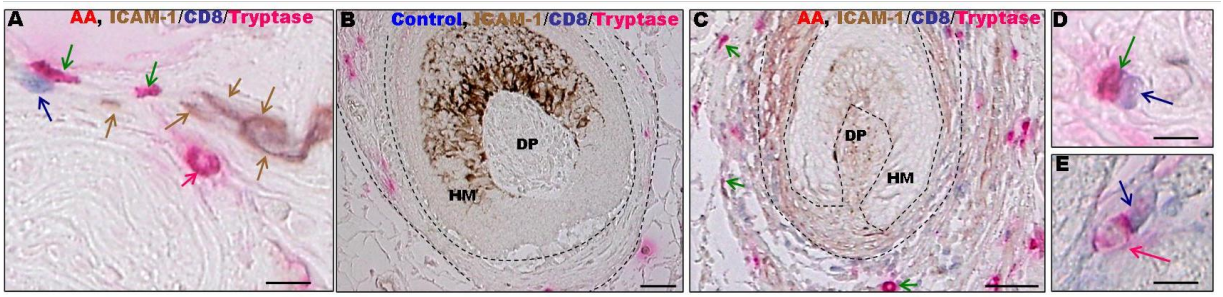

Figure 3.14: ICAM-1+ mast cells are slightly increased in alopecia areata lesional skin.
Representative pictures showing the expression pattern of ICAM-1 within MCs in AA lesional skin detected using ICAM-1/CD8/Tryptase triple staining (A). Immunohistochemical identification of ICAM-1+/tryptase+ MCs and ICAM-1-/tryptase+ MCs, detected using ICAM-1/CD8/Tryptase staining around the HF bulb of control (B) and lesional skin of an AA patient (C) showing higher ICAM-1+/tryptase+ MCs in AA lesional skin. Representative pictures of ICAM-1+/tryptase+ (D) and ICAM-1-/tryptase+ (E) MCs interacting with CD8+ T-cells. Brown cells are ICAM-1+ cells (brown arrows), blue cells are CD8+ T-cells (blue arrows), pink cells are tryptase+ MCs (pink arrows) and brown-pink cells are ICAM-1+/tryptase+ MCs (green arrows). Of note, the brown colour in panel B, in the HM is the melanin content of melanocytes, not CD8+ T-cells.This staining was observed in one section/subject of 6 healthy individuals and non-lesional skin from 4 AA patients and lesional skin from 11 AA patients. Scale bars: 20µm (A), 50µm (B,C) and 10µm (D,E). Dermal papilla (DP), hair matrix (HM).

In control skin and non-lesional skin from AA patients, only few ICAM-1+ cells and ICAM-1+ MCs were found **(Figure 3.14B).** In striking contrast, ICAM-1+ IR tremendously increased in AA lesional skin around affected hair bulbs **(Figure 3.14C)**, confirming previously published results (Mcdonagh et al. 1993). Moreover, a slight increase in the number of ICAM-1+ MCs was also found in AA lesional skin **(Figure 3.14C)**. However, only very few ICAM-1+ MCs were seen in close proximity to CD8+ T-cells **(Figure 3.14D)**.

Therefore, ICAM-1+ MCs are up-regulated in AA lesional skin and it is conceivable that the ICAM-1/LFA-1 signalling pathway may promote pathogenic MC-CD8+ T-cell interactions during AA pathogenesis.

Collectively, the above results further support the concept that MCs in AA are skewed towards pro-inflammatory activities and that namely OX40/OX40L, but possibly also 4-1BB/4-1BBL and/or ICAM-1/LFA-1 signalling pathways might be involved in regulating abnormal MC-CD8+ T-cell interactions in AA.

3.1.4 Immuno-inhibitory mast cells appear to be defective in alopecia areata lesional skin

Under physiological conditions, MCs can release immuno-inhibitory molecules and thus maintain peripheral tolerance and IP (Chan et al. 2013, Voehringer 2013, Gan et al. 2012, Kalesnikoff and Galli 2011, Tsai et al. 2011, Frossi et al. 2010, de Vries et al. 2009a, Sayed et al. 2008, Lu et al. 2006, Waldmann 2006), e.g. by releasing immunosuppressive cytokines such as IL-10 and TGFβ1 (Sayed et al. 2008, Waldmann 2006, Maurer et al. 2003). Considering the reduced TGFβ1 expression of perifollicular MCs *in situ* AA reported above

(Figure 3.1), it was interesting to check other MC immune-inhibitory markers are also expressed at reduced levels in AA skin.

Therefore, triple immunostainings were established in order to visualize MCs (either using c-Kit or tryptase), CD8+ T-cells and MCs positive for three selected immuno-inhibitory molecules known to dampen MC-CD8+ T-cells interactions: IL-10, PD-L1 and CD200 (Chan et al. 2013, Gan et al. 2012, Gri et al. 2012, Chacon-Salinas et al. 2011, Harvima and Nilsson 2011, de Vries et al. 2009b, Lu et al. 2006, Nakae et al. 2006). As explained before, the MC expression of these antigens was investigated only after having confirmed that the analyses of the total number of MCs, CD8+ T-cells and MC-CD8+ T-cell interactions gave similar results to the ones obtained with the CD8/Tryptase double-staining technique. Again, for each triple-staining the correct tissue for positive control was stained together with sample sections (see Material and methods, **Figure 2.6**).

3.1.4.1 The number of IL-10+ mast cells is significantly decreased in lesional and non-lesional alopecia areata skin

IL-10, a predominantly type II cytokine, is a potent inhibitor of antigen presentation and proinflammatory cytokine production (Soyer et al. 2013, Mosser and Zhang 2008, Groux et al. 1998). Depending on the environment and functional context, MCs can produce IL-10 and regulate peripheral tolerance (Chan et al. 2013, Gan et al. 2012, Gri et al. 2012, Chacon-Salinas et al. 2011, Harvima and Nilsson 2011, de Vries et al. 2009b, Lu et al. 2006).

To investigate the expression pattern of IL-10 within perifollicular MCs and whether IL-10 expression on MCs is changed in AA lesional skin or during the interaction with CD8+ T-cells, an IL-10/C-Kit/CD8 triple immunostain was established and executed in control healthy skin, lesional and non-lesional skin from AA patients: IL-10+ cells were labelled as red cells, IL-10-/c-Kit+ MCs as blue cells, CD8+ T-cells as brown cells, and IL-10+/c-Kit+ MCs as red-blue cells **(Figure 3.15A-C)**.

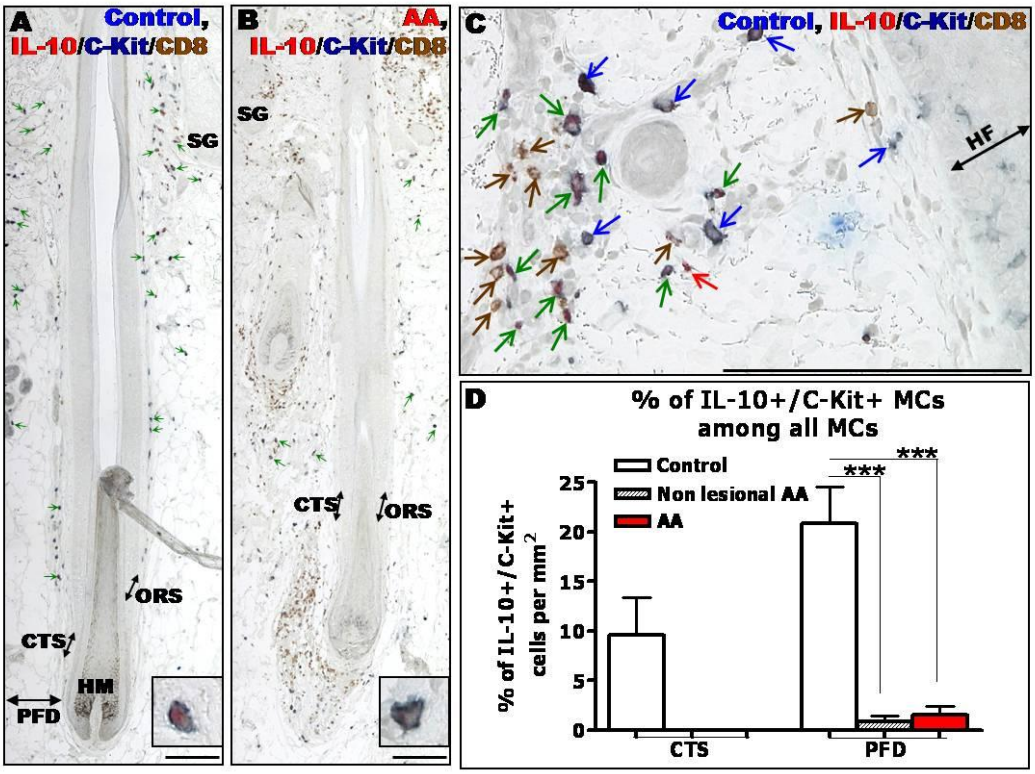

Figure 3.15: The number of IL-10+ mast cells is significantly decreased in lesional and non-lesional alopecia areata skin compared to healthy controls.
Immunohistochemical identification of IL-10+/c-Kit+ MCs and CD8+ T-cells in human scalp skin of controls (A) and AA lesional skin (B), higher magnification of a single MC in inserted panels showing the decreased expression of IL-10 in AA MCs. Higher magnification of IL-10/CD8/c-Kit triple staining (C). Blue cells are c-Kit+ MCs (blue arrows), blue-red cells are c-Kit+/IL-10+ MCs (green arrows), red cells are IL-10+ cells (red arrows) and brown cells are CD8+ T-cells (brown arrows) (C). Of note, the brown colour in panel A, in the HM is the melanin content of melanocytes, not CD8+ T-cells. Scale bars: 200µm. Quantitative analysis of IL-10+ MCs (detected by c-Kit) in lesional skin compared to non-lesional skin from AA patients and control skin from healthy individuals revealing a down-regulation in IL-10+ MCs in AA patients (D). Analysis derived from 14-20 areas of 5-16 HFs of 7 healthy controls and 7 AA patients (for non lesional skin only from 3 AA patients), ***p≤0.001, ±SEM, Kruskal-Wallis test followed by Dunn´s multiple comparison test. Connective tissue sheath (CTS), hair follicle (HF), hair matrix (HM), outer root sheath (ORS), perifollicular dermis (PFD), sebaceous glands (SG).

Interestingly, most of the cells demarcated with this immunostaining technique were seen to express prominent IL-10 IR in healthy human skin were in fact MCs, namely in the CTS and PFD **(Figure 3.15A,C)**. However, the number of IL-10+ MCs was significantly decreased in lesional and non-lesional AA skin compared to healthy controls **(Figure 3.15A-D)**. The few IL-10+ MCs which remained visible in AA lesional skin were localized around sweat glands, SG and blood vessels, and were seen only rarely in the perifollicular mesenchyme (i.e CTS or PFD) **(Figure 3.15B)**. Moreover, IL-10 protein expression of individual MCs was decreased in AA skin compared to healthy controls (**Figure 3.15A,B**, see higher magnification insert).

Generally, MCs that interacted with CD8+ T-cells did not express IL-10 IR, neither in healthy nor in AA skin **(Figure 3.16A-E)**. We could identify only one incident of IL-10+ MCs interacting with CD8+ cells in an AA patient (lesional skin) and two in healthy controls

(Figure 3.15B,C). Obviously, this does not provide a basis for any meaningful quantitative analysis.

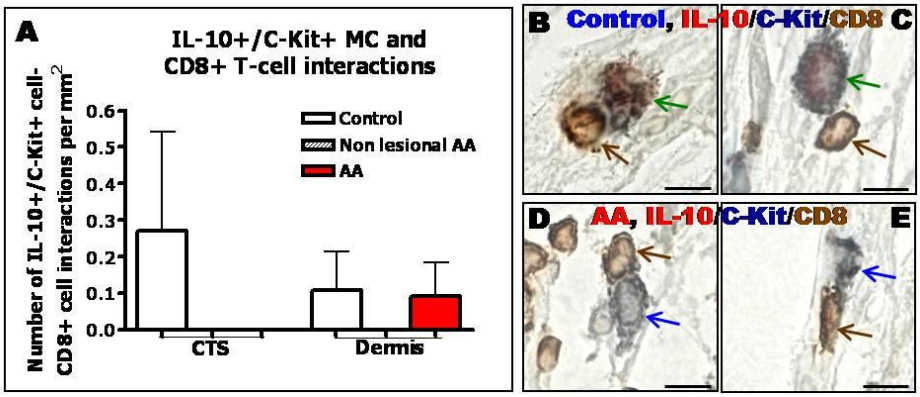

Figure 3.16: The number of IL-10+ mast cells interacting with CD8+ T-cells does not change in alopecia areata lesional skin compared to non-lesional alopecia areata and healthy controls skin.
Quantitative analysis of IL-10+ MCs (detected by c-Kit) interacting with CD8+ T-cells in lesional skin compared to non-lesional skin from AA patients and control skin from healthy individuals (A) which reveals no change between those cases. Analysis derived from 14-20 areas of 5-16 HFs of 7 healthy controls and 7 AA patients (for non-lesional skin only from 3 AA patients), ns, ±SEM, Kruskal-Wallis test followed by Dunn´s multiple comparison test. Representative pictures of IL-10+ (B,C) and IL-10- (D,E) MCs (detected by c-Kit) interacting with CD8+ T-cells in control skin (B,C) and AA lesional skin (D,E). Blue cells are c-Kit+ MCs (blue arrows), blue-red cells are c-Kit+/IL-10+ MCs (green arrows) and brown cells are CD8 + T-cells (brown arrows). Scale bars: 10µm. Connective tissue sheath (CTS), perifollicular dermis (PFD).

Thus, many IL-10+ MCs populate healthy control skin, while their number drastically decreases in AA lesional skin. However, the expression of IL-10+ within MCs may not be directly related to their interaction with CD8+ T-cells, because only seldomly IL-10+ MCs appear to be interacting with CD8+ T-cells.

3.1.4.2 The number of PD-L1+ mast cells seems to be reduced in lesional alopecia areata skin

Mouse bone marrow derived MCs constitutively express PD-L1 and its expression is slightly increased after IgE and Ag-stimulation (Nakae et al. 2006). However no data on PD-L1 expression is available for human MCs. Therefore, a triple immunostaining for PD-L1/C-Kit/CD8 was established and performed in healthy control, non lesional and lesional AA skin in order to explore if MCs in human skin express PD-L1. Moreover, evidence for any changes of PD-L1 IR in AA MCs was searched, together with a possible interaction between PD-L1+ MCs and CD8+ T-cells. The corresponding staining revealed PD-L1+ cells as red cells, c-Kit+ MCs as blue cells, CD8+ T-cells as brown cells and PD-L1+/c-Kit+ cells as red-blue cells **(Figure 3.17A-E)**.

These analyses provided the first evidence in the literature that primary human MCs can express varying levels of PD-L1 *in situ* in healthy human skin **(Figure 3.17A-D).** Qualitatively, the number of PD-L1+ MCs appeared to be reduced in lesional AA compared to healthy skin

(Figure 3.17E). However the total number of PD-L1+ cells was too low to permit a quantitative analysis.

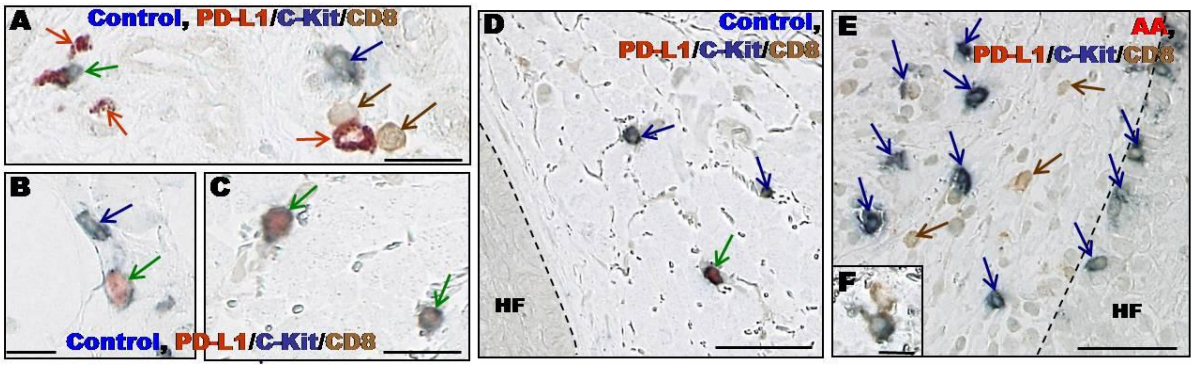

Figure 3.17: The number of PD-L1+ mast cells appears to be reduced in lesional alopecia areata compared to non-lesional alopecia areata and healthy skin.
Immunohistochemical identification of PD-L1+/c-Kit+ MCs and CD8+ T-cells in human healthy skin (A). Representative pictures showing low (B) and high (C) PD-L1 IR of human healthy skin MCs *in situ*. Immunohistochemical identification of perifollicular PD-L1+/c-Kit+ MCs and CD8+ T-cells in human healthy skin (D) and AA patient (E) enlightening the decreased PD-L1+ MC numbers in AA lesional skin. Higher magnification showing PD-L1- MC interacting with CD8+ T-cells (F). Brown arrows indicate CD8+ T-cells, blue arrows indicate c-Kit+ MCs, red arrows indicate PD-L1+ cells, green arrows indicate PD-L1+/c-Kit+ MCs. This staining was carefully observed in one section/subject of 8 healthy individuals and non-lesional skin from 4 AA patients and lesional skin from 12 AA patients. Scale bars: 20µm (A-C) and 50µm (D-E). Hair follicle (HF).

Overall, PD-L1+ MCs were not found to interact with CD8+ T-cells, neither in healthy nor in AA skin **(Figure 3.17A-E)**.

Shortly, PD-L1+ MCs inhabit healthy control skin and their number seems to decrease in AA lesional skin. However, PD-L1+ MCs do not appear to directly contact CD8+ T-cells, since these two cell populations were not found to be physically interacting.

3.1.4.3 Human skin displays almost no CD200+ mast cells

The final functional MC marker we examined in this series of experiments, was the important immuno-inhibitory “no danger-signal”, CD200, which plays a key role in HF-IP maintenance (Harries et al. 2013, Meyer et al. 2008, Rosenblum et al. 2006) and whose interaction partner is expressed on T-cells (Rygiel and Meyaard 2012). Therefore, a CD200/CD8/Tryptase triple staining was established and performed in healthy control, non-lesional and AA lesional skin with the intention of examining whether CD200 is expressed by MCs in skin, if its IR changes in AA lesional skin and if CD200+ MCs are interacting with CD8+ T-cells. As shown in **Figure 3.18A-D**, CD200+ cells were labeled as brown cells, CD8+ T-cells as blue cells, tryptase+ MCs as pink cells.

This staining revealed that almost no CD200+ MCs were found, neither in healthy human skin nor in AA lesional skin **(Figure 3.18A-D)**, in line with a previous report (Cherwinski et al.

2005). For this reason, it was not possible to detect CD200+ MCs double-positive cells which should have been depicted as pink-brown cells.

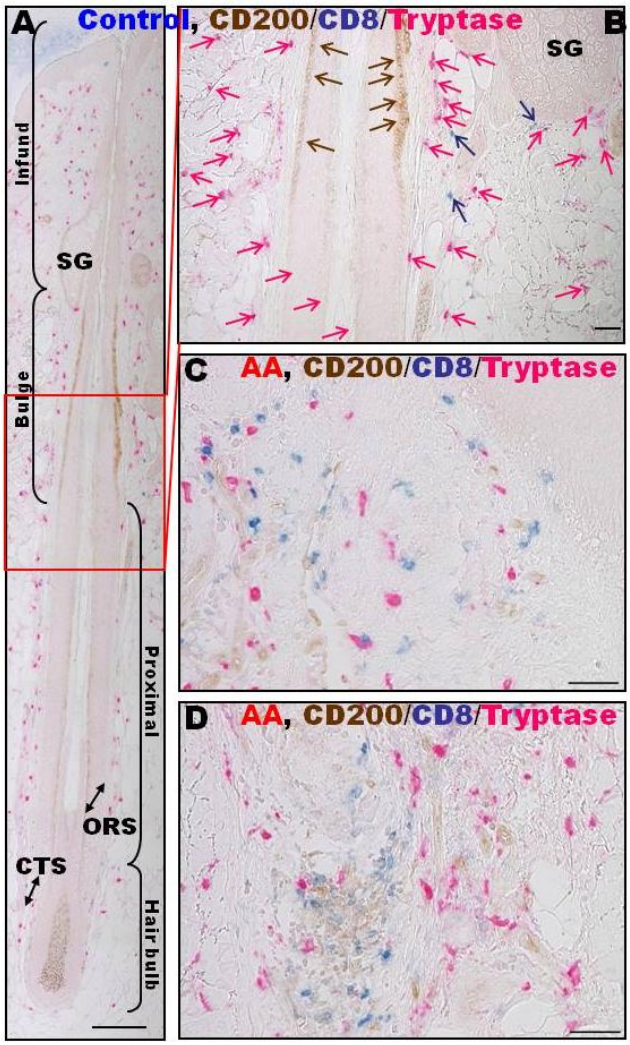

Figure 3.18: There are almost no CD200+ mast cells in healthy or alopecia areata-afflicted human skin.
Representative picture of a control HFs showing the expression pattern of CD200 in the bulge and blood vessels, tryptase+ MCs and CD8+ T-cells detected by CD200/CD8/tryptase triple immunohistochemistry (A). Higher magnification of the bulge area delineated in the red rectangle in panel A (B). Immunohistochemical identification of CD200+, tryptase+ and CD8+ T-cells in AA lesional skin (C,D). Brown cells are CD200+ cells (brown arrows), blue cells are CD8+ T-cells (blue arrows) and pink cells are tryptase+ MCs (pink arrows). Scale bars: 200µm (A) and 50µm (B-D). Connective tissue sheath (CTS), infundibulum (Infund), outer root sheath (ORS), perifollicular dermis (PFD), sebaceous glands (SG).

Briefly, the observation that MCs in healthy skin express classical immuno-inhibitory molecules (IL-10, PD-L1) supports the hypothesis that, physiologically, perifollicular MCs mainly have tolerance-promoting functions (Waldmann 2006). Furthermore, that MC expression of selected immuno-inhibitory proteins was reduced in AA skin further suggest that MCs in AA are skewed towards pro-inflammatory activities and that MC-CD8+ T-cells interactions in AA are predominantly pro-inflammatory.

Taken together these phenomenological *in situ* results support the working hypothesis that under physiological conditions, immune-inhibitory MCs are able to control CD8+ T-cells, preserving the HF-IP and peripheral tolerance, while under pathological conditions, such as in the skin of AA patients, abnormal, pro-inflmmatory MCs can activate pathogenic CD8+ T-cells and contribute to HF-IP collapse, break of peripheral tolerance, and thus the onset of AA.

3.2 *Ex-vivo* organ culture experiments do not allow one to functionally probe mast cell and CD8+ T-cell interactions *in situ*

Given that the above results suggested a role for abnormal MC-CD8+ T-cell interactions in AA pathogenesis, development of a functional model was desirable in order to further probe the working hypothesis summarized above. Considering the well accepted use of SP and compound 48/80 in the modulation of MC activities, also in a hair research context (Sugawara et al. 2012, Peters et al. 2007, Siebenhaar et al. 2007, Arck et al. 2005, Ito et al. 2005, Maurer et al. 1997), human HF and full-thickness skin organ culture were taken into consideration as possible models, using SP and compound 48/80 as MC secretagogues. SP is an endogenous MC secretagogue which also promotes human HF-IP collapse (Peters et al. 2007) and activates CD8+ T-cells in mice (Siebenhaar et al. 2007). Compound 48/80 is the most frequently employed exogenous MC secretagogue (Sugawara et al. 2012) whose effects on human CD8+ T-cells remain to be explored. Therefore, the usage of these two MC activators might allow one to functionally manipulate MC-CD8+ T-cell interactions (utizing SP as an endogenous activator of both cell populations, and compound 48/80 as a selective exogenous stimulator of MC degranulation).

3.2.1 Hair follicle organ culture is not suitable to investigate mast cell-CD8+ T-cell interactions

In order to probe the effects of MC secretagogues on MC-CD8+ T-cell interactions, human HF organ culture was performed as previously described (Sugawara et al. 2012, Peters et al. 2007), treating HFs for 24 hours with SP (10^{-8}M and 10^{-10}M) or compound 48/80 (5µg/ml) **(Figure 3.19A-D)**. After embedding in cryomatrix, HFs were cut and double-immunostained for Tryptase/CD8 and C-Kit/CD8, with MCs demarcated as green cells and CD8+ T-cells as red cells **(Figure 3.19A-D)**.

As expected (Sugawara et al. 2012, Peters et al. 2007), many MCs populated the HF CTS, and their number increased after SP treatment **(Figure 3.19B,C)** (qualitative observation). Moreover, SP promoted catagen involution **(Figure 3.19B,C**) (qualitative observation), as previously described (Peters et al. 2007, Siebenhaar et al. 2007, Maurer et al. 1997). Unfortunately, the total number of detectable CD8+ T-cells in the HF's CTS was extremely low under assay conditions (i.e. a total of only 4 CD8+ cells could be visualized in 26 analyzed HFs) so that their interactions with MCs could not be meaningfully investigated **(Figure 3.19A-D)**.

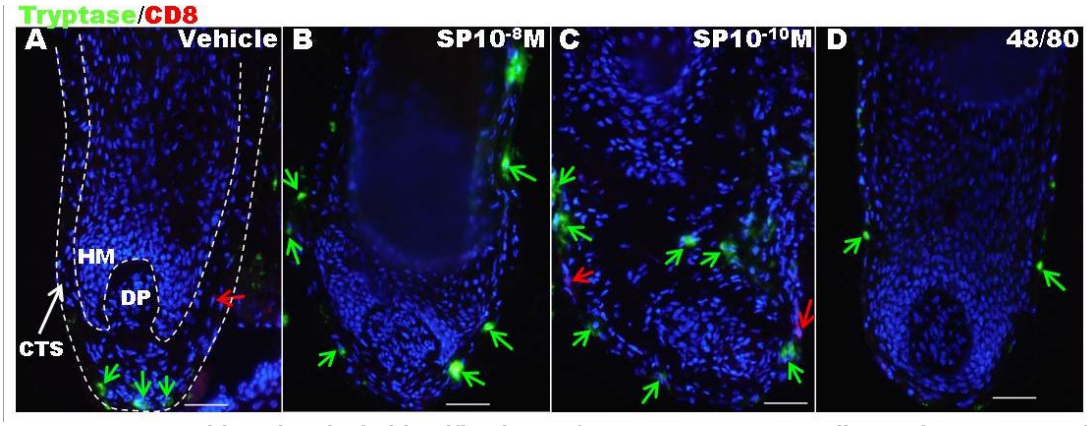

Figure 3.19: Immunohistochemical identification of tryptase+ mast cells and CD8+ T-cells in microdissected human HFs treated with endogenous or synthetic mast cell secretagogues.
Representative pictures of Tryptase/CD8 double-staining showing that in the CTS of HFs treated with vehicle (A), $SP10^{-8}M$ (B), $SP10^{-10}M$ (C) and compound 48/80 (D), the number of CD8+ T-cells is too low to study the effect of MC secretagogues on MC-CD8+ T-cell interactions. Green cells are tryptase+ MCs (green arrows) and red cells are CD8+ T-cells (red arrows). Scale bars: 50µm. Connective tissue sheath (CTS), dermal papilla (DP), hair matrix (HM).

Therefore, the current human HF organ culture assay cannot be used for further studying and manipulating MC-CD8+ T-cell interactions.

3.2.2 In healthy human skin organ culture, the effects of MC secreragogues on mast cell-CD8+ T-cell interactions show major interindividual differences

In view of the extremely low CD8+ T-cell numbers in the mesenchyme of microdissected HFs, next, full thickness healthy human scalp skin organ culture (Lu et al. 2007) was employed as an alternative method to functionally manipulate MC-CD8+ T-cell interactions in human skin. In healthy human scalp skin fragments, higher number of CD8+ T-cells was detectable (i.e. 9,26±1,98 positive cells/mm^2 after 3 day culture in vehicle group). Therefore, 4mm punches from healthy human scalp skin were organ-cultured and treated with the endogenous MC secretagogue, SP ($10^{-8}M$ and $10^{-10}M$), or the exogenous standard secretagogue, compound 48/80 (5µg/µl), for 24 or 72 hours. After the treatment, the skin punches were fixed in 4% formalin and embedded in paraffin. The skin sections were then stained for for Ki-67/C-Kit and for CD8/Tryptase by which tryptase+ cells were detected as pink cells and CD8+ T-cells as brown cells **(Figure 3.20A-D and 3.21A-D)**.

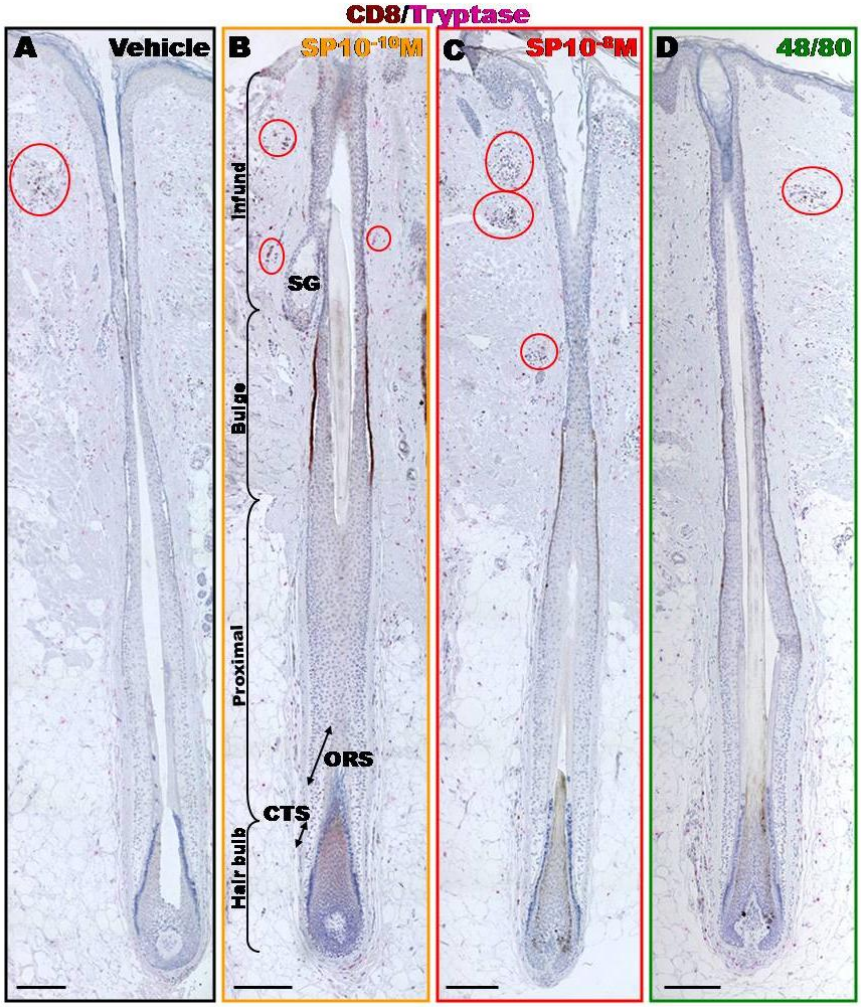

Figure 3.20: Immunohistochemical identification of tryptase+ mast cells and CD8+ T-cells around hair follicles from secretagogue-treated, organ-cultured healthy human skin fragments (full-thickness).
Representative pictures showing tryptase+ MCs and CD8+ T-cells stained by CD8/Tryptase double-IHC of HFs deriving from human healthy scalp skin punches treated with vehicle (only medium) (A), $SP10^{-10}M$ (B), $SP10^{-8}M$ (C) and compound 48/80 (D). Pink cells are tryptase+ MCs and brown cells are CD8+ T-cells. Of note, the brown colour in the HF bulge are mostly cytokeratin 15+ cells, not CD8+ T-cells. Red circles indicate aggregation of tryptase+ MCs and CD8+ T-cells in which occasionally is possible to find MC-CD8+ T-cell interactions. Scale bars: 200µm. Connective tissue sheath (CTS), infundibulum (infund), outer root sheath (ORS), sebaceous glands (SG).

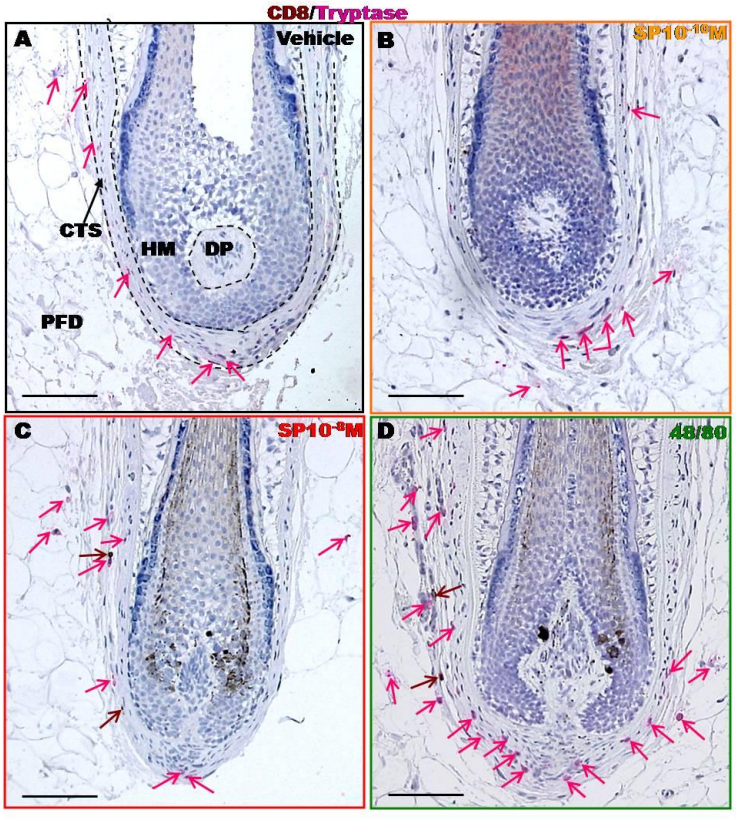

Figure 3.21: Immunohistochemical identification of tryptase+ mast cells and CD8+ T-cells around hair bulbs of secretagogue-treated human skin fragments.
Higher magnification of HF peribulbar area showing tryptase+ MCs and CD8+ T-cells stained by Tryptase/CD8 double-IHC of HFs deriving from human healthy scalp skin punches treated with vehicle (only medium) (A), $SP10^{-10}M$ (B), $SP10^{-8}M$ (C) and compound 48/80 (D). In panels C and D is possible to visualize MC-CD8+ T-cell interactions. Pink cells are tryptase+ MC (pink arrows) and brown cells are CD8+ T-cells (brown arrows). Of note, the brown colour in the HM is the melanin content of melanocytes, not CD8+ T-cells. Scale bars: 100µm. Connective tissue sheath (CTS), dermal papilla (DP), hair matrix (HM), perifollicular dermis (PFD).

The number of tryptase+ MCs, c-Kit+ MCs, CD8+ T-cells, MC-CD8+ T-cell complexes and MC degranulation were evaluated in the HF CTS and PFD. In skin from the first patient SP, but not compound 48/80, was found to slightly increase the number of tryptase+ (mature) **(Figure 3.22A)** and c-Kit+ (mature and immature) **(Figure 3.22B)** MCs, either in CTS or PFD, after 24 hours of treatment. However, this effect was not significant. Also, under these assay conditions, non significant influence on human skin MC degranulation *in situ* could be seen **(Figure 3.22C)**.

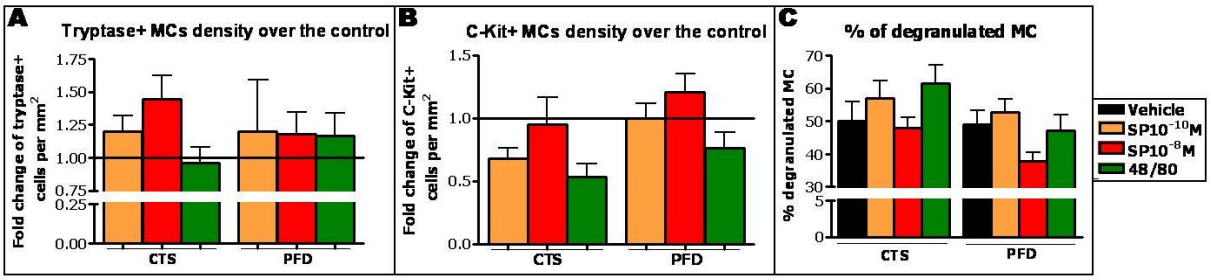

Figure 3.22: The number, but not the degranulation, of mast cells is slightly increased after 24 hours of treatment with substance P.
Quantitative analysis of tryptase+ MCs showing that the number of mature MCs is up-regulated after the treatment with SP but not after compound 48/80 treatment (A). Black line indicates vehicle. Analysis deriving from 22-32 areas from 14-25 HFs/group divided in upper dermis, dermis and subcutis, ns, ±SEM, Kruskal-Wallis test followed by Dunn´s multiple comparison test. Quantitative analysis of c-Kit+ MCs number showing that the number of mature and immature MCs is slightly up-regulated after the treatment with SP but not after compound 48/80 treatment (B). Black line indicates vehicle. Analysis deriving from 19-22 areas from 10-16 HFs/group divided in upper dermis, dermis and subcutis, ns, ±SEM, One-Way ANOVA followed by Bonferroni´s comparison test. Quantitative analysis of the percentage of tryptase+ MCs showing that the degranulation status of mature MCs does not change after SP or compound 48/80 treatment (C). Analysis deriving from 22-32 areas from 14-25 HFs/group divided in upper dermis, dermis and subcutis, ns, ±SEM, One-Way ANOVA followed by Bonferroni´s comparison test. Connective tissue sheath (CTS), perifollicular dermis (PFD).

The treatment with SP, whose receptor is also expressed by lymphocytes (Siebenhaar et al. 2007), also increased the number of CD8+ T-cell number while no change was detected after treatment with compound 48/80 **(Figure 3.23A)**.

Surprisingly, SP and compound 48/80 treatments decreased the number of MC-CD8+ T-cell interactions in the CTS and only SP up-regulated them in the PFD **(Figure 3.23B).** However, again, the frequency of detectable MC-CD8+ T-cell contacts was too low to obtain results that were statistically significantly different.

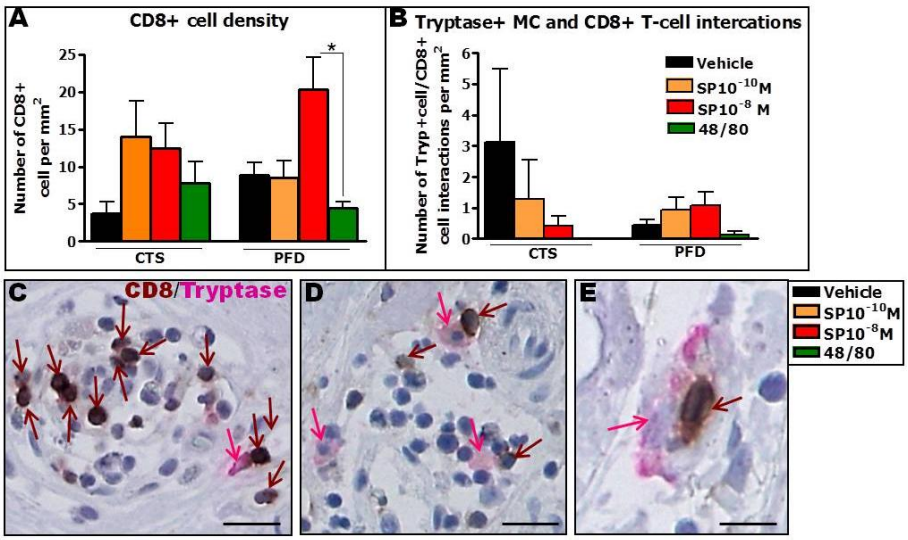

Figure 3.23: CD8+ T-cells are increased while the number of tryptase+ mast cell-CD8+ T-cell interactions is decreased in the coonective tissue sheath after 24 hours of substance P treatment. Quantitative analysis of CD8+ T-cell number showing that their number is up-regulated after SP, but not after compound 48/80 treatment (A). Quantitative analysis of MC-CD8+ T-cell interactions showing that their number decreases in the HF CTS after SP and compound 48/80 treatments (B). Analysis deriving from 22-32 areas from 14-25 HFs/group divided in upper dermis, dermis and subcutis, *p≤0.05, ±SEM, Kruskal-Wallis test followed by Dunn´s multiple comparison test. Representative pictures of Tryptase/CD8 double-IHC showing MC-CD8+ T-cell interactions. Pink cells are tryptase+ MCs (pink arrows) and brown cells are CD8+ T-cells (brown arrows) (C-E). Scale bars: 20µm (C,D) and 10µm (E). Connective tissue sheath (CTS), perifollicular dermis (PFD).

The numbers of Ki-67+/tryptase+ cells and Ki-67+/c-Kit+ cells were also evaluated to investigate whether SP or compound 48/80 might stimulate human skin MC proliferation *in situ*. These double-immunostainings revealed MCs as pink cells, Ki-67+ cells as black/brown cells and Ki-67+/tryptase+ or c-Kit+ cells as pink-black/brown cells **(Figure 3.24C)**. However, any detectable proliferation events were extremely rare **(Figure 3.24A-B),** so that the results obtained for test and control groups failed to reach a significant level of difference and thus remained inconclusive.

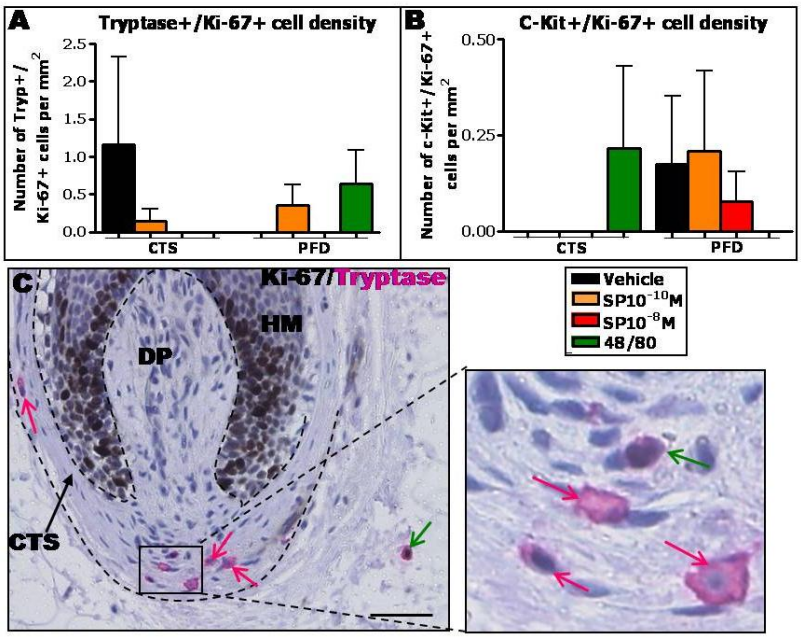

Figure 3.24: The number of Ki-67+/tryptase+ mast cells and Ki-67+/c-Kit+ mast cells were too low for obtaining meaningful quantitative data. Quantitative analysis of Ki-67+/tryptase+ MCs showing that their number was low in vehicle and treated groups (A). Analysis deriving from 13-15 areas from 5-9 HFs/group divided in upper dermis, dermis and subcutis, ns, ±SEM, Kruskal-Wallis test followed by Dunn´s multiple comparison test. Quantitative analysis of Ki-67+/c-Kit+ MCs showing that their number was low in vehicle and treated groups (B). Analysis deriving from 19-22 areas from 10-16 HFs/group divided in upper dermis, dermis and subcutis, ns, ±SEM, Kruskal-Wallis test followed by Dunn´s multiple comparison test.Representative pictures of Ki-67/Tryptase double-IHC showing Ki-67+/tryptase+ MCs around a hair bulb. Pink cells are tryptase+ MCs (pink arrows) and black cells are Ki-67+ cells, pink-black cells are Ki-67+/tryptase+ MCs (green arrows) (C). Scale bar: 50µm. Connective tissue sheath (CTS), hair matrix (HM), perifollicular dermis (PFD).

Since SP stimulates both catagen and HF-IP collapse (Peters et al. 2007, Siebenhaar et al. 2007, Maurer et al. 1997), the expression of MHC class I and TGFβ1 were analyzed *in situ* in HFs from skin punches treated with SP and compound 48/80 for 24 hours, after performing the corresponding staining. Both SP and compound 48/80 up-regulated MHC class I protein expression (green signal) in the HM, CTS and ORS of organ-cultured human HFs **(Figure 3.25A-C)**. TGFβ1 protein expression in the HF's ORS remained unchanged, though **(Figure 3.25D)**. Therefore, in the present assay not only SP, but also compound 48/80, promote HF-IP collapse.

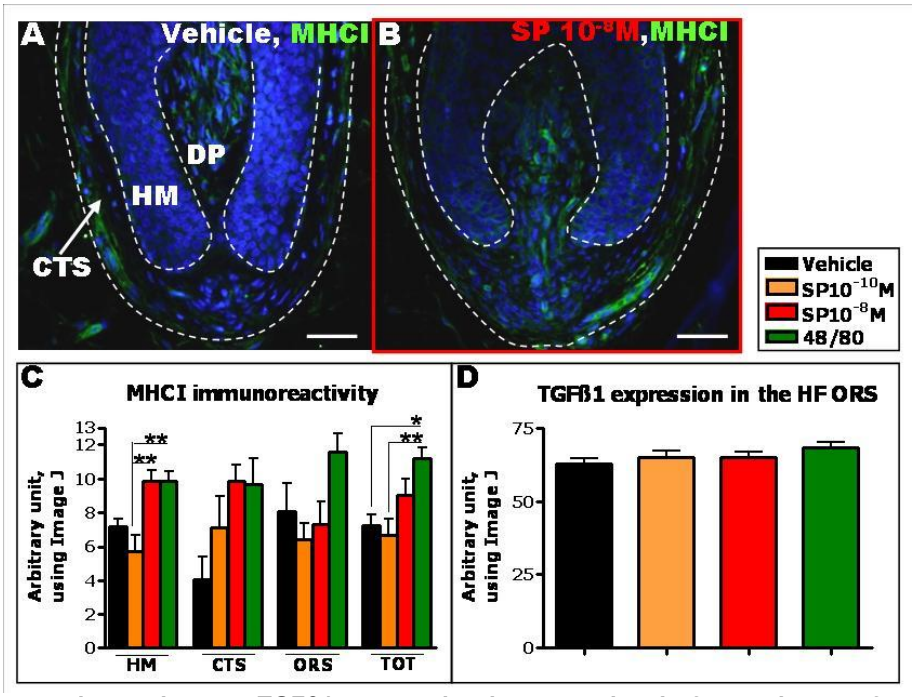

Figure 3.25: MHC class I, but not TGFβ1, expression is up-regulated after 24 hours of treatment with substance P and compound 48/80.
Representative pictures of MHCI IF showing increased MHC class I expression (green signal) in the HF bulb of SP10^{-8}M (B) compared to vehicle (A) treated skin. Scale bars: 50µm.The quantitative analysis of MHC class I IR revealed an increase of MHC class I expression in HF bulb of treated skin compared to vehicle (C). Analysis deriving from 4-5HFs/group, *p≤0.05, **p≤0.01, ±SEM, One-Way ANOVA followed by Bonferroni´s comparison test. Quantitative analysis of TGFβ1 IR revealed no change of TGFβ1 expression in the HF ORS in treated skin compared to vehicle (D). Analysis deriving from 33-42 areas from 18-28 HFs/group divided in upper dermis, dermis and subcutis, ns, ±SEM, Kruskal-Wallis test followed by Dunn´s multiple comparison test. Connective tissue sheath (CTS), hair matrix (HM), outer root sheath (ORS).

In skin from the same subject, 72 hours of treatment with SP or compound 48/80 did not significantly change the number of perifollicular tryptase+ **(Figure 3.26A)** or c-Kit+ **(Figure 3.26B)** MCs or MC degranulation **(Figure 3.26C**) compared to vehicle controls.

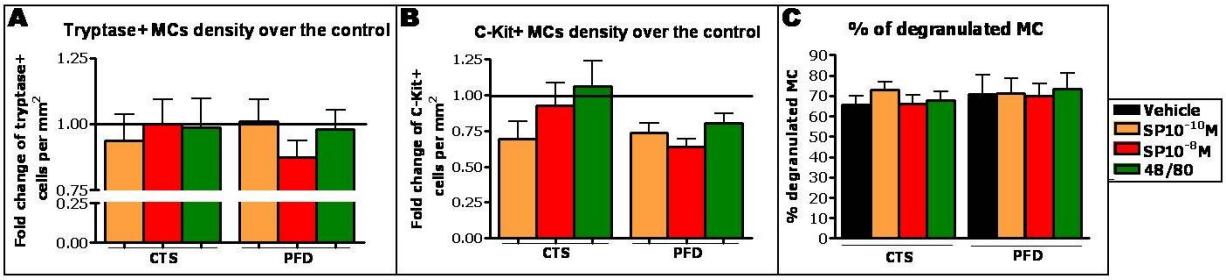

Figure 3.26: The number of mast cells and their degranulation do not change after 72 hours of treatment with substance P or compound 48/80.
Quantitative analysis of tryptase+ MCs showing that the number of mature MCs does not change after SP and compound 48/80 treatments (A). Analysis deriving from 45-58 areas from 20-51 HFs/group divided in upper dermis, dermis and subcutis, ns, ±SEM, One-Way ANOVA followed by Bonferroni´s comparison test. Quantitative analysis of c-Kit+ MCs showing that the number of mature and immature MCs does not change after SP and compound 48/80 treatments (B) Analysis deriving from 20-35 areas from 10-29 HFs/group divided in upper dermis, dermis and subcutis, ns, ±SEM, One-Way ANOVA followed by Bonferroni´s comparison test. Quantitative analysis of the percentage of tryptase+ MCs showing that the degranulation status of mature MCs does not change after SP and compound 48/80 treatments (C). Analysis deriving from 45-58 areas from 20-51 HFs/group divided in upper dermis, dermis and subcutis, ns, ±SEM, One-Way ANOVA followed by Bonferroni´s comparison test. Connective tissue sheath (CTS), perifollicular dermis (PFD).

Again, after 72 hours of treatment, Ki-67+/tryptase+ **(Figure 3.27A)** and Ki-67+/c-Kit+ **(Figure 3.27B)** MCs were extremely rare in vehicle and test skin, precluding meaningful quantitative evaluation and comparison.

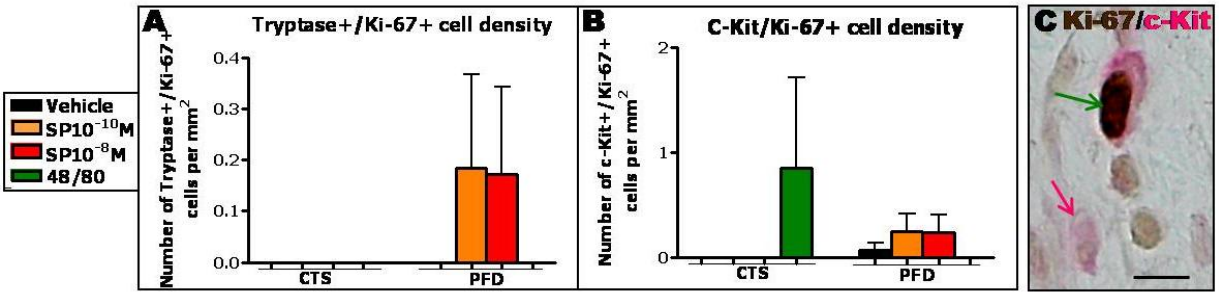

Figure 3.27: The number of Ki-67+/tryptase+ mast cells and Ki-67+/c-Kit+ mast cells were too low for obtaining meaningful quantitative data, after 72 hours of treatment with mast cell secretagogues.
Quantitative analysis of Ki-67+/tryptase+ MC number showing that their number was low in vehicle and treated groups (A). Analysis deriving from 22-24 areas from 9-21 HFs/group divided in upper dermis, dermis and subcutis, ns, ±SEM, Kruskal-Wallis test followed by Dunn´s multiple comparison test. Quantitative analysis of Ki-67+/c-Kit+ MC number showing that their number was low in vehicle and treated groups (B). Analysis deriving from 20-35 areas from 10-29 HFs/group divided in upper dermis, dermis and subcutis, ns, ±SEM, Kruskal-Wallis test followed by Dunn´s multiple comparison test. Representative picture of Ki-67/c-Kit double-IHC showing Ki-67+/c-Kit+ MCs. Pink cell is c-Kit+ MC (pink arrow) and pink-brown cell is Ki-67+/c-Kit+ MCs (green arrow) (C). Scale bar: 10µm. Connective tissue sheath (CTS), perifollicular dermis (PFD).

The number of perifollicular CD8+ T-cells was slightly, but non-significanty up-regulated after 72 hours of SP treatment **(Figure 3.28A).** Also, the fact that the number of CD8+ T-cells is reduced with increasing organ culture duration **(Figure 3.23A)** suggests that T-cell viability declines progressively under the current human skin organ culture conditions (possibly because the culture medium favors HF growth but may disfavor lymphocyte survival).

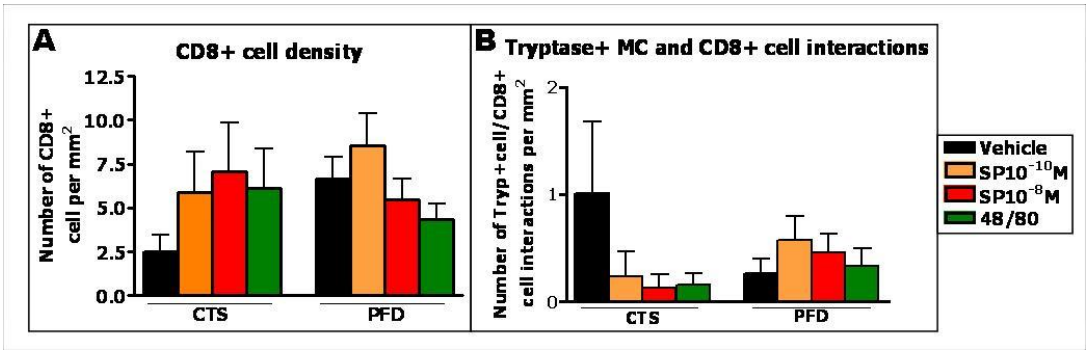

Figure 3.28: The number of CD8+ T-cells is increased after 72 hours of treatment with substance P treatment, while the interactions between tryptase+ mast cell and CD8+ T-cell in connective tissue sheath decline.
Quantitative analysis of CD8+ T-cells showing that their number is slightly up-regulated after 72 hours of treatment with SP but not with compound 48/80 (A). Quantitative analysis of MC-CD8+ T-cell interactions showing that their number decreases in the HF CTS after SP and compound 48/80 treatments after 72 hours (B). Analysis deriving from 45-58 areas from 20-51 HFs/group divided in upper dermis, dermis and subcutis, ns, ±SEM, Kruskal-Wallis test followed by Dunn´s multiple comparison test. Connective tissue sheath (CTS), perifollicular dermis (PFD).

Nevertheless, the number of visualized MC-CD8+ T-cell interactions after 72 hours of treatment with SP or compound 48/80 revealed the same decreased trend in HF CTS and increased trend in HF PFD **(Figure 3.28B)**, as it was seen after 24 hours **(Figure 3.23B)**.

Regarding the previously assessed IP markers (MHC class I and TGFβ1), MHC class I revealed not change between HF bulbs of treated and vehicle groups **(Figure 3.29A)** while TGFβ1 expression was found to be decreased in the HF ORS of treated groups **(Figure 3.29B)** after 72 hours of treatments.

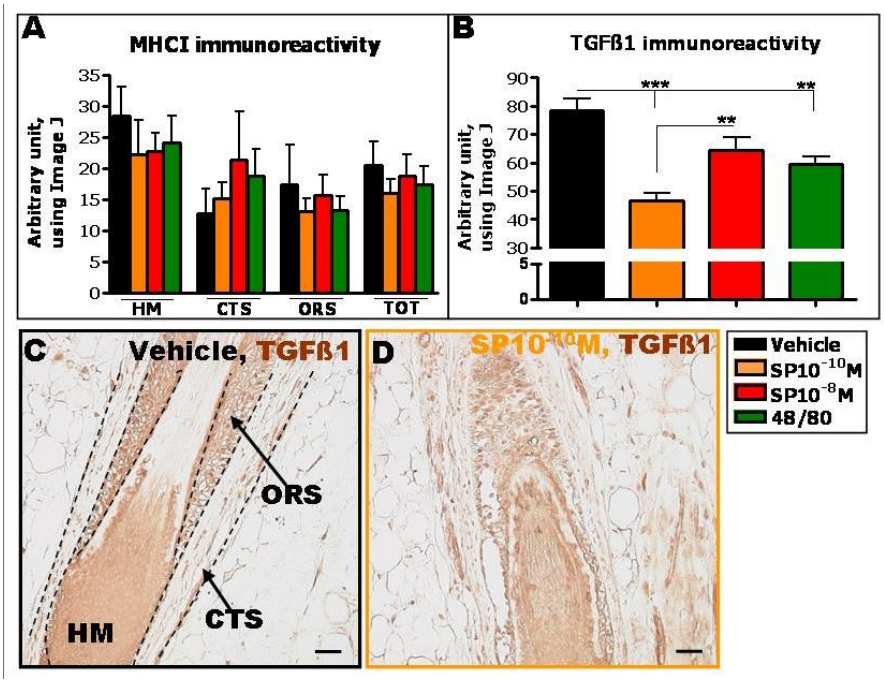

Figure 3.29: TGFβ1, but not MHC class I, expression is down-regulated after 72 hours of treatment with substance P or compound 48/80.
The quantitative analysis of MHC class I IR revealed no change of MHC class I in HF bulb of treated skin compared to vehicle after 72 hours of treatment (A). Analysis deriving from 4-5HFs/group, ns, ±SEM, One-Way ANOVA followed by Bonferroni´s comparison test. Quantitative analysis of TGFβ1 IR revealed decrease TGFβ1 expression in the HF ORS of treated skin compared to vehicle (B). Analysis deriving from 28-51 areas from 19-36 HFs/group divided in upper dermis, dermis and subcutis, **p≤0.01, ***p≤0.001, ±SEM, One-Way ANOVA followed by Bonferroni´s comparison test. Representative pictures of TGFβ1 IHC showing decreased TGFβ1 expression (brown colour) in the HF ORS of SP10^{-10}M treated skin (D) compared to vehicle (C). Scale bars: 50µm. Connective tissue sheath (CTS), hair matrix (HM), outer root sheath (ORS).

On the basis of these preliminary results two additional full-thickness human scalp skin organ cultures were performed using skin fragments from two distinct individuals, and the concentration of compound 48/80 was increased to 50µg/µl. Unfortunately, however, these additional skin organ culture experiments failed to reproduce the results of the first pilot organ culture experiment, possibly due to large interindividual variations in the skin response to stimulation with SP or compound 48/80 (note that the only anonymised patient information we were allowed to have according to our ethics license was the age, sex, and location of skin specimen so that there was no chance to attribute any differences in the results to major variables such as concomitant diseases or medication).

For example, in skin from a second individual, the number of tryptase+ cells was slightly up-regulated around the HFs **(Figure 3.30A)** as in skin from the first subject **(Figure 3.22A)** after 24 hours of treatment with SP and compound 48/80, but only in PFD, together with a slightly increased MC degranulation **(Figure 3.30B)**. Contrary to the skin from the first subject **(Figure 3.23A)**, the number of CD8+ T-cells did not increase after the treatment with SP but only after the one with higher concentration of compound 48/80 (50µg/µl) **(Figure 3.30C)**. Overall, the number of CD8+ T-cells in this second individual **(Figure 3.30C)** was lower compared to the first treated subject **(Figure 3.23A)**. At the same time, totally opposite results were found regarding the number of MC-CD8+ T-cell interactions, in fact in the skin from the second individual, their number increased in the CTS but not in the PDF after the treatment with SP and compound 48/80 **(Figure 3.30D)**.

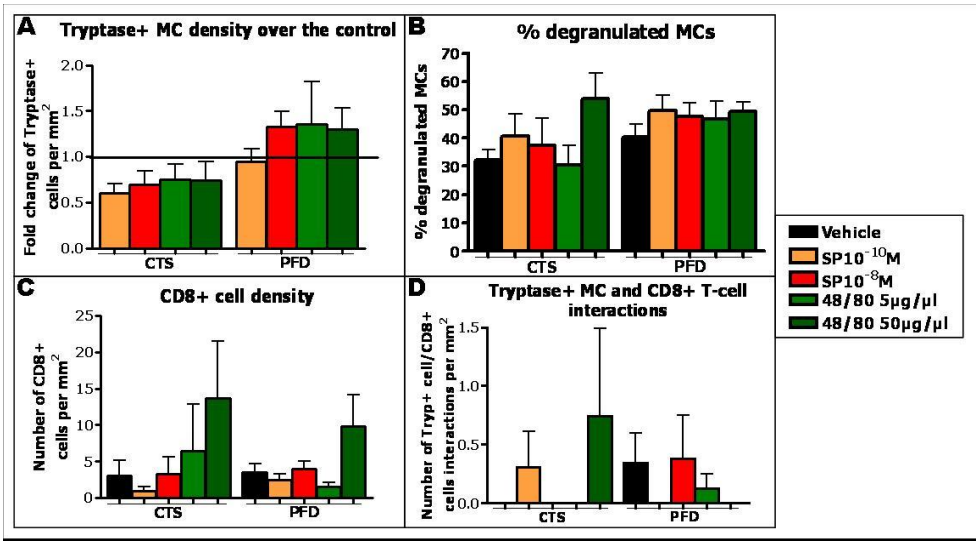

Figure 3.30: Skin from a second subject treated for 24 hours with substance P or compound 48/80 revealed major interindividual results variations. Quantitative analysis of tryptase+ MCs showing that the number of mature MCs is slightly up-regulated after SP and compound 48/80 treatments (A). Quantitative analysis of the percentage of tryptase+ MCs showing that the percentage of degranulated MCs is slightly up-regulated after SP and compound 48/80 treatments (B). Quantitative analysis of CD8+ T-cell number showing that their number is up-regulated only after the treatment with higher dose of compound 48/80 (C). Quantitative analysis of MC-CD8+ T-cell interactions showing that their number are increased in the HF CTS after SP and compound 48/80 treatments (D). Analysis deriving from 18 areas from 6-11 HFs/group divided in upper dermis, dermis and subcutis, ns, ±SEM, One-Way ANOVA or Kruskal-Wallis test followed respectively by Bonferroni´s or Dunn´s multiple comparison tests. Connective tissue sheath (CTS), perifollicular dermis (PFD).

Since the results between three patients were highly variable, it was not permissible or meaningful to pool them. Therefore, even the full-thickness scalp skin organ culture failed as a suitable and instructive model for functional studies (e.g. modulation of MC-CD8+ T-cells interactions after treatment with MC secretagogues), even though many HFs were evaluated.

However, if any conclusions at all can be drawn from these preliminary experiments, one may speculate that, in predisposed individuals (such as the first subject), excessive release of SP, which could occur e.g. in connection with psychoemotional stress (Arck et al. 2005, Arck et al. 2006), may impair the physiological control of CD8+ T-cells by MCs.

3.2.3 In full thickness lesional alopecia areata skin organ culture, mast cell-CD8+ T-cell interactions are influenced by MC secratogues and stabilizers

For evident reasons, large AA skin biopsies needed for organ culture are essentially unobtainable and are ethically difficult to justify. However, unexpectedly, it became possible during the course of this PhD project to obtain, with written patient consent, one larger strip of alopecic scalp skin for full thickness skin organ culture from a female patient (age 67) with long-standing AA totalis (duration >10 years) who underwent cosmetic facelift surgery. Due to the long duration of the disease, only very few miniaturized HFs **(Figure 3.31A-B)**, and a very discrete inflammatory cell infiltrate was seen, as expected from the literature (Whiting 2003).

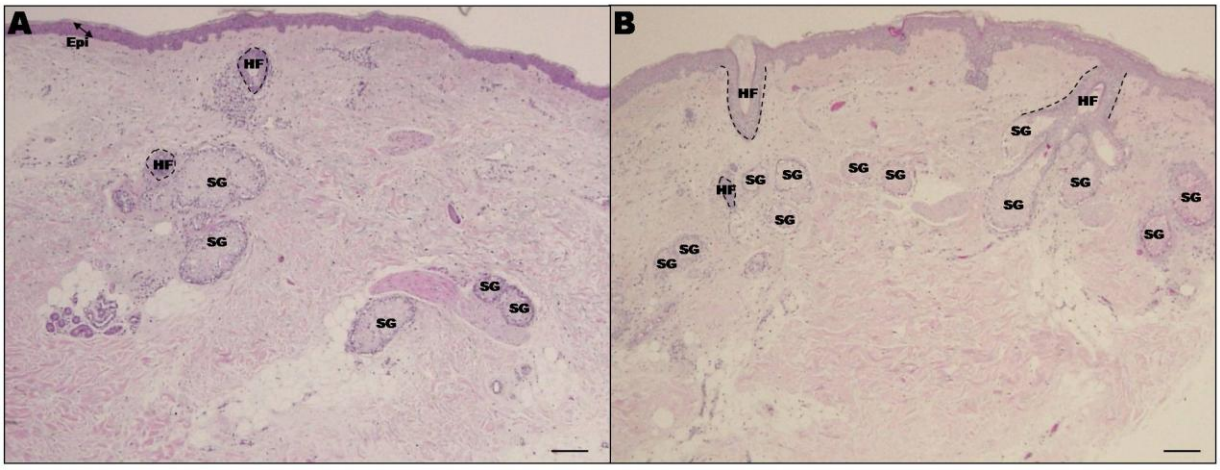

Figure 3.31: Histopathology of the organ-cultured skin from a female patient with extremely chronic alopecia areata.
HE staining showing miniaturized HFs in untreated skin sections of a patient characterized by long-standing AA totalis.The skin form this patient was used to run the full-thickness AA organ culture. Scale bars: 200µm. Epidermis (Epi), hair follicle (HF), sebaceous gland (SG).

This unique opportunity to organ-culture chronic human AA skin was grasped and the effect of MC secretagogues (SP, compound 48/80) and of the MC stabilizer, cromoglycate, on MCs, CD8+ T-cells and MC-CD8+ T-cell interactions was investigated in AA human scalp skin, 4mm punches were treated with SP (10^{-8}M and 10^{-10}M), compound 48/80 (5µg/µl), or

cromoglycate (10^{-4}M and 10^{-7}M) for 24 hours. After the treatment, the skin punches were fixed in 4% formalin, embedded in paraffin, and then stained for CD8/Tryptase by which tryptase+ cells were detected as pink cells and CD8+ T-cells as brown cells **(Figure 3.32A-G)** and Ki-67/C-Kit by which c-Kit+ MCs were labeled as pink cells, Ki-67+ cells as brown cells and Ki-67+/c-Kit+ MCs as pink-brown cells **(Figure 3.33A-B)**.

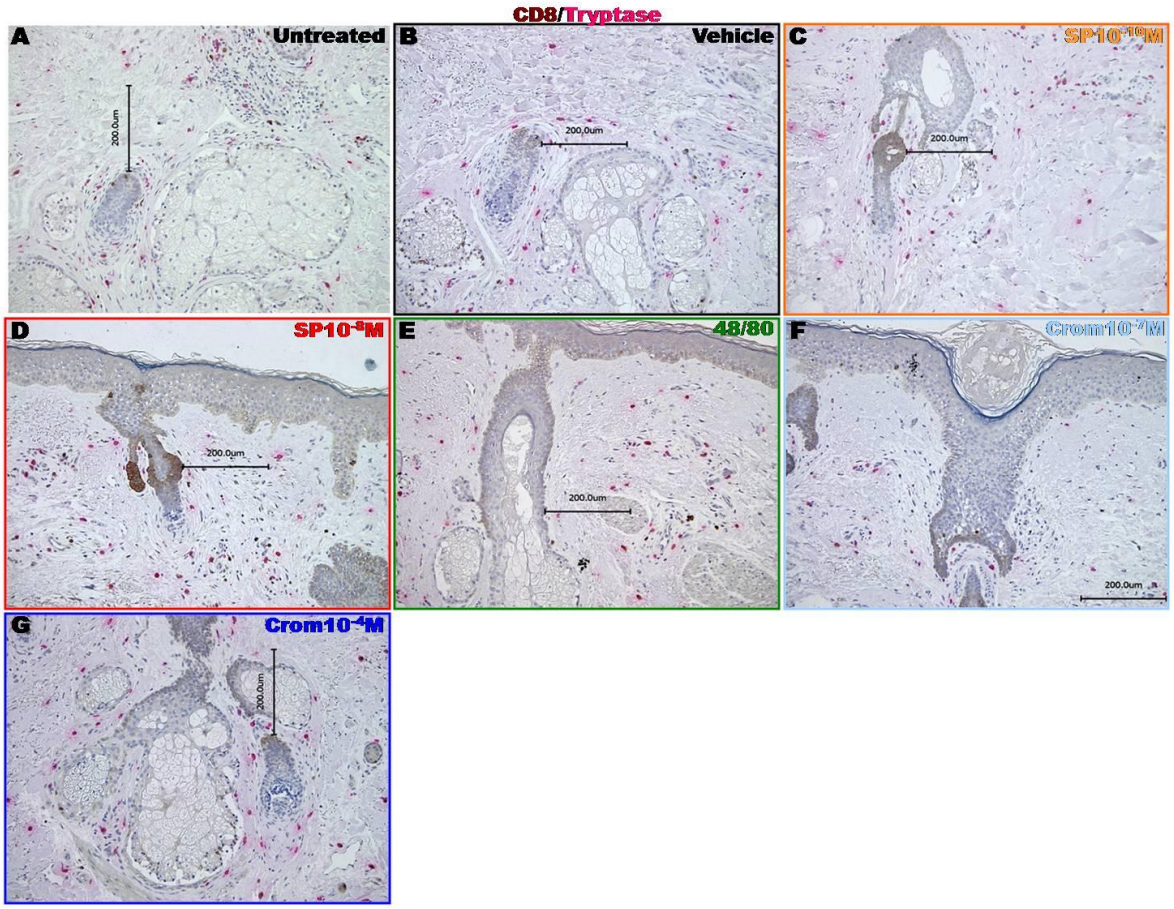

Figure 3.32: Immunohistochemical identification of tryptase+ mast cells and CD8+ T-cells around hair follicle of organ-cultured human alopecia areata skin fragments.
Representative pictures showing tryptase+ MCs and CD8+ T-cells stained by Tryptase/CD8 double-IHC of HFs deriving from human AA scalp skin punches untreated (A) and treated with vehicle (only medium) (B), $SP10^{-10}$M (C), $SP10^{-8}$M (D), compound 48/80 (E), cromoglycate 10^{-7}M (F) and 10^{-4}M (G). Pink cells are tryptase+ MC and brown cells are CD8+ T-cells. Of note, the brown colour cells in the HF bulge are mostly cytokeratin 15+ cells, not CD8+ T-cells. Scale bars: 200µm.

Interestingly, the treatment with SP slightly increased the number of c-Kit+ MCs **(Figure 3.33A-C)** and tryptase+ MCs **(Figure 3.34A)** in chronic AA skin, at least in PFD, even though significance was not reached. Tendentially, also compound 48/80 treatment increased the number of c-Kit+ MCs in the PFD of lesional AA HFs **(Figure 3.33C)**. Surprisingly, treatment with cromoglycate, a MC stabilizer, also slightly increased the number of c-Kit+ MCs **(Figure 3.33C)** but not of tryptase+ MCs in AA samples compared to vehicle **(Figure 3.34A)**.

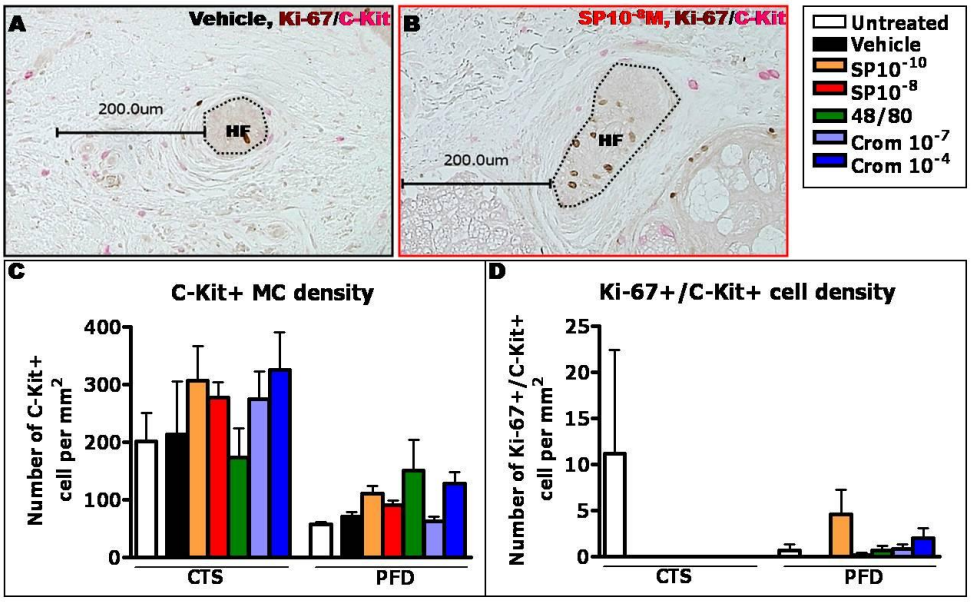

Figure 3.33: The number of perifollicular c-Kit+ mast cells is tendentially up-regulated in alopecia areata skin by substance P, compound 48/80 and cromoglycate treatments.
Representative pictures showing c-Kit+ MCs and Ki-67+ cells stained by Ki-67/C-Kit double-IHC of HFs deriving from human AA scalp skin punches treated with vehicle (only medium) (A) and SP10^{-10}M (B). Pink cells are c-Kit+ MCs, brown cells are Ki-67+ cells and pink-brown cells are Ki-67+/c-Kit+ MCs. Scale bars: 200µm. Quantitative analysis of c-Kit+ MCs showing that the number of MCs is slightly increased in AA skin after SP, compound 48/80 and cromoglycate treatments (C). Quantitative analysis of Ki-67+/c-Kit+ MCs showing that their number is extremely low in vehicle and treated groups (D). Analysis deriving from 9-22 HFs/group, ns, ±SEM, One-Way ANOVA or Kruskal-Wallis test followed respectively by Bonferroni´s or Dunn´s multiple comparison tests. Connective tissue sheath (CTS), perifollicular dermis (PFD).

As previously shown in full-thickness healthy skin organ culture, the total number of proliferating MCs, detected by Ki-67/C-Kit double-immunostain, was very low in all groups **(Figure 3.33D)**. No significant changes of MC degranulation were seen **(Figure 3.34B)**.

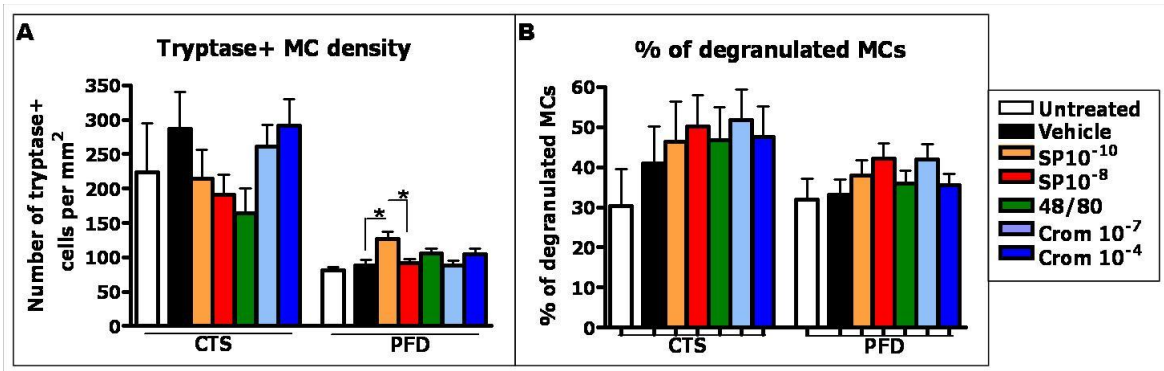

Figure 3.34: The number of tryptase+ mast cells is slightly up-regulated in alopecia areata skin after substance P treatment.
Quantitative analysis of tryptase+ MCs showing that the number of mature MCs did not change after compound 48/80 and cromoglycate treatments but is slightly up-regulated after SP treatment (A). Quantitative analysis of the degranulation status of tryptase+ mature MCs which is vaguely increased after SP, compound 48/80 and cromoglycate treatments (B). Analysis deriving from n.6-20 HFs/group, *p≤0.05, ±SEM, One-Way ANOVA followed by Bonferroni´s comparison test. Connective tissue sheath (CTS), perifollicular dermis (PFD).

The quantification of CD8+ T-cells revealed that the infiltrate around AA HFs was further reduced by loss of immunocytes after 3 days of organ-culture, in fact their number decreased in vehicle compared to untreated HFs **(Figure 3.35A-B)**. However, the treatment of AA skin

with SP tended to up-regulate perifollicular CD8+ T-cells, although not significantly **(Figure 3.35A-B)**.

The treatment with SP showed an (again, non-significant) tendency towards increased MC interactions with CD8+ T-cells, while no effect were seen with compound 48/80 **(Figure 3.35A,C)**. Slightly decreased number of MC interactions with CD8+ T-cells was observed with the treatment cromoglycate, but only in PFD **(Figure 3.35A,C)**.

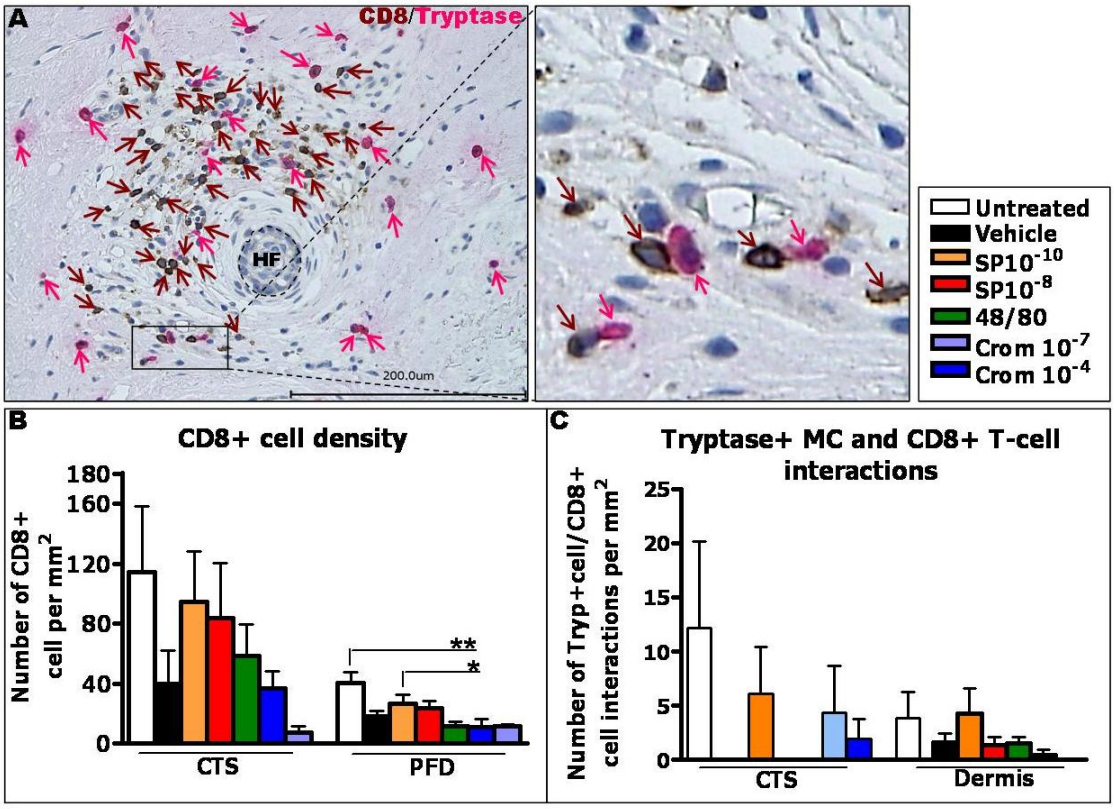

Figure 3.35: The number of CD8+ T-cells and tryptase+ mast cell-CD8+ T-cell interactions are tendentially increased in alopecia areata skin after substance P treatment.
Representative picture showing tryptase+ MCs (pink cells, pink arrows) and CD8+ T-cells (brown cells, brown arrows) stained by Tryptase/CD8 double-IHC and in higher magnification tryptase+ MC-CD8+ T-cell interactions (A). Scale bar: 200µm. Quantitative analysis of CD8+ T-cells showing that their number is slightly up-regulated in AA skin after the treatment with SP but not with compound 48/80 or cromoglycate (B). Quantitative analysis of MC-CD8+ T-cell interactions showing that their number is tendentially increased in AA skin after SP and compound 48/80 treatments and decreased after cromoglycate treatment, at least in PFD (C). Analysis deriving from n.6-20 HFs/group, *p≤0.05, ** p≤0.01, ±SEM, One-Way ANOVA or Kruskal-Wallis test followed respectively by Bonferroni´s or Dunn´s multiple comparison test. Connective tissue sheath (CTS), perifollicular dermis (PFD).

Briefly, in AA treated skin, SP treatment tendentially augments MC number, degranulation, CD8+ T-cells and MC-CD8+ T-cell interactions while compound 48/80 had essentially no effect. Considering that the chance to ever be able to repeat this experiment is dismally low, obtaining significant results appears impossible.

However, given that this is the first time that such an organ culture experiment has ever been performed with human AA skin, the limited pilot observations that could be made

encourage at least this speculation: under conditions of SP-dependent neurogenic inflammation (e.g. in the context of stress or trauma), SP may further increase pathogenic MC-CD8+ T-cell interactions and consequently worsen the the course of AA.

Taken together, pilot experiments that attempted to functionally probe MC-CD8+ T-cells interaction in organ-cultured intact human scalp HFs or skin *in vitro* remained inconclusive due to major methodological constraints that could not be overcome.

3.3 Mast cell-CD8+ T-cell interactions are enhanced and abnormal also *in vivo*

3.3.1 The subcutaneous injection of IFNγ does not accelerate spontaneous development of alopecia areata in C3H/HeJ mice

Given the failure to establish an organ culture model in which to functionally dissect MC-CD8+ T-cell interactions using skin organ culture, an *in vivo* approach had to be taken.

C3H/HeJ mice are known to develop spontaneously AA lesions in older age (King et al. 2008). In these mice AA onset can be accelerated by intravenous injection of INFγ (Gilhar et al. 2005). It has been hypothesized that the local up-regulation of INFγ or other pro-inflammatory signals may be one of the reasons why AA lesions in humans and other mammals are appearing predominantly as focal (patchy lesions) (McElwee et al. 2013). Therefore, 42 C3H/HeJ mice were subcutaneously injected near the neck on the back side either with 10000 UI of INFγ or vehicle once a week, in order probe whether a local increase in INFγ accelerates AA development and to investigate how this impacts on MC-CD8+ T-cell interactions.

This experiment showed that the subcutaneous injection of INFγ did not accelerate AA onset in the treated C3H/HeJ mice, because only few mice developed AA, and this independently of the treatment and at very different time points during or after treatment. Therefore, it was not possible to kill the mice all at the same day. However, in order to keep the experiment as standardized as possible, mice were killed when sign of AA appeared and always together with one non-alopecic control mouse (injected only with vehicle), as reported in **Figure 3.36A-L** and **3.37A-L**.

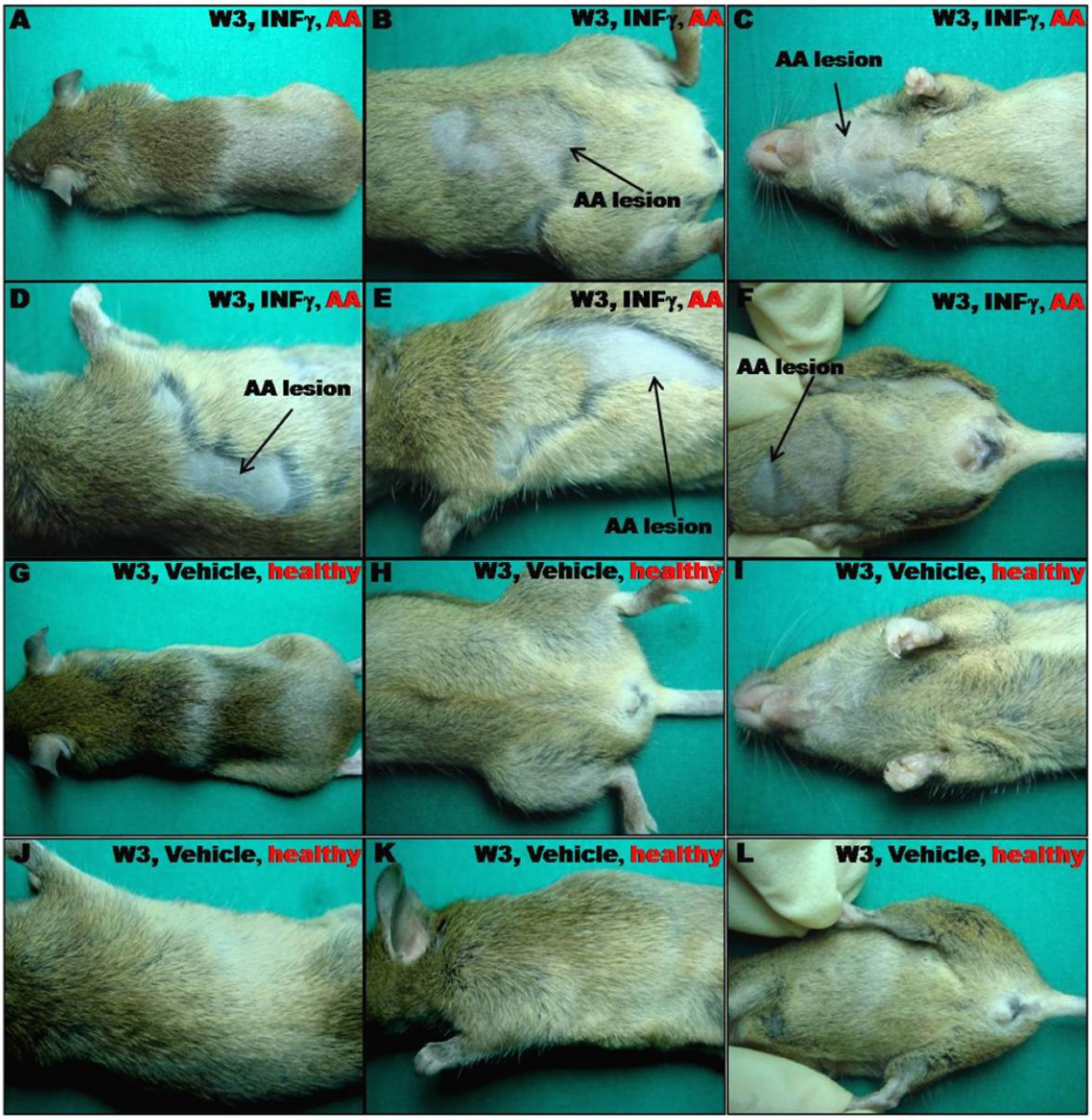

Figure 3.36: Photo-documentation of alopecia areata lesions in alopecia areata-affected C3H/HeJ mice after three weeks of vehicle or IFNγ injection.
Representative pictures showing AA lesions in C3H/HeJ mouse affected by AA previously injected subcutaneously with INFγ (A-F) and healthy C3H/HeJ mouse previously injected subcutaneously with vehicle (G-L), sacrificed after three weeks of treatment. Of note, the treatment with INFγ did not accelerate the disease onset in C3H/HeJ mice because only the 14% of 42 mice developed the disease and independently if injected with INFγ or vehicle.

AA lesions in C3H/HeJ started to appear mostly in ventral/lateral skin **(Figure 3.36B-F** and **Figure 3.37B-E)** as well as on the snout **(Figure 3.37F)**, and only in one case also occurred on the back skin near the injection site, but in a vehicle-treated mouse **(Figure 3.37A)**.

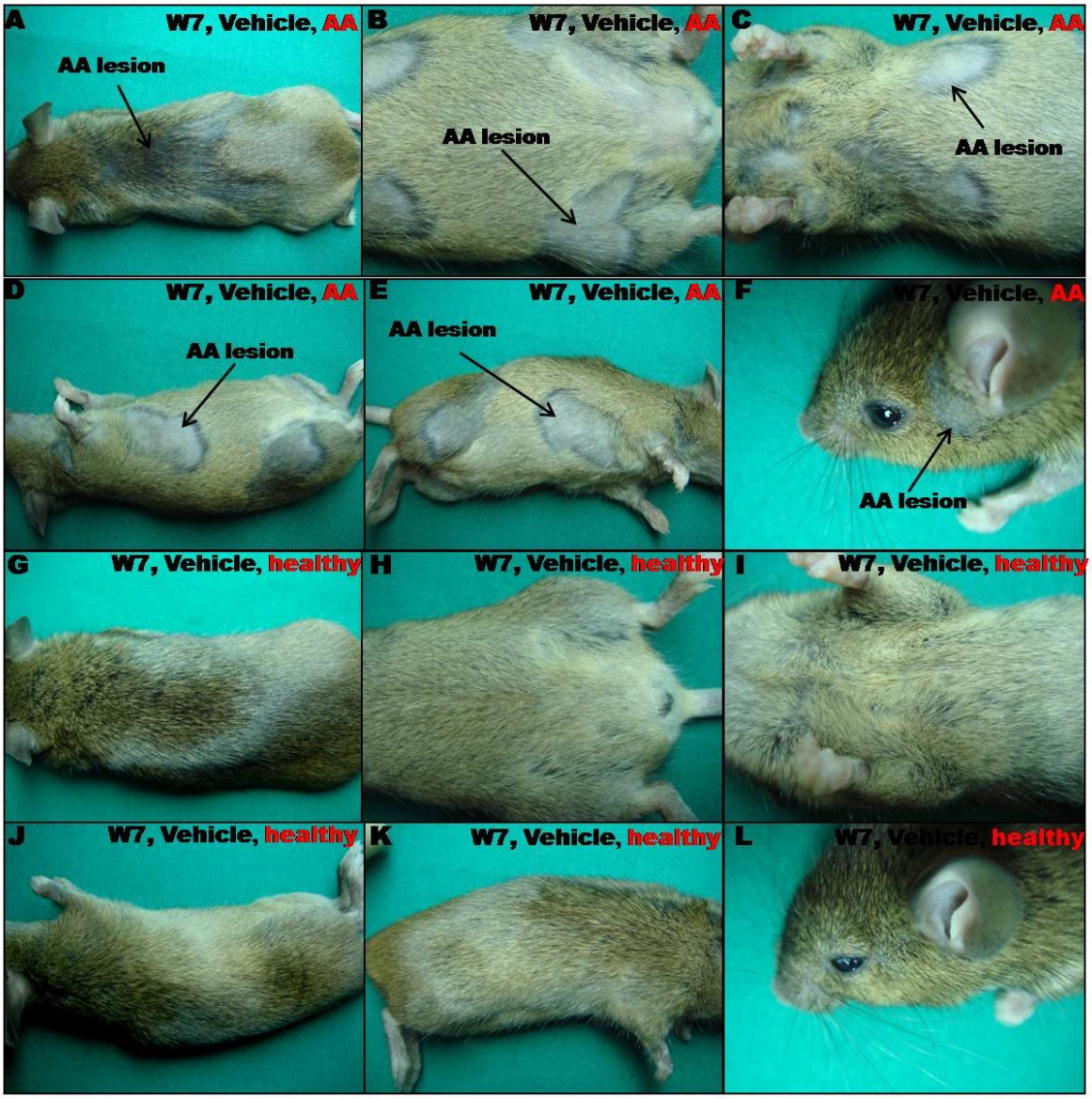

Figure 3.37: Photo-documentation of AA lesions in alopecia areata-affected C3H/HeJ mice after seven weeks of vehicle injection.
Representative pictures showing AA lesions in C3H/HeJ mouse affected by AA previously injected subcutaneously with vehicle (A-F) and healthy C3H/HeJ mouse previously injected subcutaneously with vehicle (G-L), sacrificed after seven weeks of treatment.

Overall, 2 mice treated (week 3 and 23 of treatment) with INFγ and 4 mice injected with vehicle (week 7, 6, 15 and 23 of treatment) developed AA. However, 6 INFγ and 1 vehicle treated mice died during the course of thos long-term experiment. At week 44 of treatment, when 26 mice were still alive, the experiment was terminated and all mice were killed. Generally, about only the 14% of the mice developed AA, value even below the 20% threshold reported in the literature (King et al. 2008). However, to obtain nevertheless as much information as possible from these mice, considering that peri-lesional and lesional skin from each affected mouse had been harvested, CD8/C-Kit double immunostaining was performed in AA and non-alopecic control mice in order to assess whether MCs and CD8+ T-

cell numbers and MC-CD8+ T-cell interactions were increased in peri-lesional AA skin in these C3H/HeJ mice **(Figure 3.38 A-I)**.

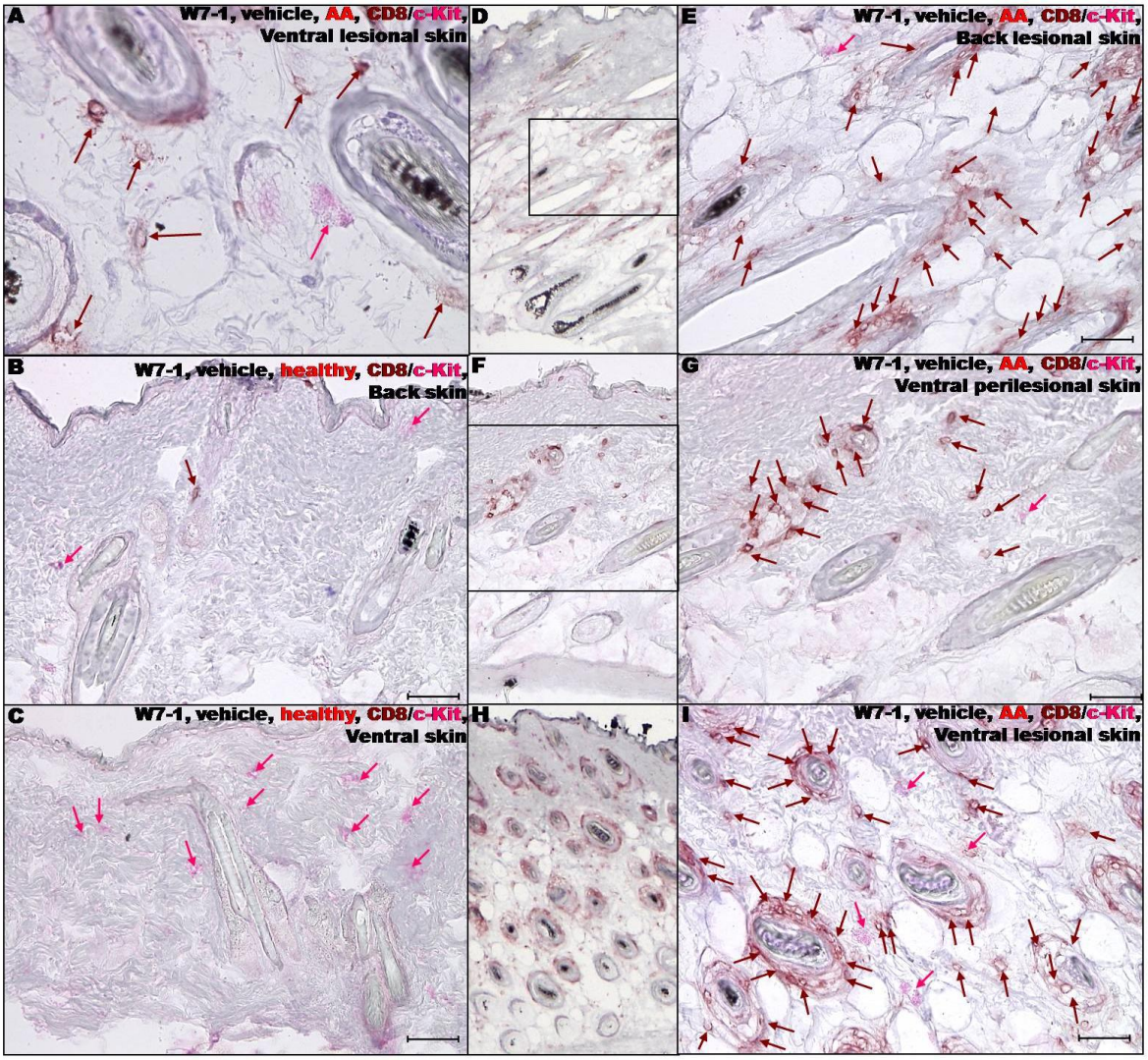

Figure 3.38: Immunohistology of a vehicle injected C3H/HeJ mouse affected by alopecia areata.
Higher magnification showing CD8+ T-cells and c-Kit+ MCs stained by CD8/C-Kit double-IHC (A). Back (B) and ventral (C) skin from a healthy mouse with rare CD8+ T-cells and few MCs. Lesional back skin (D,E) and perilesional ventral skin (F,G) from an AA affected mouse revealed increased CD8+ T-cell numbers. Lesional ventral skin from an AA affected mouse showing increased number of CD8+ T-cells and MCs (H,I). Brown cells are CD8+ T-cells (brown arrows) and pink cells are c-Kit+ MCs (pink arrow). Scale bars: 50µm.

As expected (Paus et al. 1998), in non-alopecic mouse skin, rare CD8+ T-cells were found in the HF infundibulum, and a few c-Kit+ MCs were depicted around the HFs **(Figure 3.38B,C)**. Preliminary results showed higher CD8+ T-cells not only in lesional AA skin **(Figure 3.38D,E,H,I)** but also in perilesional ventral skin **(Figure 3.38F,G)**. However, MCs were found to be increased only in lesional ventral skin **(Figure 3.38H,I)**, which probably reflects later stage of AA development **(Figure 3.37B-E)** compared to the back lesions **(Figure 3.37A)**. Moreover, few MC-CD8+ T-cell interactions were appreciated in these samples.

Therefore, due to the fact that only few mice developed AA at all, the classical C3H/HeJ model of spontaneously developing AA turned out to be unsuitable for evaluating and manipulating MC-CD8+ T-cell interactions in vivo.

3.3.2 The number of mast cell-CD8+ T-cell interactions is in the grafted C3H/HeJ mouse model

To increase the likelihood of success of AA development in C3H/HeJ mice to 100% and to speed up disease development, collaborators have established a grafted C3H/HeJ mouse model (Silva and Sundberg 2013, Wang et al. 2013, King et al. 2008). In this model, lesional AA skin from old C3H/HeJ mice is transplanted onto young mice. Presumably this transfers pathogenic T-cells from the AA donor skin lesions to the recipient mice, thus accelerating the occurrence of AA lesions dramatically. Therefore, thanks to an excellent collaboration with Prof. Kevin McElwee (University of British Columbia, Vancouver, Canada), it was possible to probe whether selected key findings on abnormal MC-CD8+ T-cell interactions that had been obtained in human AA skin are also present in this best-established mouse model for AA (Wang et al. 2013, King et al. 2008).

Skin samples from 4 mice/group of normal (NM), sham-grafted (mSH), failed-grafted (fAA) and AA mice (mAA) were received from Prof. McElwee, and double-immunohistology for CD8/C-Kit and mMCP6 (mouse tryptase)/CD8 was performed. Since not all the skin samples revealed anagen HFs, considering the hair cycle-dependent changes in MC numbers and function in mice (Paus et al. 1998, Paus et al. 1994c), in order to use exclusively anagen skin, only the skin from 2 NM and mSH mice and 3 fAA could be analysed nd compared with the skin samples from 4 mAA mice.

As shown in **Figure 3.39A-D** and **3.40A-D**, NM, mSH and fAA mice, i.e. the three types of control mice, showed relatively few perifollicular MCs (detected by c-Kit **(Figure 3.39A-C)** and mMCP6 **(Figure 3.40A-C)**) and extremely few CD8+ T-cells **(Figure 3.39A-C** and **3.40A-C).**

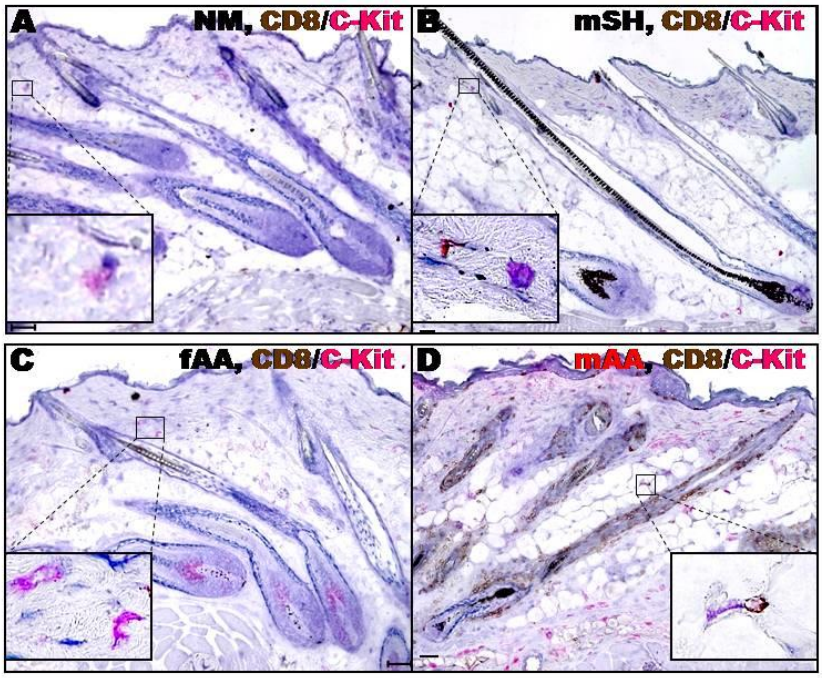

Figure 3.39: Immunohistochemical identification of c-Kit+ mast cells and CD8+ T-cells in the grafted C3H/HeJ mouse model of alopecia areata. Representative pictures of CD8/C-Kit double-staining in normal (NM) (A), sham-grafted (mSH) (B), failed-grafted (fAA) (C) and AA (mAA) (D) mice showing an increase of c-Kit+ MCs and CD8+ T-cells in AA mice compared to controls. Pink cells are c-Kit+ MCs while brown cells are CD8+ T-cells. Of note, the pink colour in the HM are c-Kit+ melanocytes not MCs. Scale bars: 50 µm.

In striking contrast, compared to control mice, lesional skin of mice affected by AA (mAA) showed significantly more perifollicular (immature) c-Kit+ MCs **(Figure 3.39D)**, while mature mMCP6+ skin MCs remained essentially unaltered **(Figure 3.40D)**. The quantification of positive cells around the HFs corroborated the qualitative observations **(Figure 3.41A** and **3.42A)**.

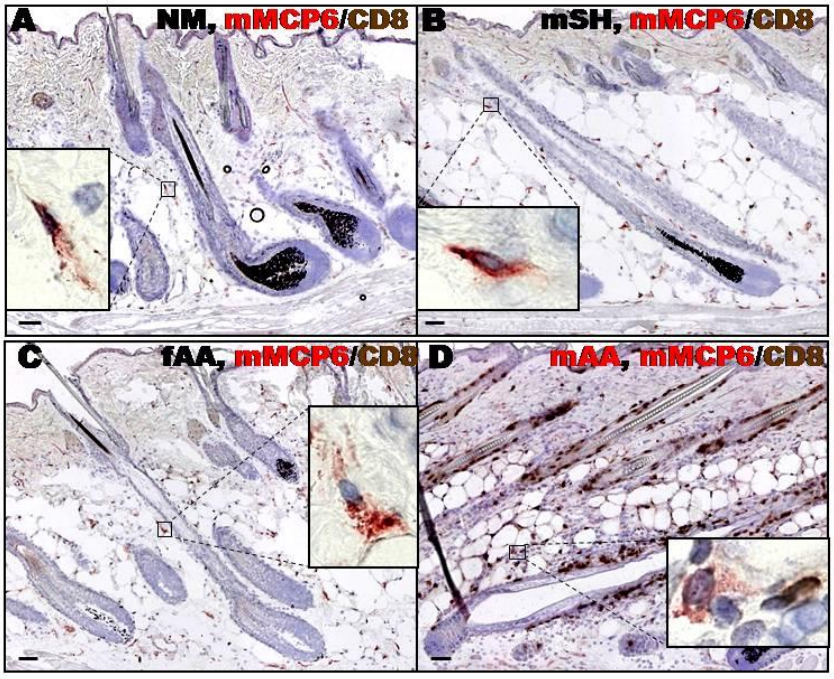

Figure 3.40: Immunohistochemical identification of mMCP6+ mast cells and CD8+ T-cells in the grafted C3H/HeJ mouse model of alopecia areata. Representative pictures of mMCP6/CD8 double-staining in normal (NM) (A), sham-grafted (mSH) (B), failed-grafted (fAA) (C) and AA (mAA) (D) mice showing an increase of mature mMCP6+ MCs and CD8+ T-cells in AA mice compared to controls. Red cells are mMCP6+ MCs while brown cells are CD8+ T-cells. Scale bars: 50µm.

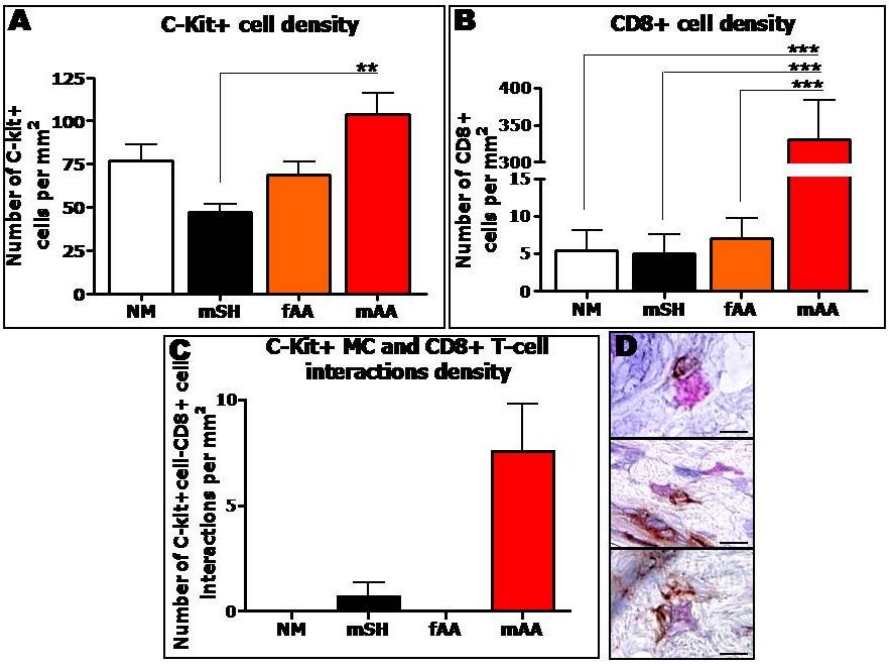

Figure 3.41: The number of c-Kit+ mast cells, CD8+ T-cells and c-Kit+ mast cell-CD8+ T-cell interactions are increased in grafted C3H/HeJ mice affected by alopecia areata.
The quantitative analysis revealed an increase of c-Kit+ MCs (A), CD8+ T-cells (B) and c-Kit+ MC/CD8+ T-cell interactions (C) in mAA compared to NM, mSH and fAA control mice. Analysis derived from 6 HFs/mouse of 2-4 mice/group, **p≤0.01, ***p≤0.001, ±SEM, One-Way ANOVA or Kruskal-Wallis test followed by Bonferroni´s test or Dunn's test. Representative pictures of c-Kit+ MC-CD8+ T-cell interactions (D). Pink cells are c-Kit+ MCs while brown cells are CD8+ T-cells. Scale bars: 10µm. AA mice (mAA), failed-grafted AA mice (fAA), normal mice (NM), sham-grafted mice (mSH).

Moreover, compared to control mice, AA mice showed a significantly increased MC degranulation **(Figure 3.41C,D)** and higher CD8+ T-cell numbers **(Figure 3.40B)** along with increased MC-CD8+ T-cell interactions, both of c-Kit+ **(Figure 3.40C,D)** and of mMCP6+ MCs **(Figure 3.41C,E)**.

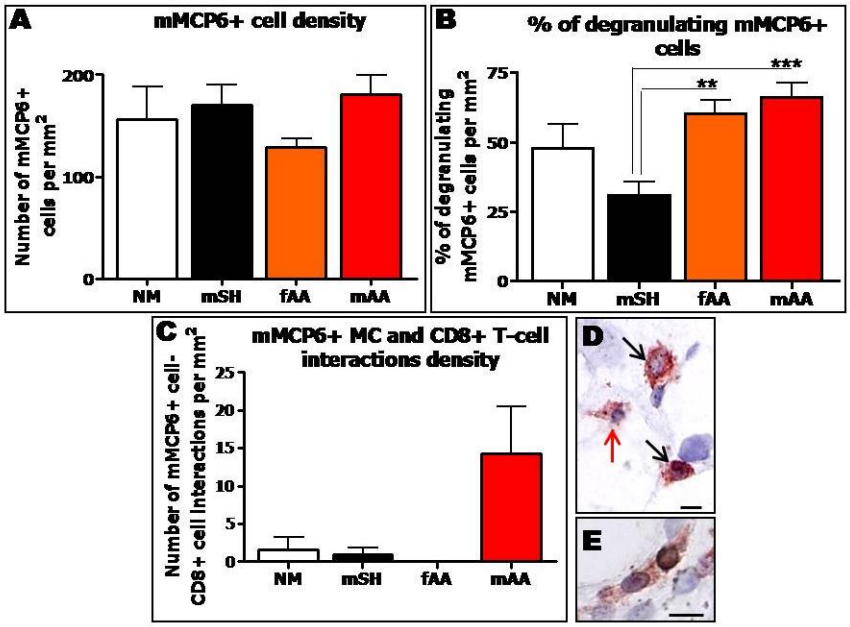

Figure 3.42: The degranulation of mMCP6+ mast cells and mMCP6+ mast cell-CD8+ T-cell interactions are increased in grafted C3H/HeJ mice affected by alopecia areata.
The quantitative analysis revealed no change of mMCP6+ MCs (A) but increase % of degranulation (B) and mMCP6+ MC-CD8+ T-cell interactions (C) in mAA compared to NM, mSH, fAA control mice. Analysis derived from 6 HFs/mouse of 2-4 mice/group, **p≤0.01, ***p≤0.001, ±SEM, One-Way ANOVA or Kruskal-Wallis test followed by Bonferroni´s test or Dunn's test. Immunohistochemical identification of non-degranulated (black arrows) and degranulated (red arrow) of mMCP6+ MCs (D) and mMCP6+MC-CD8+ T-cell interaction (E). Red cells are mMCP6+ MCs while brown cells are CD8+ T-cells. Scale bars: 10µm. AA mice (mAA), failed-grafted AA mice (fAA), normal mice (NM), sham-grafted mice (mSH).

These in vivo data from the best-accepted mouse model of AA independently suggests that abnormal MC activities and MC-CD8+ T-cell interactions are a general feature of the AA phenotype across species barriers.

3.3.3 Mast cell-CD8+ T-cell interactions are also increased in experimentally induced alopecia areata in human skin on humanized SCID mice

During the course of these experiments, a novel humanized mouse model for AA research became available, which is now thought to be the AA model that resembles human AA most closely (Gilhar et al. 2013a,b). Therefore, the final question addressed was whether abnormal MC-CD8+ T-cell interactions can also be seen in experimentally induced AA lesions in previously healthy human scalp skin that had been engrafted onto SCID mice and injected with IL-2-treated PBMCs from healthy donors that were enriched for NKG2D+/CD56+ cells (Gilhar et al. 2013a,b). This became possible thanks to a recently established collaboration with Prof. Amos Gilhar (Technion-Israel Institute, Haifa, Israel), who hosted the PhD candidate for two weeks in his lab in Haifa. During this time, the candidate also had the opportunity to participate in human scalp skin transplantation experiments and performed exploratory CD8/C-Kit double-IF on cryosections of healthy human scalp skin **(Figure 3.43A)**, lesional skin from AA patients and AA lesional human skin transplanted onto SCID mice **(Figure 3.43B).**

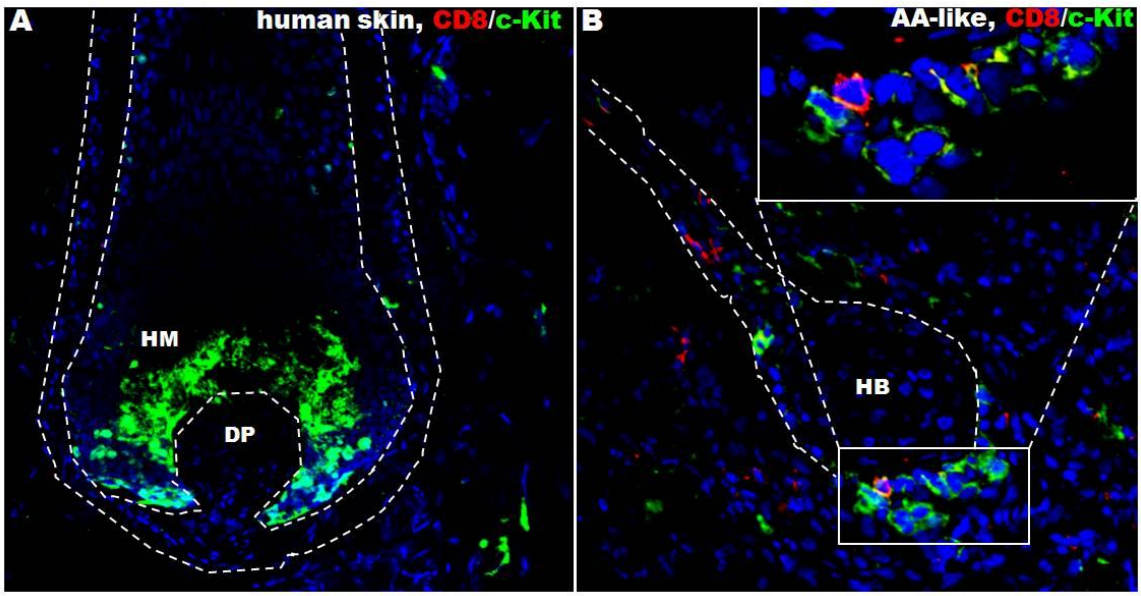

Figure 3.43: Immunohistochemical identification of CD8+ T-cells and c-Kit+ mast cells on alopecia areata-like human skin lesions (humanized mouse model of alopecia areata).
Representative confocal pictures of CD8/C-Kit double-staining in human skin (A) and in AA lesion of human skin previous transplanted on SCID mice and injected with IL-2-treated PBMCs from healthy donors that were enriched for NKG2D+/CD56+ cells (B). Red cells are CD8+ T-cells while green cells are c-Kit+ cells in AA-like. Of note, many c-Kit+ melanocytes resided within the hair matrix of a human healthy HF and many c-Kit+ MCs attached to the HF bulb of AA lesion human skin previous transplanted on SCID mice. Dermal papilla (DP), hair bulb (HB) and hair matrix (HM).

In addition, Prof. Amos Gilhar provided us AA affected and healthy human skin samples previously transplanted on SCID mice. After receiving the samples in the lab, paraffin skin sections were cut and CD8/Tryptase **(Figure 3.44A,B)** and c-Kit immunohistochemistry were performed.

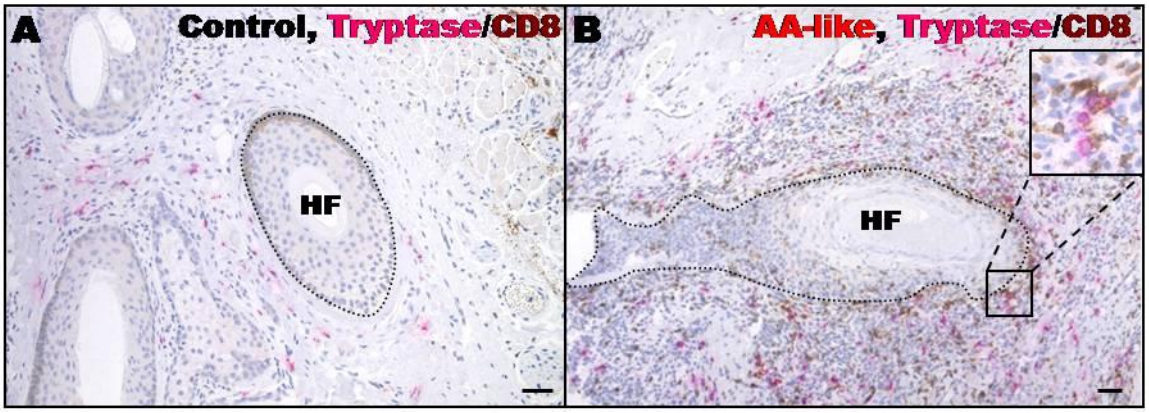

Figure 3.44: Immunohistochemical identification of tryptase+ mast cells and CD8+ T-cells in the humanized mouse model of alopecia areata.
Representative pictures of CD8/Tryptase double-staining in control (A) and AA-like (B) mice showing an increase of tryptase+ MCs and CD8+ T-cells. In panel B a higher magnification of tryptase+ MC-CD8+ T-cell interaction. Pink cells are tryptase+ MCs while brown cells are CD8+ T-cells. Of note, the brown cells in the HF bulge are mostly cytokeratin 15+ cells, not CD8+ T-cells. Size bars: 50µm. Hair follicle (HF).

Quantitative (immuno-)histomorphometry confirmed that the experimentally induced AA-like lesions in transplanted human skin show significantly perifollicular CD8+ T-cells **(Figure 3.45A)**, confirming previous observation (Gilhar et al. 2013a) compared to human skin control transplants injected with PHA-treated PBMCs enriched for NKG2D+/CD56+ cells.

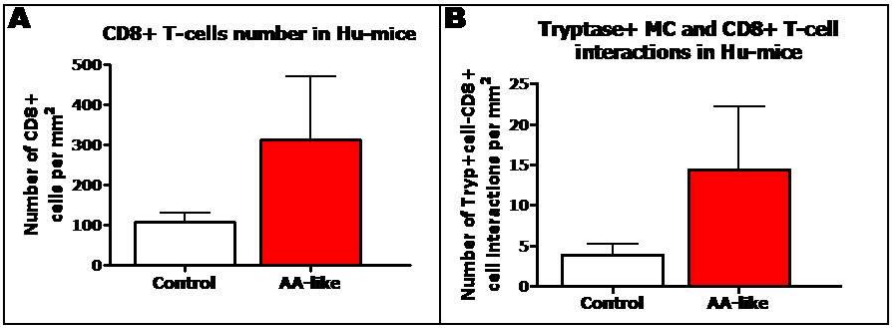

Figure 3.45: The number of CD8+ T-cells and tryptase+ mast cell-CD8+ T-cell interactions in human skin are increased in the humanized mouse model of alopecia areata.
Quantitative analysis of CD8+ cells (A) and tryptase+ MC-CD8+ T-cell interactions (B) in AA-like mice compared to control mice showing that the number of CD8+ T-cells and tryptase+ MC-CD8+ T-cell interactions are increased around AA-like HFs. Analysis deriving from 1-6 areas (HFs)/mouse of 3 mice/group from 2 experiments, ±SEM, Student t-test or Mann-Whitney-U-Test (ns).

Tendentially, more perifollicular mature and immature MCs **(Figure 3.46A,B)** were counted around AA-like HFs of human skin lesions on SCID mice compared to control mice (although significance was not reached).

However, MC degranulation was not increased in AA-like lesions compared to human skin control transplants injected with PHA-treated PBMCs enriched for NKG2D+/CD56+ cells **(Figure 3.46C)**.

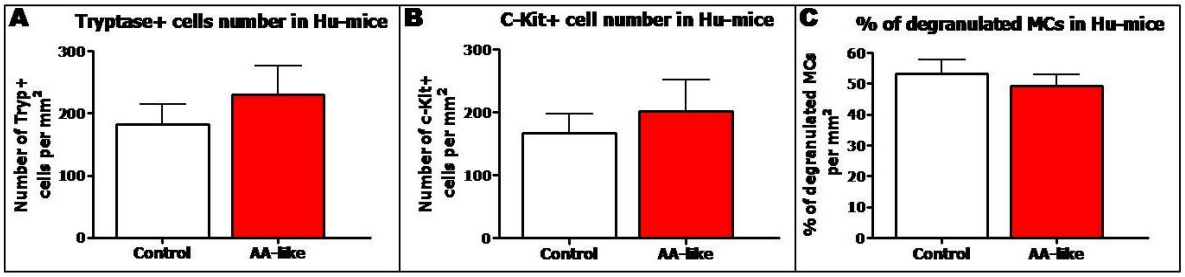

Figure 3.46: The number of mast cells, but not their degranulation, is increased in the humanized mouse model of alopecia areata.
Quantitative analysis of tryptase+ MCs (A), c-Kit+ MCs (B), % of degranulated tryptase+ MCs (C), in AA-like mice compared to control mice showing that the number of MCs, but not their degranulation, is increased around AA-like HFs. Analysis deriving from 1-6 areas (HFs)/mouse of 3 mice/group from 2 experiments, ±SEM, Student t-test or Mann-Whitney-U-Test (ns).

Most importantly, the number of MCs in physical contact with CD8+ T-cells was increased in AA-like human skin lesions on SCID mice compared to control mice **(Figure 3.44B)**. However, since the degree of inflammation presented around the HFs differed greatly between the mice, statistical significance was not reached.

Taken together, these *in vivo* experiments showed that MC and CD8+ T-cell interactions are also increased in AA affected C3H/HeJ mice and in experimentally induced AA lesions in previously healthy human skin, further suggesting that MC-CD8+ T-cell interactions are important for AA development.

4. Discussion

The concept that MCs are somehow involved in the pathogenesis of AA dates back several decades (Cetin et al. 2009, D'Ovidio et al. 1988, Finzi and Landi 1964, Baccaredda-Boy and Giacometti 1959), but has still not been systematically followed-up. However, recent reports that anti-histamines may be beneficial in at least some AA patients (Ito et al. 2013b, Ohyama et al. 2010, Inui et al. 2007) underscore the practical clinical relevance of dissecting the contribution of MCs to AA pathogenesis. The results reported above essentially confirm and then significantly extend the previous literature (e.g. Cetin et al. 2009, D'Ovidio et al. 1988, Finzi and Landi 1964, Baccaredda-Boy and Giacometti 1959) by focusing on MC interactions with CD8+ T-cells, the key immunocytes in AA pathogenesis (Gilhar et al. 2013a, Ito et al. 2013c, Alli et al. 2012, Petukhova et al. 2010, Cetin et al. 2009, McElwee et al. 2005, Yano et al. 2002, Gilhar et al. 1998, McElwee et al. 1996, Paus et al. 1993).

Specifically, this thesis project has revealed by quantitative (immuno-)histomorphometry analyses that, in AA lesional skin, perifollicular MCs show decreased TGFβ1 and IL-10 but increased tryptase immunoreactivity. This suggests that MCs switch from an immuno-inhibitory to a pro-inflammatory phenotype (**Figure 4.1**). This concept is supported by the observation that the number of IL-10+ and PD-L1+ MCs is decreased, while that of OX40L+, CD30L+, 4-1BBL+ and ICAM-1+ MCs is increased in AA.

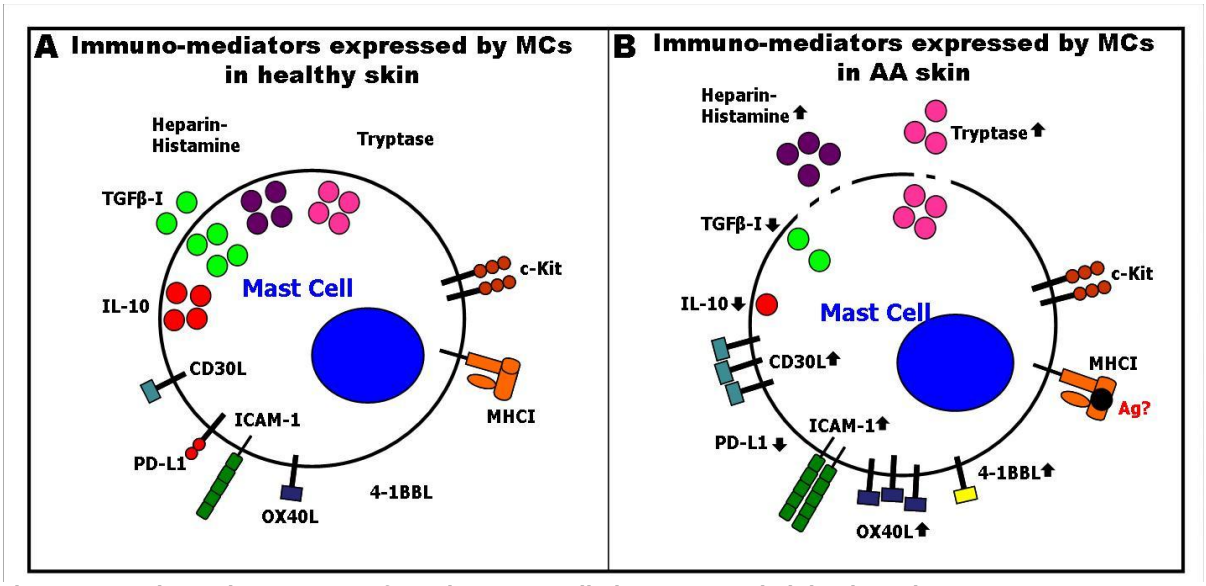

Figure 4.1: Schematic summary of results: mast cell phenotype switch in alopecia areata.
In healthy skin, MCs are mostly non-degranulated and they express the SCF receptor, c-Kit, and MHCI molecules. Most of them express IL-10, TGFβ1 while only some express OX40L and PD-L1 and very few express CD30L and ICAM-1 (A). In AA, the degranulation of MCs is increased (release of tryptase, heparin and histamine) and the expression of tryptase is increased while the contents of TGFbeta1, IL-10 are decreased. Moreover, the numbers of OX40L+, CD30L+, 4-1BBL+ and ICAM-1+ MCs are up-regulated while MCs positive for IL-10 and PD-L1 are down-regulated (B). This schematic drawing was preparing using also the Biomedical-PPT-Toolkit-Suite of Motifolio Inc., USA.

Lesional AA-HFs also display significantly more perifollicular CD8+ T-cells as well as more physical MC-CD8+ T-cell contacts than healthy or non-lesional human control skin. During their interaction with CD8+ T-cells, MCs prominently express MHC class I and OX40L, and sometimes 4-1BBL or ICAM-1, in AA skin. This observation is in line with (though it evidently does not prove yet) the concept that MC may present autoantigens and/or important co-stimulatory signals to CD8+ T-cells.

In the following, selected elements of MC pathobiology and of MC-CD8+ T cell interactions in AA are being discussed individually. Namely, we briefly discuss the pro-inflammatory MC mediators and co-stimulatory molecules examined *in situ* in AA lesional skin in regard to CD8+ T-cells and their potential role in AA pathogenesis.

First of all, the fact that in human skin and most precisely in the HF CTS and PFD, MCs are strongly expressing **TGFβ1** (**Figure 3.1A,B**) **IL-10 (Figure 3.15A,C,D)** and **PD-L1 (Figure 3.17A-C) (Figure 4.1)**, further supports the hypothesized role of MCs in HF-IP and peripheral tolerance maintenance (Waldmann 2006). This is further supported by the demonstrated declined expression of TGFβ1 (**Figure 3.1A-D**) and IL-10 (**Figure 3.15A,B**) and number of MCs positive for IL-10 **(Figure 3.15B,D)** and PD-L1 **(Figure 3.17A-D)** in AA lesional skin where the HF-IP is collapsed. Obviously, it remains to dissect whether these phenomena are critically involved in the primary pathogenesis of AA, or represents mere epiphenomena. Moreover, definitive proof for the validity of the concept that perifollicular MCs loose their HF-IP-promoting properties can only arise from functional studies, e.g. in the *in vivo* AA models that were employed also in the current thesis project (Wang et al. 2013, Gilhar et al. 2013a,b, Kang et al. 2008).

Also, the above results do not clarify the mechanism that regulates the MC expression/secretion of TGFβ1, IL-10 or PD-L1. However, from other systems, it is known that IL-10 expression in immuno cells is positively regulated by multiple transcription factors e.g. STAT3, C/EBPβ, IFN regulatory factor-1, NF-κB-related proteins (Lutay et al. 2014, Shoji et al. 2014, Mosser and Zhang 2008). Similar pathways are involved in the modulation of PD-L1 expression (Song et al. 2014, Huang et al. 2013). While integrins or proteases are responsible for the transformation of pro-TGFβ1 to active protein and its release from immuno cells (Worthington et al. 2012). Similar regulatory mechanisms may be in place in human skin MCs, and may be constitutively defective in AA patients, which could make such individuals more prone to suffering from HF-IP collapse.

In addition, it remains unclear how IL-10+ or PD-L1+ MCs are acting, namely with which other cells they are mainly interacting. The results reported above suggest that CD8+ T-cells are not the main interaction partners of IL-10+ or PD-L1+ MCs, since the few MCs that were seen to be interacting with CD8+ T-cells are rarely expressing IL-10 **(Figure 3.16A-E)** and never PD-L1 **(Figure 3.17A-D)**. Therefore, it is possible that, in human skin, IL-10 and PD-L1+ MCs interact with other cells population, among which regulatory T-cells are likely candidates since it was shown that e.g. IL-10+ MCs promote Tregs suppression of T-cells *in vitro* and thus, via the same mechanism contribute to peripheral tolerance and diminishing glomerulonephritis in autoimmune vascolitis *in vivo* (Gan et al. 2013, Frossi et al. 2010).

To clarify this further, the current results should be followed-up by triple immunostaining experiments for IL-10/C-Kit/FoxP3 and by functionally demonstrating that MCs play indeed a role in HF-IP. This may be achieved by using siRNA-based gene silencing techniques that have previously been used successfully in order to knock-down defined genes in the human HF (Vidali et al. 2014, Samuelov et al. 2012, Sugawara et al. 2012). After silencing IL-10 gene expression in organ-cultured human HFs, one could then search for evidence of a HF-IP collapse (e.g. ectopic up-regulation of MHC class I and II) after low-level stimulation with IFNγ (Ito et al. 2004) or substance P (Peters et al. 2007).

Secondly, the current data suggest that MCs in AA are skewed from a protective immuno-inhibitory to a pro-inflammatory phenotype (**Figure 4.1** and **Table 4.1**): the number of **IL-10+ (Figure 3.15A-E)** and **PD-1L+ (Figure 3.17A-C)** MCs are decreased as well as the content of immunosuppressive mediators such **TGFβ1** (**Figure 3.1A-E**) and **IL-10 (Figure 3.15A-D**). Meanwhile, potent pro-inflammatory mediators such as **tryptase** (**Figure 3.2A-E**) are up-regulated and the number of **OX40L+ (Figure 3.8A-D)**, **CD30L+ (Figure 3.11A-C)**, **4-1BBL+ (Figure 3.13A-E)**, **ICAM-1+ (Figure 3.14A-C)** MCs are increased.

Antigens	IR inside MCs	Number (or %) of positive MCs	Number of MC positive for the indicated antigen during interaction with CD8+ T-cells	Selected background references
Pro-inflammatory				
Tryptase	↑↑↑ (***)			Brown and Hatfield 2012
OX40L		↑↑↑ (***)	↑↑ (*)	Ilves and Harvima 2013
CD30L		↑↑↑(***/*)	Almost never	Fischer et al. 2006
4-1BBL		↑(rare) (n.q)	↑(rare) (n.q)	Watts 2005
ICAM-1		↑(few) (n.q)	Rare (n.q)	Gri et al.2012
Immuno-inhibitory				
TGFbeta1	↓↓			Wahl 2006
IL-10	↓(n.q)	↓↓↓(***)	Almost never	Chan 2013
PD-L1	↓(n.q)	↓ (n.q)	never	Nakae et al. 2006

Table 4.1: Mast cell phenotype in alopecia areata lesional skin, also during mast cell-CD8+ T-cell interactions.
Pro-inflammatory and immuno-inhibitory molecules which are considered to be involved in the cross-talk between MCs and CD8+ T-cells were analysed within MCs (IR inside MC). Moreover, positive MCs for these markers were counted around HFs (n. of + MCs) as well as their percentage among all MCs (% of + MCs). Finally, we investigated if MCs were positive for these markers during their interactions with CD8+ T-cells (n. of + MCs during CD8+ T-cells interactions) in AA patients. Arrows indicate increase (↑) or decrease (↓) expression and stars indicate significance (***p≤0.001, **p≤0,01, *p≤0.05), n.q. not quantified.

Interestingly, such a MC switch to a pro-inflammatory phenotype has already been reported in the experimental literature. For example, in a murine skin allograft model, MCs have been reported to induce regulatory T-cell-dependent peripheral tolerance (Lu et al. 2006); in contrast, if MCs are over stimulated (by inducing systemic MC degranulation) this tolerance can be reversed (de Vries et al. 2009b). This pleiotropic ability of MCs to modify their anti- versus pro-inflammatory activities depending on the predominant local signals they receive from their immediate signalling milieu (Kritas et al. 2014a, Fernando et al. 2013, Balato et al. 2012, Harvima and Nilsson 2011), may not only be important for for the immediate MC responses against pathogens (St. John and Abraham 2013, Frenzel and Hermine 2013, Galli et al. 2011), but also for the MC's complex contributions to maintaining tissue homeostasis, peripheral tolerance and defined IP environments (Chan et al. 2013, Voehringer 2013, Gan et al. 2012, Kalesnikoff and Galli 2011, Tsai et al. 2011, Frossi et al. 2010, de Vries et al. 2009a, Sayed et al. 2008, Lu et al. 2006, Waldmann 2006).

It is not clear yet which are the key stimuli that promote this major functional change in MC activities; but this switch is likely to be important in several autoimmune diseases (Frenzel and Hermine 2013, Brown and Hatfield 2012, Harvima and Nilsson 2011, Gilfillan and Beaven 2011, Fischer et al. 2006). The current results raise the possibility that AA ranks

among the autoimmune disorders in which an abnormal switch in the MC phenotype at least contributes to the pathogenesis. Such a MC phenotype switch might be triggered for example, by exposition of high concentration of MC secretagogues (deVries et al. 2009b) or the absence in the pathological environment of MC-relevant immunosuppressant cytokines (e.g. IL-9, TGF1β) (Fernando et al. 2013, Eller et al. 2011, Harvima and Nilsson 2011, Lu et al. 2006). The activation of vitamin D receptor on MC surface might also be involved in this phenotype modulation, since it was shown that the stimulation of vitamin D receptor on MC promotes immuno-inhibitory functions (Biggs et al. 2010). Since this important gap is not closed yet, it would be interesting to investigate the MC phenotype before and after the treatment with immuno-suppressive or pro-inflammatory cytokines/substances on microdissected HFs in culture. However, for autoimmune diseases it is also proposed that MC pro-inflammatory activities might be encouraged directly by exposure to the antigens (Christy et al. 2013).

Third, physical MC-CD8+ T-cell interactions, a fundamental prerequisite for CD8+ T-cell activation by MCs (Stelekati et al. 2009, Nakae et al. 2006), are enhanced in the perifollicular mesenchyme of lesional AA skin not only in AA patients **(Figure 3.4 A-D)** and in AA mice **(Figures 3.41C,D and 3.42C,D)**, but also in previously healthy human skin experimentally transformed into lesional AA skin **(Figure 3.45C)** (Gilhar et al. 2013a,b). The fact that enhanced MC-CD8+ T-cell interactions occur in both spontaneous and induced disease and across different mammalian species, suggests that this is not an epiphenomenon, but central to primary AA pathogenesis. Considering that MCs interacting with CD8+ T-cells in AA are strongly pro-inflammatory, they may promote pathogenic CD8+ T-cell responses against HFs which culminates in an enhanced **IFNγ** cell secretion from CD8+ T-cells (Stelekati et al. 2009), the recognized key cytokine in AA pathogenesis **(Figure 4.2)** (McElwee et al. 2013, Gilhar et al. 2012, Freyschmidt-Paul et al. 2006, Ito et al. 2004).

IFNγ is expected to then trigger two key events in AA pathogenesis (McElwee et al. 2013, Gilhar et al. 2012): 1. the stimulation of premature HF regression (catagen) (Ito et al. 2005b), and 2. the induction of HF-IP collapse in human and mice (Ito et al. 2004, Ruckert et al. 1998). In addition, IFNγ may be directly cytotoxic to epithelial and/or mesenchymal HF cells. **Figure 4.2** summarizes a plausible hypothetical how abnormal MC-CD8+ T-cell interactions may substantially promote AA pathogenesis.

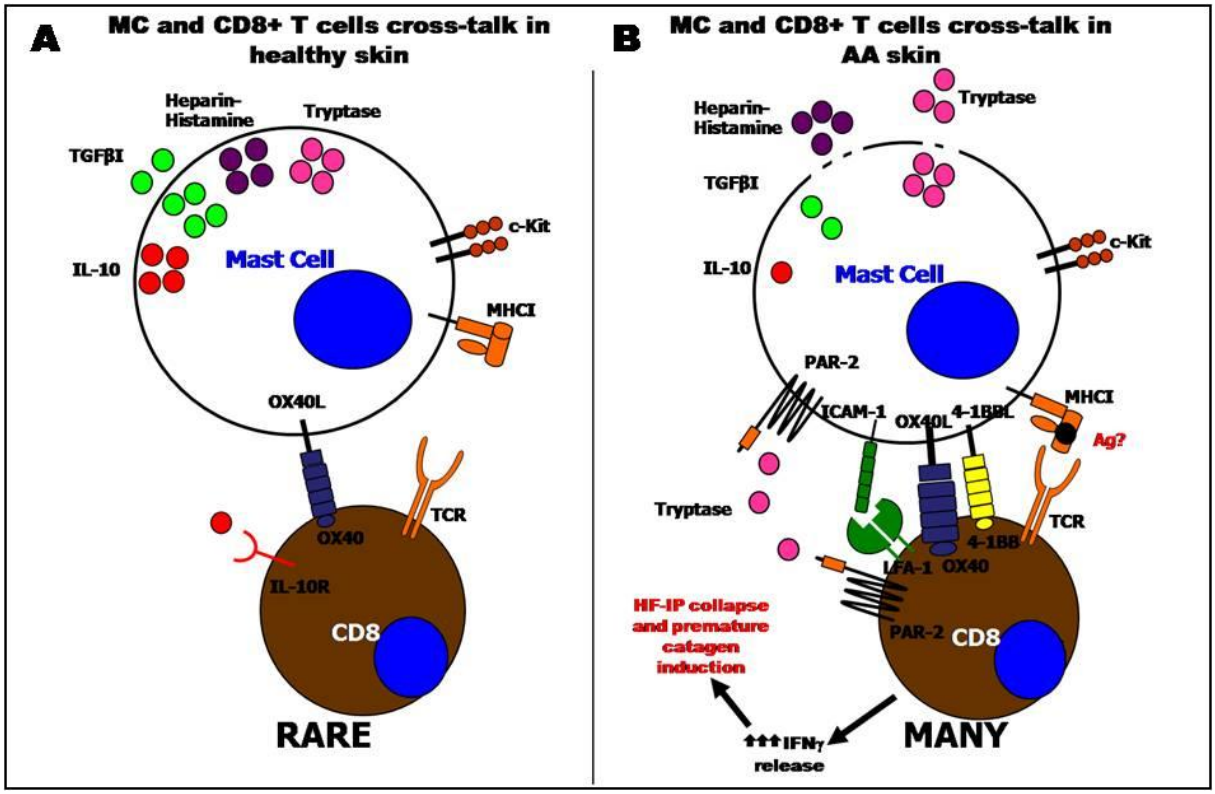

Figure 4.2: Schematic summary of results: mast cell-CD8+ T-cell interactions in healthy human skin compared to lesional human alopecia areata skin.
In healthy skin, rare MCs are found in close contact with CD8+ T-cells and most of them are expressing OX40L. Therefore, we hypothesised that OX40/OX40L might mediate this interaction. Rarely, we could find IL-10+ MCs interacting with CD8+ T-cells (A). In AA, many MCs were interacting with CD8+ T-cells. During this cross-talk, most MCs express OX40L but instead, in some rare cases, 4-1BBL and ICAM-1 were expressed. These ligands might stimulate the activation and proliferation of CD8+ T-cells. Since MCs during this interaction, are also degranulating we hypothesize an activation of PAR-2 (tryptase receptor) on CD8+ T-cells. Finally, we suggest that MCs may operate as autoantigen-presenting cells (B). This schematic drawing was preparing using also the Biomedical-PPT-Toolkit-Suite of Motifolio Inc., USA.

During MC-CD8+ T-cells interactions, in fact, half of MCs are degranulated **(Figure 3.2A-E**) therefore, the massive release of pre-formed MC granules containing e.g. neutral proteases (tryptase, chymase), histamine, proteoglycans (heparin and chondroitin sulphate E) and cytokines like TNF-alpha (Christy et al. 2013, Frenzel and Hermine 2013, St John and Abraham 2013, Gri et al. 2012, Harvima and Nilsson 2011, de Vries et al. 2009b, Pejler et al. 2010, Lu et al. 2009, Shin et al. 2009, Kashiwakura et al. 2004, Krejci-Papa et al. 1998, Maurer et al. 1997), together with the substantial increase in the total number of MCs in lesional AA skin (Bertolini et al. 2014), is expected to create a strongly pro-inflammatory perifollicular environment. This may promote the pathogenesis cascade leading to the AA phenotype (McElwee et al. 2013) and CD8+ T-cell activation and stimulation (Stelekati et al. 2009, Nakae et al. 2006).

Namely, among the investigated mediators, MC-derived **tryptase** can stimulate CD8+ T-cells (Harvima and Nilsson 2011, Li and He 2006). Moreover, tryptase play a key role in MC-directed collagenolysis (Magarinos et al. 2013, Sayed et al. 2008, Krejci-Papa et al. 1998).

Together, excessive tryptase release lead to a disruption of the HF's collagen-rich basement membrane, which could facilitate immigration of immunocytes into the – normally relatively shielded – HF epithelium, and could activate CD8+ T-cells in the course of AA pathogenesis. Moreover, tryptase can promote the release of immuno-mediators from keratinocytes (Harvima and Nilsson 2011, Steinhoff et al. 1999, Lohi et al. 1992) and T-cells (Harvima and Nilsson 2011, Spinnler et al. 2010, Li and He 2006), enhance MC activation (Hernandez-Hernandez et al. 2012, Harvima and Nilsson 2011, Moormann et al. 2006) via activation of PAR-2 receptor (Kempkes et al. 2014). Moreover, this receptor is expressed on sensory nerve fiber (Kempkes et al. 2014, Steinhoff et al. 2003, 2000), thus tryptase could contribute to the neurogenic inflammation in AA.

A key role for MC-dependent neurogenic inflammation is now well-appreciated in psoriasis (Hunter et al. 2013, Balato et al. 2012, Hernandez-Hernandez et al. 2012) and may also apply to AA, especially in AA patients that credibly report a triggering or exacerbating influence of psychoemotional stress on the appearance of AA lesions (Paus and Arck 2009). It is tempting to speculate that excessive release of tryptase from perifollicular MCs plays a cardinal role in this context, not the least since MC have already been documented to be indispensable for the perifollicular inflammatory infiltrates, neurogenic inflammation and subsequent hair growth inhibition/catagen induction that can be induced by perceived stress in mice (Paus and Arck 2009, Arck et al. 2005)

This thesis project has shown that MCs can express **OX40L** in both human healthy and AA skin *in situ* **(Figure 3.7A-F**). This is the first evidence that confirm *in vivo* the *physiological* relevance of previous corresponding *in vitro*-results that had been obtained with cultured human and mouse MCs (Zeng et al. 2013, Sibilano et al. 2012, Nakano et al. 2009, Gri et al. 2008, Fujita et al. 2006, Kashiwakura et al. 2004) and in chronic GVHD (Kotani et al. 2007).

While OX40L can be found in the nucleus, cytoplasm or cell membrane of human skin MCs, eventually, OX40L becomes preferentially localized at one side of the cells, thus facilitating juxtacrine signalling (Biggs et al. 2011, Tsuboi et al. 1999), As shown in **Figure 3.7A-F**, this phenomenon is also seen in human skin MCs. OX40L expression on the surface of MCs, may therefore facilitate MC-CD8+ T-cell interactions in both healthy human skin and AA skin lesions, since OX40L appeared to be the most prominently expressed co-stimulatory molecule on MCs that are in physical contact with CD8+ T-cells **(Figure 3.9A-C)**.

However the activation of CD8+ T-cells through OX40 in AA might be not only facilitated only via the cross-talk of OX40L+ MCs with OX40+ CD8+ T-cells (Ilves and Harvima 2013, Zhang

et al. 2013a, Weinberg et al. 2011, Croft 2010, Frossi et al. 2010, Ishii et al. 2010, Kober et al. 2008, Nakae et al. 2006, Kashiwakura et al. 2004) but also by other OX40L+ immuno cells, e.g. through CD4+ T-cells, although it is still unclear how this last phenomenon is regulated (Croft 2010, Humphreys et al. 2007). This last observation may be relevant to AA considering on the one hand that the pathogenesis of AA involves an antigen-specific, CD8+ T-cell immune response for which help from CD4+ T-cells is important, though not a *conditio sine qua non* (McElwee et al. 2013, Gilhar et al. 2012, Gilhar et al. 1998). This indirect scenario is suggested by the current finding that also MCs not interacting with CD8+ T-cells express OX40L.

In any case, OX40L+ MCs can enhance T-cell activation, proliferation, survival and cytokine production *in vitro* and regulates the accumulation of T-cells in primary and secondary responses (Zeng et al. 2013, Frossi et al. 2010, Gri et al. 2012, Kashiwakura et al. 2004, Tsuboi et al. 1999, Croft 2010, Sibilano et al. 2012). Therefore, in AA OX40L-OX40 interactions may be important in modulating pro-inflammatory MC-CD8+ T-cell contacts. However, as mentioned before, during the rare MC-CD8+T-cell interactions in healthy skin MCs are also expressing OX40L. Considering the well known OX40/OX40L cross-talk between Tregs and MCs necessary to preserve peripheral tolerance (Frossi et al. 2011, Frossi et al. 2010), most likely the CD8+ T-cells interacting with OX40L+ MCs in healthy skin may well be regulatory T-cells. Therefore, it would be interesting to stain normal healthy human skin for OX40L/Tryptase/FoxP3 in order to support this hypothesis.

The up-regulation of the number of **CD30L**+ MC found here in AA skin **(Figure 3.11A-C)** has already been reported in several pathological conditions, such as Hodgkin lymphoma (Molin et al. 2001), cancer (Diaconu et al. 2007), atopic dermatitis and psoriasis (Fischer et al. 2006). The latter observation is interesting since psoriasis shares some features with AA, e.g. both represent Th1-mediated inflammatory processes, and not only psoriasis (Ariza et al. 2013, Girolomoni et al. 2012) but also AA may exhibit a Th17 phenotype (Adami et al. 2014, Malakouti et al. 2014, Ito et al. 2013c, Tanemura et al. 2013, Tojo et al. 2013, Lew et al. 2012, Gilhar et al. 2003, Ranki et al. 1984). Although CD30-CD30L interactions provide a co-stimulatory signal for T-cells and stimulate T-cell proliferation and cytokine production (Croft et al. 2014, Cabrera et al. 2013, Weinberg et al. 2011, Ishii et al. 2010, Molin et al. 2001, Gruss et al. 1996), CD30L+ MCs were almost never found in close contact with CD8+ T-cells in AA skin **(Figure 3.12A-D)**.

While previous observations suggested a preferential enrolment of CD30 in Th2-mediated immunity of both CD4+ and CD8+ T-cells (Horie and Watanabe 1998, Del Prete et al. 1995),

more recently, CD30 has been shown to mediate the activation of T-cells prone to a Th1-type response (Cabrera et al. 2013, Fischer et al. 2006, Hamann et al. 1996). This suggests that CD30L+ MCs in AA skin may interact with other immune cells (e.g. CD4+ T-cells) or may signal via the release of sCD30 (Velasquez et al. 2013), which is produced by the cleavage of membrane-bound CD30 by metalloproteinase (Hansen et al. 1995).

4-1BBL enhances survival, proliferation, memory and cytolytic activities of T-cells and augments Th1-immune responses (Croft et al. 2014, Chacon et al. 2013, Vinay and Kwon 2012, Wang et al. 2009, Watts 2005, Sayama et al. 2002) and co-stimulated MC antigen presentation to CD8+ T-cells, at least in mice (Stelekati et al. 2009). Therefore, despite the relative paucity of 4-1BBL+ MCs interacting with CD8+ T-cells **(Figure 3.13A-E),** these rare events could be very important for AA pathogenesis, if one takes into account that 4-1BBL+ MCs are predominantly found in close contact with CD8+ T-cells at the proximal part of the HF around which the typical, dense inflammatory cell infiltrate is localized in AA, namely the anagen hair bulb (McElwee et al. 2013, Gilhar et al. 2012, Cetin et al. 2009, Whiting 2003).

In addition, the expression of 4-1BB is induced only after TCR activation, thus maintaining primed CD8+ T-cells after the inciting antigen is no longer available (Vinay and Kwon 2012). Therefore, our limited protein expression *in situ* data are well in line with the concept that the 4-1BB/4-1BBL pathway may be important regulating MC and CD8+ T-cell interactions in AA in pathogenesis. Evidently, this and the above hypotheses all await exploration and confirmation in subsequent functional *in vivo* studies, using the AA models employed here.

Only relatively few data are available regarding the interaction between **ICAM-1+** MCs and LFA-1+ T-cells (Gri et al. 2012, Nagai et al. 2009, Galli et al. 2005, Brill et al. 2004, Skokos et al. 2001, Inamura et al. 1998), and most of these studies have only considered the effect of activated LFA-1+ T-cells on ICAM-1+ MCs (Nagai et al. 2009, Brill et al. 2004, Inamura et al. 1998), rather than vice versa. Therefore, the results of the current thesis project, namely that ICAM-1+ MCs are interacting with CD8+ T-cells **(Figure 3.14A-E)** contribute to close an important gap in immunobiology research and encourage one to further investigate in follow-up studies the functional importance of ICAM-1/LFA-1 signalling pathways in the priming of CD8+ T-cells by MCs, namely in human skin and during AA pathogenesis. Ideally, one could up-regulate the expression of ICAM-1 on MCs *in vitro* (e.g. stimulating them with kinins (Figueroa et al. 2014)) and co-culture them with CD8+ T-cells. This would allow one to investigate at least which cytokines or pro-inflammatory receptors are expressed by CD8+ T-cells, once activated by MCs.

Methodologically, the major shortcoming of the current study is its purely phenomenological nature, despite its rigorous quantitative (immune-)histomorphometry approach as well as the use of complex triple-immunostaining techniques and mutually complementary AA *in vivo*-systems. However, although several potential fuctional *in vitro* studies were attempted in the current thesis project in order to further investigate and functional dissect the role of MCs and CD8+ T-cell interactions, none of them were successful due to methodological constraints.

Specifically, the **HF organ culture** was first engaged as a possible model in which functionally dissect the roles of MCs and CD8+ T-cell interactions. To overcome the problem that the number of CD8+ T-cells around the anagen HF bulb is very low (Christoph et al. 2000), we employed SP as a treatment which not only stimulates MCs (Sugawara et al. 2012, Peters et al. 2007) but also CD8+ T-cells (Siebenhaar et al. 2007). According to our findings, indeed SP up-regulates the number of MCs, as already shown (Sugawara et al. 2012, Kumamoto et al. 2003) **(Figure 3.19A-D),** but it does not amplify the number of CD8+ T-cells **(Figure 3.19A-D)**. This experiment was strongly supported by the fact that had the number of MCs and CD8+ T-cells been enough, and MC-CD8+ T-cell interactions had been found around microdissected HFs, it would have been possible to obtain immediate results about the role of MC-CD8+ T-cells in HF-IP maintenance. Moreover, having the compound 48/80 as control substance which seemed to activate only MCs, would have helped to discern if the MC-CD8+ T-cell interactions were promoted by MCs activation or CD8+ T-cells stimulation.

Since the treatment with SP and compound 48/80 of healthy human skin was well established to investigate MC activities (Sugawara et al. 2012), the second attempt made here to establish a model for investigating and manipulating MC-CD8+ T-cells in human skin was the organ culture of full-thickness healthy human skin (Langan et al 2012, Sugawara et al. 2012, Lu et al. 2007).

However, the effect of SP and compound 48/80 treatments on MC degranulation reveals inter-individual differences. In fact, in some of the examined individuals the treatment failed to increase MC degranulation **(Figure 3.22C** and **3.26C)**. At the same time, in those examined individuals that indeed respond to SP and compound 48/80, MC degranulation is only slightly promoted **(Figure 3.30B)**. This is in contrast to previous findings from our lab (e.g. Sugawara et al. 2012, Peters et al. 2007, Maurer et al. 1997, Paus et al. 1994b,c) and that of others labs in human or murine skin treated with SP or compound 48/80 (Chatterjea et al. 2013, Liu et al. 2013, Siebenhaar et al. 2007).

This discrepancy was further investigated during the write-up phase of the current thesis by analyzing the number of degranulated MCs in different cutaneous reference areas (i.e. upper dermis, dermis and subcutis). This pilot experiment suggested that MC degranulation in the skin treatment with SP is promoted mostly in the dermal part of human skin (data not shown), thus confirming previously observation (Sugawara et al. 2012). Therefore, considering that functionally and phenotypically distinct MC sub-populations populations exist in human skin (Sugawara et al. 2012, Buckley et al. 1999, Algermissen et al. 1994), as yet ill-defined sub-populations of dermal MCs may be more sensitive to stimulation by neuropeptides such as SP than other MC populations in human skin.

Same interindividual differences were seen also regarding the activation of CD8+ T-cells with SP and compound 48/80. In fact, while CD8+ T-cells are strongly activated by SP in the skin organ cultures from both examined patients **(Figure 3.23A and 3.23C)**, compound 48/80 increased CD8+ T-cell number only in one patient **(Figure 3.23C)**. This last observation further suggests that compound 48/80 is not specific for MCs activation and can also stimulate CD8+ T-cells. The mechanisms by which compound 48/80 might stimulate CD8+ T-cells could well be the depletion of intracellular calcium (WoldeMussie and Moran 1984), as in the case of MC activation.

In addition, after 3 days of culture the number of CD8+ T-cells declines **(Figure 3.23A** and **3.28A)**. Therefore, it would be interesting to establish and performed a double-staining TUNEL/CD8 in order to investigate if this decrease of CD8+ T-cells number is due to an apoptosis-mediated phenomenon.

The analysis of MC-CD8+ T-cell interactions number in SP or compound 48/80 treated healthy skin in CTS and PFD is also characterized by inter-individual differences and may reflect also the different activation of MCs and CD8+ T-cells in each examined individual **(Figures 3.23B, 3.28B** and **3.30D)**. In skin treated from the first investigated individual, MC-CD8+ T-cell interactions are decreased in the CTS after secretagogue treatment, but are increased in the PFD **(Figure 3.23B** and **3.28B).** This is quite interesting, as it invites one to speculate that perifollicular MCs in the CTS of healthy HFs may play a particularly prominent role in HF-IP preventing the activation of CD8+ T-cells. Therefore, MCs in PFD could belong to a different subpopulation compared to the one of CTS. PFD-MCs, for example, may favor the activation of intracutaneous CD8+ T-cells which is necessary during anti-microbial defences against pathogen invasion of the skin (Frenzel and Hermine 2013, St. John and Abraham 2013, Galli et al. 2011). However, this could be just a *mere speculation* since this result could not be replicated in treated skin form other individuals.

However, especially regarding MC-CD8+ T-cell interactions, the data have to be interpreted with caution due to the fact that only rare MC-CD8+ T-cell interactions events are found in vehicle and treated healthy skin. Therefore, a single event could dramatically change the variation of the results in each group. As well, the CTS area is always much smaller compared to the one of PFD, reason why the standard deviation is higher in analyses performed in CTS compared the ones done in PFD.

The final intriguing point that deserves discussion here is the observed up-regulation of MHC class I IR after 24 hours **(Figure 3.25C)**, while a decrease in TGFβ1 IR occurs only after 72 hours **(Figure 3.29B)** of MC secretagogues treatment. First of all, this confirms that SP promotes HF-IP collapse, as previously reported (Peters et al. 2007), and suggests that the skin organ culture experiment was performed appropriately despite of the odd results of MC degranulation. It is of note that the expression of TGFβ1 is down-regulated long after the up-regulation of MHC class I, suggesting that the IP collapse is composed by a sequence of events which chronology is still unknown. Therefore, it would give a great contribution to the IP field to understand this deeply, maybe via stimulating the HF-IP collapse either with IFNγ (Ito et al. 2004) or SP (Peters et al. 2007) and analysing classical IP markers (MHC class I, class II, TGFβ1,…) (Meyer et al. 2008, Paus et al. 2005) at specific time points (e.g. 1, 3, 6, 10, 15, 20 and 24 hours after the treatment).

Taken together, the very limited currently available pilot data from human skin organ culture suggest that this experimental system, in principle, permits one to test concrete hypotheses on physiological MC-CD8+ T-cell interactions in full-thickness human skin organ culture as long as one can overcome problems encountered in the current pilot studies, such as the large interindividual differences and the very low numbers of relevant cells found after extended skin organ culture. However, these technical challenges should be manageable, eventually.

However, the treatment of the single available sample human AA skin with SP, compound 48/80 or cromoglycate in an additional functional pilot assay further support the concept that abnormal MC-CD8+ T-cell interactions play a role in HF-IP collapse and AA pathogenesis. In fact, in **organ-cultured AA skin** where the environment is strongly pro-inflammatory and HF-IP has already collapsed, SP appears to up-regulate the number of MC-CD8+ T-cell interactions in the CTS **(Figure 3.35B*)***. Also in the organ culture of AA lesional skin as for the organ culture of healthy skin, the number of CD8+ T-cells is tremendously reduced during the culture **(Figure 3.35A)**, further suggesting that CD8+ T-cells might die for apoptosis.

Unexpectedly, the treatment with cromoglycate in this experiment seems not to inhibit MCs **(Figure 3.33A** and **3.34A).** There might be two possible explanations for this outcome. Firstly, this data might be a result of the fact that cromoglycate has little or no effect on human connective tissue MCs compared to mucosal MCs (Bradding and Saito 2014). In fact, since few years, the efficacy and selectivity of cromoglycate to inhibit not only human but also mouse MCs is questioned (Oka et al. 2012, Weng et al. 2012). Unfortunately, the experiments were performed before that this important debate started. Weng and colleagues support quercetin instead of cromoglycate, because it is more efficient in inhibiting cytokine release from MCs and can even inhibit contact dermatitis and photosensitivity in human (Weng et al. 2012). So, for future experiments which aim to block MCs, one should consider to use quercitin instead of cromoglycate. The second possible explanation why cromoglycate does not reduce MC number in AA skin culture is that it may act on MCs, not by decreasing their number but by changing their phenotype so that anti-inflammatory MCs are up-regulated after the treatment. If this is true, cromoglycate treatment in AA skin may up-regulate protective- MC-CD8+ T-cell interactions **(Figure 3.35B)**. Unfortunately, no data in literature support this last hypothesis because in most of the case the effect of cromolyn on MCs is investigated at the level of pro-inflammatory cytokines and not anti-inflammatory cytokines (Weng et al. 2012). So, to confirm this hypothesis, one could study the phenotype of MCs on AA skin treated with cromoglycate.

However, organ culture of human AA skin is a highly unpragmatic experimental tool for probing pathological MC-CD8+ T-cells interaction, as (for evident reasons) human AA skin is almost never available in sufficient quantity for satisfactory experimental analyses.

To overcome these organ culture limitations in the human system, the research direction was moved towards an *in vivo* model. However, contrary to what had been hypothesized, the subcutaneous injection of INFγ did not accelerate AA development in **aging C3H/HeJ mice** in our hands (**Figure 3.36A-L** and **3.37A-L**). This may have been due to the INFγ relatively low dose and subcutaneous administrations schedule employed here, since previous studies demonstrated disease induction by injection of 2x10000U intravenously (Gilhar et al. 2005).

Overall, the experiment also failed since only very few aging C3H/HeJ mice spontaneously developed AA at all over several months, which made it impossible to investigate MC-CD8+ T-cell interactions in lesional AA skin in this model. Moreover, in the few animals that did develop AA lesions, the number of MCs does not increase during the early stage of AA development, contrary to the human condition (Bertolini et al. 2014, Bertolini et al. 2010a,b,

Cetin et al. 2009), and MC-CD8+ T-cell interactions are only rarely up-regulated in spontaneous developed AA lesions **(Figure 3.38A-I)**.

In addition, the immunophenotype and location of the perifollicular infiltrate of human AA are not reproduced in C3H/HeJ mice **(Figure 3.38A-I)** (King et al. 2008, McElwee et al. 1998a) and they are really difficult to be cross-bred with other immunologically interesting mouse mutants (Reber et al. 2012). This constitutes a major handicap in applying the full spectrum of mouse genomics to further dissect the molecular mechanisms that underlie development of the AA phenotype (Gilhar et al. 2013a). Moreover, these mice carry an important abnormality in toll like receptor 4, which is never seen in AA patients (cf. Gilhar et al. 2012). Taken together, this strongly questions how good this much-investigated mouse strain really is as a model for studying human AA **(Table 1.7)** (Gilhar et al. 2013a).

However, in the **grafted C3H/HeJ mouse model** of AA, were AA lesions occur much more rapidly and predictably (Silva and Soundberg 2013, Wang et al. 2013, King et al. 2008), the current thesis project could show that AA mice do exhibit an up-regulation of MC-CD8+ T-cell interactions **(Figure 3.41A-D** and **3.42A-D)**. This suggests that establishing an experimental design which standardizes the AA development is very useful, if not mandatory, provided that one wishes to adhere to the suboptimal C3H/HeJ mouse model of AA.

In the face of these considerations, **the humanized mouse-model** of AA **(Figure 3.44A,B**, **3.5A,B** and **3.46A-C)** is definitely a better model in which to study AA pathogenesis, because it clearly mimics the AA human condition. Moreover, in sharp contrast to the C3H/HeJ model, in this model it is human immunocytes that attack human HFs that are growing perfectly well within their natural tissue habitat *in vivo* (human scalp skin transplanted onto SCID mice) (Gilhar et al. 2013a,b). Moreover, SCID mice can be cross-bred with other immunologically interesting mouse mutants, allowing application of the full spectrum of mouse genomics to further dissect the molecular mechanisms that underlie development of the AA phenotype **(Table 1.7)**.

In order rigorously probe the hypothesis put forward in this thesis project, one needs to *selectively* deplete or modulate MCs activities in order to dissect the role of MC-CD8+ T-cells in AA pathogenesis. However, unfortunately, satisfactory functional experiments to definitely confirm or refute the basic hypothesis proposed here **(Figure 4.2)** can currently not be performed for the following compelling reasons:

1) None of the routinely used MC-deficient or MC-overexpressing mouse models develop classical AA lesions.
2) It is not yet possible to selectively eliminate or exclusively modulate *only* MCs *in vivo* (mice) or in transplanted or organ-cultured human skin *without also* eliminating or damaging other cutaneous cell populations (e.g. c-Kit+ melanocytes and hair matrix keratinocytes, or FceR+ Langerhans cells).
3) Frequently employed inhibitors of MC degranulation such as cromolyn or luteolyn are not effective for all MC subsets and or not MC-specific, as other cell populations, incl. CD8+ T-cells and sensory neurons and their axons, are affected, as well (Finn and Walsh 2013, Vieira Dos Santos et al. 2010, Karra et al. 2009, Theoharides et al. 2007).
Therefore, the concept that abnormal MC-CD8+ T-cell interactions play a functionally important role in AA pathogenesis remains hypothetical at this time, and the underlying mechanisms of action can only be speculated upon on the basis of our phenomenological evidence – until the methodological handicaps sketched above have been overcome.

However, since the management of AA in clinical medicine remains overall quite unsatisfactory (Gilhar et al. 2012, Harries et al. 2010) and in view of the psychoemotional stress this disease imposes on affected patients (Matzer et al. 2011, Paus and Arck 2009, Gupta et al. 1997), it is a matter of urgency to develop more effective AA management strategies. Therefore, the phenomenological evidence presented here and the plausible pathogenesis scenario deduced from it **(Figure 4.1** and **4.2)** are translationally important, since they help to identify novel candidate targets for future therapeutic intervention in AA management.

Namely, our findings predict that treatment regimen which promote an immuno-inhibitory phenotype and/or suppress the switch towards a pro-inflammatory MC phenotype, should down-regulate undesired CD8+ T-cell responses against human HFs. This novel treatment strategy should inhibit AA progression and may facilitate hair regrowth in established AA lesions by assisting in the restoration of HF IP.

Specifically, our data invite one to test the effects of blocking OX40L/OX40, e.g using oxelumab, which was first developed for the treatment of allergic asthma (Catley et al. 2011, Weinberg et al. 2011). Alternatively or in addition, 4-1BB/4-1BBL interactions could be blocked (Gizinski et al. 2010, Wang et al. 2009) with a blocking or antagonist antibody (Le et al. 2013, Tu et al. 2012) or the PAR-2 activation by tryptase could be antagonized (Crilly et al. 2012, Michael et al. 2013). These novel approaches in AA therapy could be explored in the two distinct AA mouse models employed here.

However, these signalling molecules are broadly expressed on immunocytes; therefore, more specific therapies need to be contemplated, including the use of bi-specific antibodies for selectively blocking MCs (Harvima et al. 2014, Karra et al. 2009). On the other hand, one need to keep in mind that MCs are important for maintaining tissue homeostasis and peripheral tolerance (Chan et al. 2013, Voehringer 2013, Gan et al. 2012, Kalesnikoff and Galli 2011, Tsai et al. 2011, Frossi et al. 2010, de Vries et al. 2009a, Sayed et al. 2008, Lu et al. 2006, Waldmann 2006) so instead of blocking MCs activities, the best would be to promote their immuno-inhibitory phenotype (e.g. using vitamin D (Biggs et al. 2010)). Alternatively, antagonists/blockers that modulate undesired MC-CD8 T-cell interactions should be applied only externally to the skin, using HF targeting vehicles such as nanoparticles and liposomes that are capable of enriching these agents after topical application inside the pilosebaceous unit (Knorr et al. 2014, Patzelt et al. 2013, Mak et al. 2012, Liu et al. 2011). With these recent methodological advances in dermatopharmacology in mind, the current study underscores that the therapeutic modulation of perifollicular MCs human skin deserves to be systematically explored as a novel therapeutic strategy in the future management of AA.

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6. Abstract

Alopecia areata (AA) is a CD8+ T-cell dependent autoimmune disease of the hair follicle (HF) in which the collapse of HF-immune privilege (IP) plays a key role. Mast cells (MCs) are crucial immunomodulatory cells implicated in the regulation of T-cell-dependent immunity, IP, and hair growth which number and activities are increased in AA. Given that CD8+ T-cells are considered to be the key effector cells in AA pathogenesis, the current thesis project aim is to dissect the role of MC interactions with CD8+ T-cells in both human and mouse skin with AA lesions.

Quantitative (immuno-)histomorphometry revealed that in AA lesional skin, perifollicular MCs showed decreased TGFβ1 and IL-10 but increased tryptase immunoreactivity, suggesting that MCs switch from an immuno-inhibitory to a pro-inflammatory phenotype. This concept was supported by a decreased number of IL-10+ and PD-L1+ MCs, while OX40L+, CD30L+, 4-1BBL+ and ICAM-1+ MCs were increased in AA.

Lesional AA-HFs also displayed significantly more perifollicular CD8+ T-cells as well as more physical MC/CD8+ T-cell contacts than healthy or non-lesional human control skin. During the interaction with CD8+ T-cells, AA MCs prominently expressed MHC class I and OX40L, and sometimes 4-1BBL or ICAM-1, suggesting that MC may present autoantigens to CD8+ T-cells and/or co-stimulatory signals.

Preliminary experiments towards the establishment of a model where to functionally modulate MCs, revealed that the treatment of microdissected HFs with endogenous (substance P) and exogenous (compound 48/80) MC secretagogues is not suitable for this purpose. An improvement was obtained with the full-thickness healthy scalp skin organ-cultures in which 4mm punches from healthy skin were treated with MC secretagogues. These experiments suggested that in predispose individuals, the excessive release of substance P may impair the physiological control of CD8+ T-cells by MCs. Following an unique opportunity to test MC secretagogues and stabilizer (cromoglycate) in a full-thickness AA scalp skin organ-culture, it was observed that MC-CD8+ T-cells are increased in AA lesional skin after the treatment with substance P while almost no effect was visible with compound 48/80 and a slight decreased was detected with cromoglycate.

Nevertheless, abnormal MC numbers, activities, and interactions with CD8+ T-cells were also seen in AA lesions of spontaneously developed in C3H/HeJ mice, of grafted C3H/HeJ mouse model and in a new humanized mouse model for AA.

These phenomenological *in vivo* data suggest the novel AA pathobiology concept that perifollicular MCs are skewed towards pro-inflammatory activities that facilitate cross-talk with CD8+ T-cells in this disease, thus contributing to triggering HF-IP collapse in AA. If confirmed, MCs and their interactions with CD8+ T-cells could become a promising new therapeutic target in the future management of AA.

7. Zusammenfassung

Alopecia areata (AA) ist eine CD8+ T-Zell-abhängige Autoimmunerkrankung des Haarfollikels (HF), bei welcher der Zusammenbruch des Immunprivilegs eine Schlüsselrolle spielt. Mastzellen (MC) sind wichtige immunmodulatorische Zellen, die für die Regulation der T-Zell-abhängigen Immunität, für das Immunprivileg und für das Haarwachstum eine zentrale Rolle spielen. Die Anzahl und die Aktivität der MC sind in der AA erhöht. Aufgrund der Annahme, dass CD8+ T-Zellen eine Schlüsselrolle als Effektorzelle in der Pathogenese der AA spielen, ist das Ziel der vorliegenden Arbeit, die Rolle der MC Interaktion mit den CD8+ T-Zellen sowohl in menschlicher als auch in Maushaut zu untersuchen.

Quantitative (Immun-)Histomorphometrie legte dar, dass in läsionaler Haut von AA die perifollikulären MC eine reduzierte Immunreaktivität für TGFβ1 und IL-10 aufwiesen, diese jedoch für Tryptase erhöht war. Dies deutet darauf hin, dass MC von einem immuninhibitorischen zu einem pro-inflammatorischen Phänotyp wechseln. Die Annahme wurde weiterhin durch eine verminderte Anzahl an IL-10+ und PD-L1+ MC unterstützt, wohingegen OX40L+, CD30L+, 4-1BBL+ und ICAM-1+ MC in AA erhöht waren.

Im Vergleich zu gesunder und nicht-läsionaler humaner Kontrollhaut wiesen läsionale AA HF sowohl signifikant mehr perifollikuläre CD8+ T-Zellen auf als auch mehr MC/CD8+ T-Zell-Kontakte. Während der Interaktion mit CD8+ T-Zellen exprimierten AA MC bedeutend mehr MHC Klasse I und OX40L und manchmal auch mehr 4-1BBL und ICAM-1. Dies legt nahe, dass MC Autoantigene gegenüber CD8+ T-Zellen präsentieren und/oder ko-stimulatorische Signale.

Erste Vorläufer-Experimente zur Etablierung eines Modells, um funktionell MC zu modulieren, zeigten, dass die Behandlung von mikrodissezierten HF mit endogenen (Substanz P) und exogenen (Compound 48/80) MC Sekretagoga nicht geeignet sind für dieses Vorhaben. Eine Verbesserung dieses Vorhabens konnte erreicht werden, indem Vollhaut von gesunder Skalphaut in Organkultur genommen wurde. Hierbei wurden 4 mm Stanzen von gesunder Haut mit MC Sekretagoga behandelt. Diese Experimente wiesen darauf hin, dass in prädispositionierten Individuen die exzessive Freisetzung von Substanz P die physiologische Kontrolle von CD8+ T-Zellen durch MC beeinträchtigt. Es bot sich zudem die einzigartige Möglichkeit MC Sekretagoga und Stabilisatoren (Cromoglycate) an einer Vollhaut-Organkultur von Skalphaut eines AA Patienten zu testen. Es konnte dabei beobachtet werden, dass MC-CD8+ T-Zellen in läsionaler AA Haut nach Behandlung mit Substanz P erhöht waren, wohingegen fast kein Effekt mit Compound 48/80 zu sehen war und nur eine kleine Verringerung der Anzahl mit Cromoglycate erreicht werden konnte.

Gleichwohl konnten abnorme MC Zahlen, Aktivitäten und Interaktionen mit CD8+ T-Zellen in AA Läsionen beobachtet werden, die spontan in C3H/HeJ Mäusen, einem C3H/HeJ-Transplantat-Mausmodell und auch in einem humanisierten Mausmodell für AA auftraten.

Diese neuen *in vivo* gewonnenen Daten legen ein neues pathobiologisches Konzept nahe, nämlich dass perifollikuläre MC in Richtung pro-inflammatorische Aktivitäten bei dieser Krankheit agieren und eine Interaktion mit CD8+ T-Zellen fördern und somit daran beteiligt sind, ein Kollabieren des HF IP in

AA auszulösen. Wenn es bestätigt werden kann, MC und ihre Interaktion mit CD8+ T-Zellen, könnte dies eine neue erfolgversprechende Therapieoption in der Therapie der AA dar stellen.

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List of figures

List of tables

Publication arising from this thesis project

Bertolini M, Zilio F, Rossi A.,Kleditzsch P, Emelianov VE, Gilhar A, Keren A, Meyer KC, Wang E, Funk W, McElwee K, Paus P. Abnormal interactions between perifollicular mast cells and CD8+ T-cells may contribute to the pathogenesis of alopecia areata. ***PLoS One***. 2014 May 15;9(5):e94260. IF: 3.73.

Abstracts arising from this thesis project

Bertolini M, Kleditzsch P, Emelinov VE, Sugawara K, Meyer KC, Paus R (2010): Comparative analysis of perifollicular mast cells in alopecia areata and normal human scalp skin. Exp Dermatol 18(2):166-232. (Abstract n. p341, Poster presentation at 37th Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), Lübeck, Germany)

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Bertolini M, Kleditzsch P, Emelinov VE, Arakawa A, Sugawara K, Meyer KC, Paus R (2011): Do abnormal interactions between perifollicular mast cells and CD8+T-cells contribute to the pathogenesis of alopecia areata? (5th International Conference on Autoimmunity: Mechanisms and Novel Treatments, Crete, Greece, oral presentation)

Bertolini M, Kleditzsch P, Emelinov VE, Sugawara K, Arakawa A, Meyer KC, Paus R (2012): Dissecting mast cell-CD8+ T-cell interactions in the pathogenesis of human autoimmune disease: Pointers from alopecia areata (Poster n.6, 7th ENII EFIS/EJI Spring School in Advanced Immunology, Sardinia, Italy)

Bertolini M, Kleditzsch P, Emelinov VE, Sugawara K, Arakawa A, Meyer KC, Paus R (2012): Dissecting mast cell-CD8+ T-cell interactions in the pathogenesis of human autoimmune disease: Pointers from alopecia areata (Poster n.79, 8th International Congress on Autoimmunity, Granada, Spain)

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Paus R, Bertolini M. The role of hair follicle immune privilege collapse in alopecia areata: status and perspectives. ***J Investig Dermatol Symp Proc***. 2013 Dec;16(1):S25-7. IF: 3.733

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Curriculum vitae

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11/2009-03/2011	University of Lübeck, Germany, Post-graduate training fellowship
2007-2009	University of Padua 10/2009: Masters Degree in Pharmaceutical Biotechnologies (Faculty of Pharmacy) (score 110/110)
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2004	High school diploma in scientific subjects, Liceo Scientifico "Leonardo da Vinci," Cerea, Verona, Italy

WORKING EXPERIENCE

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12/2011	Guest scientist at the research laboratory of Prof. Amos Gihar, Technion- Israel Institute of Technology, Haifa, Israel

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2007	Five months training at the research laboratory of the Department of Microbiology, Histology and Medical Biotechnology of University of Padua for the Bachelor's thesis project 'Study of the interaction of Nef (HIV-1 accessory protein) and Dynamin-2 (cellular GTPasi) by the two Hybrid system in yeast' (evaluation attached)
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- 5th International Conference on Autoimmunity: Mechanisms and Novel Treatments, Crete, Greece (2011)
- **Co-organized** the International Workshop on “alopecia areata as a model for T cell- specific autoimmune disease” at the University of Lübeck, Germany (2011)
- 7th ENII EFIS/EJI Spring School in Advanced Immunology, Alghero, Italy (2012)
- 8th International Congress on Autoimmunity, Granada, Spain (2012)
- 42nd Annual Meeting of the European Society for Dermatological Research (ESDR), Venice, Italy (2012)
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- 41st Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), Cologne, Germany (2014)
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PRIZES AWARDED

- **2nd Publication Award 2013** of GRK 1727/1 “Modulation of autoimmunity” for the publication entitled: The immune system of mouse vibrissae follicles: cellular composition and indications of immune privilege, *Exp Dermatol* 2013
- **5th Autoimmunity Travel Award**: Mechanisms and Novel Treatments
- **Poster prize** awarded during the 42nd ESDR (European Society of Dermatological research) conference in Venice, 09/2012.

OTHERS

- FELASA licence to perform experiments with laboratory animals (January 2010).

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