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Immune privilege collapse and epithelial stem cell damage during hair follicle inflammation in murine epidermolysis bullosa acquisita (EBA)

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Dedicated to my Parents, Teachers and Friends

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ABBREVIATIONS

AA	Alopecia areata
ABC	Avidin-biotin complex
ACTH	Adrenocorticotropic hormone
APM	Arrector pili muscle
BM	Basement membrane
BP	Bullous pemphigoid
eSC	Epithelial stem cell
CA	Cicatricial alopecia
CCCA	Central centrifugal cicatricial alopecia
CCLE	Chronic cutaneous lupus erythematosus
CD	Cluster of differentiation
COL7	Collagen type VII
CTS	Connective tissue sheath
DAB	3,3'diaminobenzidine
EBA	Epidermolysis bullosa acquisita
Fc	Fragment, crystallizable
FcγRIV	Fragment, crystallizable receptor type IV
Fig	Figure
FITC	Fluorescein isothiocyanate
GST	Glutathione-S-transferase
HF	Hair follicle
ICAM	Intraepithelial cell adhesion molecule
IDO	2,3 indoleamine-dioxygenase
IR	Immuno reactivity
IF-	Immunofluorescence
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IL15	Interleukin
ILTAC	Intralesional triamcinolone acetonide
IP	Immune privilege
IR	Immunoreactivity

IRS	Inner root sheath
Ko	Knockout
LC	Langerhans cells
LFA	Leukocyte facilitating antigen
LPP	Lichen planopilaris
µM	micrometer
ml	Milliliters
MC	Mast cells
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institute of Health
NR-IgG	Normal rabbit immunoglobulin G
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ORS	Outer root sheath
PBS	Phosphate buffered saline
PCA	Primary cicatricial alopecia
PPAR-γ	Peroxisome proliferator-activated receptor gamma
SEM	Standard error of the mean
SG	Sebaceous gland
TB	Toluidine blue
TBS	Tris buffered saline
TGF-β	Transforming growth factor β
Th1	T helper response type 1
TSA	Tyramide signal amplification
TNT	Tris-NaCl-Tween

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1.0 INTRODUCTION

1.1 Project overview

Alopecias represent a group of hair follicle (HF) disorders in which the HF`s ability to grow is stunted, diminished, or abrogated, leading to hair loss. Alopecia areata (AA) and cicatricial alopecia (CA) represent the major subgroups of inflammatory alopecia. CA is thought to occur as a result of an inflammatory attack on bulge epithelial stem cells of the HF, which may lose their immunologically privileged status, eventually leading to HF destruction. However, until today, the exact pathobiology of all forms of CA remains.

HF undergo constant cycling which impacts on their immune privilege (IP). The HF bulb and bulge are privileged as they express low levels of MHC class I molecules (MHCI) and constitutively express CD200 in the outer root sheath (ORS) and since anagen follicles secrete various immunoinhibitors such as α MSH, TGF-β, IDO and MIF. IP in the HF is a relative state that is down regulated or collapses in diseases like alopecia areata (AA) and cicatricial alopecia/scarring alopecia (CA).

CA is also presented in passive and active disease mouse models of epidermolysis bullosa acquisita (EBA), an acquired autoimmune bullous skin disease caused by autoantibodies directed against collagen type VII (COL7) that can be associated with CA, due to damage to HF stem cells in the bulge. Taking advantage of these mouse models of EBA, the current thesis addresses three general questions: a) Are there indications of HF immune privilege (IP) in murine telogen follicles (as these are seen to be primarily attached in murine EBA models)? b) Which mechanisms may lead to irreversible destruction of murine bulge stem cells, and is there any evidence that a collapse of bulge IP is involved in this? and, finally, c) Can this destructive process be therapeutically antagonized?

These questions were mainly addressed by qualitative and quantitative (immuno-) histomorphometry and by administering blocking antibodies to the FcγRIV receptor. The current project demonstrates that murine EBA models serve as an

excellent tool to dissect general principles of antibody- and/or inflammation-induced irreversible stem cell damage in the follicular bulge, leading to HF IP collapse and HF destruction and the development of a clinical CA phenotype. These models can also be exploited to further explore whether CA development and/or progression, at least in EBA, may be treated by targeting FcγRIV receptor with appropriate antibodies.

1.2 Hair follicle biology

HFs, sebaceous glands (SG), and sweat glands form important cutaneous appendages. These help in maintaining skin homeostasis by actively adopting both physiological and protective roles (Paus and Cotsarelis 1999; Stenn and Paus 2001, Schneider et al. 2009). The HF is a complex mini organ that is loaded with diverse pools of epithelial, mesenchymal and melanocyte stem cells and that is connected with the adjacent SG and arrector pili muscle to form an intact pilosebaceous unit (Ohayama 2006, Goldstein and Horsely 2012, Nishimura 2011). Like other organs, it is also surrounded by complex networks of blood vessels and nerve fibers and has its own stroma, the connective tissue sheath (CTS) (**Fig. 1**).

The HF has an independent ability to regenerate very effectively, largely due to the presence of many different stem cell populations (Tiede et al. 2007a,b , Schneider et al. 2009, Rompolas et al. 2012.). The most important epithelial stem cells of the HF in mice and man are lodged in a special, immunologically privileged zone of the HF epithelium, called the bulge (Cotsarelis 2006, Kloepper et al. 2008, Goldstien and Horsely 2012, Nishimura 2011) (**Fig.1**). Though the stages of HF cycling are the same in mice and humans, the dynamics of regeneration are species- and location-dependent. For example, HF cycling in mice takes place in a wave-like fashion from the head to the tail (Sten and Paus 2001, Sundberg et al. 2005, Schneider et al. 2009), while in humans, the pattern is more random or mosaic (Kligman 1959). Apart from protection, the hair follicle also play a role in promoting skin regeneration and remodeling of the skin during wound healing (Lau et al. 2009, Jiang et al 2010,Ansell et al. 2011,Mardayeve et al.2011).

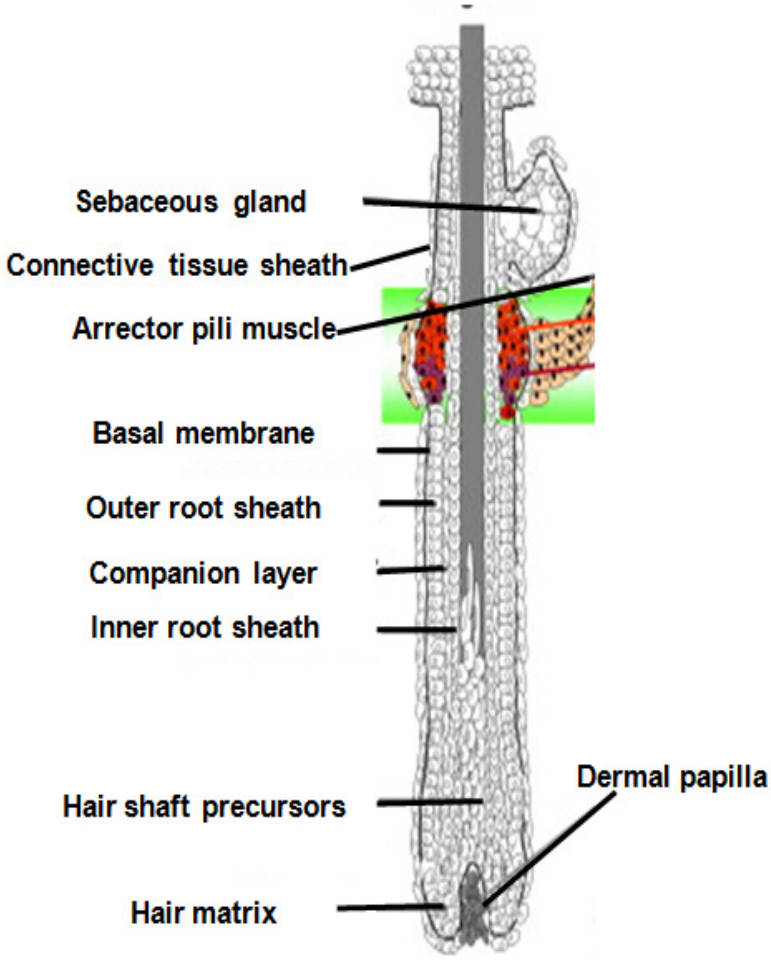

Fig.1: Schematic drawing depicting the structural outline of the anagen VI hair follicle. *The picture shows the outer root sheath, inner root sheath, basal membrane connective tissue sheath, hair matrix and dermal papilla. It also depicts the bulge (red) and associated arrector pilli muscle (orange) (Fig modified from Tiede et al. 2007)*

1.2.1 Functional anatomy of the hair follicle

It is estimated that there are about 5 million HFs distributed over the human integument, with the scalp contributing to about a million of them (Paus and Cotsarelis 1999; Paus and Foitzik 2004, Lee and Tumbar 2012). Under the control of inductive fibroblasts of the HF's dermal papilla, rapidly proliferating cells in the hair matrix generate the bulk of keratinocytes from which a hair shaft is generated. The terminally differentiating keratinocytes from the hair shaft's cortex are guided and packaged by the IRS, with the companion layer serving as slippage plane. In the precortical hair matrix, these keratinocytes receive melanin via melanosomes

that are transferred to them by specialized melanocytes of the HF's pigmentary unit (Botchkarev et al. 2003,Kasuer et al. 2010,Nishimura et al. 2011) (**Fig.2,3**).

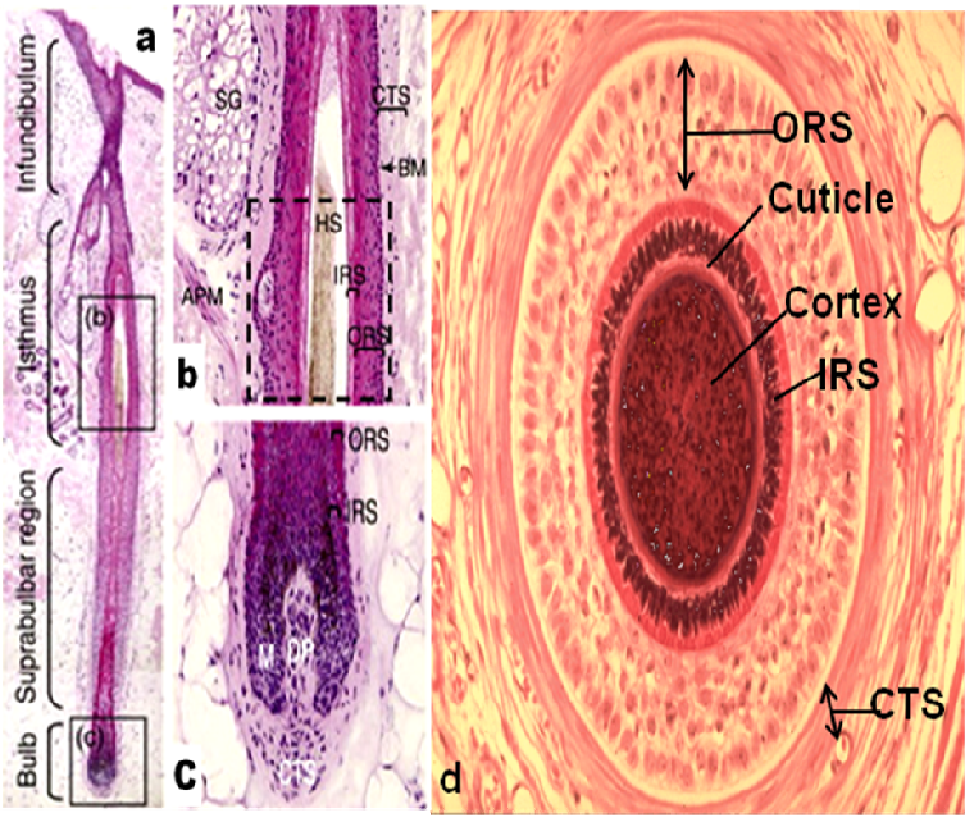

Fig.2: Structure of the hair follicle.*Longitudinal cross section of the hair follicle representing various regions of the hair follicle. Infundibulum is the portion of the hair follicle between the sebaceous gland and epidermis. Isthmus is the region that lies below the infundibulum and harbors the bulge that contains eSHs that can give rise to new follicles upon induction during catagen.The hair root or the bulb is the portion that harbours the dermal papilla and hair matrix, the size of the dermal papilla determines the length of the hair follicle (a) Higer magnification of the bulge region (b) and dermal papill (c) Schematic picture representing transverse cross section of the hair root or hair bulb (d).APM-arrector pili muscle, CTS-cutaneous tissue sheath, DP-dermal papilla, HS-hair shaft, IRS-inner root sheath, ORS-outer root sheath and SG-sebaceous gland. fig.a,b,c modifiedfrom Schneider et al.2009,2b modified from http://www.udel.edu/biology/Wags/histopage/colorpage/cin/cinhft2.gif)*

Horizontal sections through the hair follicle show eight concentric ring-like structures : the cortex, medulla and cuticle of the hair shaft (HS), the cuticle of the IRS, Huxley's layer (IRS), Henle's layer (IRS), companion layer, and the ORS. Interestingly, each of these above-mentioned layers has distinct lineages of

epithelial differentiation and shows differences in their enzymes, structural or adhesion molecules (Powell and Rogers 1995; Stenn and Paus 2001).

The different layers such as the ORS, IRS, hair matrix and the HS arise from a pool of epithelial stem cells (eSCs) that are label-retaining cells (Cotsarelis 2006, Myung and Ito 2012). They are located at the region of the insertion of the arrector pili muscle (APM), well below the insertion of the SG duct. In mice (**Fig.3**), the bulge can be visualized by a slight protrusion of the ORS but in the humans this region is difficult to visualize and hence the APM, SG and the newly found region of the 'follicular trochanter'' serve as guidance points to locate the bulge (Cotsarelis 2006, Tiede et al. 2007, Goldstein and Horsely 2012*)*

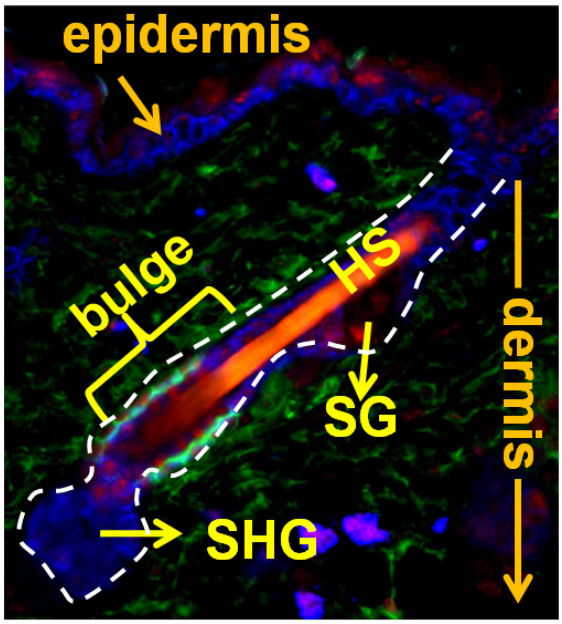

Fig.3: Structure of the murine telogen follicle. *The picture depicts the localisation of CD34+ stem cells in the bulge of a mouse HF (FITC labeled antibody, nucleus labeled in blue [DAPI]).HS-hair shaft,SHG-secondary hair germ,SG-Sebaceous gland (modified from Lander et al.2012)*

It is believed that the primary group of eSCs reside in the ORS and gives rise to secondary pool of cells that move away from the bulge to the secondary hair germ and gives rise to the IRS, hair matrix and HS (Cotsarelis et al. 1990, Panteleyev et al. 2001; Ohyama et al. 2006; Tiede et al. 2007b, Hsu and Fuchset al. 2012). The

stem cell niche is regulated through complex sets of regulatory mechanisms (Beck and Blanpain. 2012, Singh et al.2012a,b). In this regard it was recently shown that ADAM17 is needed for regulation of the stem niche (Nagao et al. 2012b). In terms of alopecia, it was recently shown that mutation in Gsdma 3 causes stem cell loss as a consequence of inflammatory attack (Zhou et al.2012). The stem cells isolated from engineered mice gives rise to cells of various lineages that eventually gave rise to the IRS, hair matrix and hair shaft (HS) (Blanpain et al. 2004). Apart from forming new hair follicles, stem cells also promote epithelial regeneration during wound healing (Lau et al. 2009,Ansell et al.2011)

The bulb of the anagen HF consists of a prominent epithelial region called the matrix that gives rise to the hair shaft (**Fig.2**) (Paus et al 1999b, Whiting 2004). The precortex located is above the tip of the DP and serves as a region where the keratinocyte differentiation to trichocytes is initiated. The further differentiation of keratinocytes gives rise to the cortex, medulla and the cuticle of the hair shaft. The volume of the DP determines the size of the hair bulb and thus the diameter of the hair shaft and impacts of the length of anagen and thus the length of the hair shaft (Reynolds and Jahoda 1996; Reynolds et al. 1999). During the hair cycle, there is also considerable bidirectional trafficking of fibroblasts between the DP and to the CTS (Tobin et al. 2003).

The angle of the HF is determined by the APM which, under adrenergic influence, leads pull up the hair shaft (Paus and Peker, 2003) In humans, each APM surrounds the HF unit that consists of 2-3 vellus HF or 4-5 terminal HF (Headington 1984). The bulge of the HF is surrounded by a tuft of sensory, autonomic and Merkel cells (humans) (Botchkarev et al. 1997). The HF constantly receives signals from neurons, both during stress and normal conditions (Paus et al. 2007,Paus et al.1997b; Powell and Rogers, 1995, Botchkarev et al. 1999; Peters et al. 2007). Additionally, the HF is escorted by a basket-like network of vessels that form arterioles, capillaries, and venules (Mecklenburg et al. 2000; Yano et al. 2001). In human HFs, some of these capillaries end up in the DP and participate in the constant nourishment of the HF, while the DP of murine HFs reportedly is avascular (Mecklenburg et al. 2000).

1.2.2 Hair follicle cycling

The HF undergoes synchronized cycles of growth regression and tissue remodeling throughout the lifecycle of an organism (**Fig.4**). This synchronised cycling is, however, thought to be dependent on seasons and procreational activity (Stenn and Paus 2001).The pelage follicles in mice cycle in a wave-like fashion from the head to the back(Muller-Rover et al. 2001**)**, while in humans and most domesticated animals this process is quite randomized after the birth ("mosaic" hair cycle) (Muller-Rover et al. 2000, Plikus & Chuong 2008a, Pilikus et al. 2008b, Mecklenburg 2009).

The telogen stage that is thought to represent the so-called "resting" stage of the folliclelasts for about 2 weeks in young mice, while the anagen stage that represents the active stage of follicular growth lasts for 2-3 weeks; catagen, the involution phase of HF cyclinglasts for 2 days. Soon after morphogenesis, the first cycle of catagen-telogen-anagen is quite short and starts as early as on week 2, 3 and 4 after morphogenesis, respectively.The second hair cycle starts with the initiation of catagen in week 6, telogen in week 7, while the second anagen sets in starting from week 12. With increasing age, an ever-larger percentage of HFs in murine skin resides in telogen, and HF cycling becomes progressively less synchronized. In pigmented mice, the skin colour reflects the stages of cycling with pink,grey and black reflecting telogen,catagen and anagen stages, since essentially all melanogenic activity of truncal skin is restricted to anagen HFs (**Fig.4**) (Paus 2011,Paus et al.1999a, Muller-Rover et al. 2001).

In humans, the catagen stage lasts a few weeks, while telogen lasts for several months, and anagen 1-6 years (on the scalp). The human adult hair grows at the rate of 0.3mm -0.4 mm per a day and is totally dependent on the proliferation of the hair matrix keratinocytes (Paus 2007; Stenn 2001). The anagen phase is subdivided into six individual stages. The anagen stage is developed from the secondary hair germ cells below the bulge and is characterised by proliferation and differentiation of keratinocytes of the matrix, organ remodeling, and follicular melanogenesis (Paus 2011,Muller-Rover et al. 2001; Paus et al. 1999a; Peters et al. 2001).

The catagen is the regression stage of the HF that is driven by apoptosis and terminal differentiation of the proximal HF epithelium (Lindner et al. 1997). This stage is also characterised by retraction of melanocyte dendrites, the cessation of all melanogenesis, and the formation of a fibrotic strand between the condensing DP and the retracting HF epithelium (Paus and Foitzik 2004, Slominski et al. 2005). This process finally transforms the HF into a so-called “resting” stage, telogen (**Fig.4**). However, recent research shows that this phase is not at all quiescent, and has been postulated to be much more active than previously thought (Geyfman et al.2012).

HF cycling results from bidirectional molecular signaling between HF keratinocytes and inductive fibroblasts of the DP, which acts as a mesenchymal control centre for the growth initiation and termination of anagen. This causes controlled changes in the intracutaneous, intra- and perifollicular signaling milieu that drive the HF from one hair cycle stage to the next (Stenn and Paus 2001, Schneider et al. 2009). Hair growth-modulatory growth factors like IGF-1, HGF, FGF7, and TGFß play a key role in these epithelial-mesenchymal interactions that drive HF cycling. For example, TGFß1 and TGFß2 are potent inducers of catagen, while IGF-1 prolongs anagen (Alonso and Fuchs 2006, Fuchs 2009, Schneider et al. 2009). The contact between keratinocytes of the secondary hair germ (SHG) and DP fibroblasts are thought to signal to the eSC in the bulge of telogen HFs to initiate the production of daughter cells (transit amplifying epithelial cells) that migrate towards the DP to give rise to a new anagen follicle (Hsu and Fuchs et al. 2012).

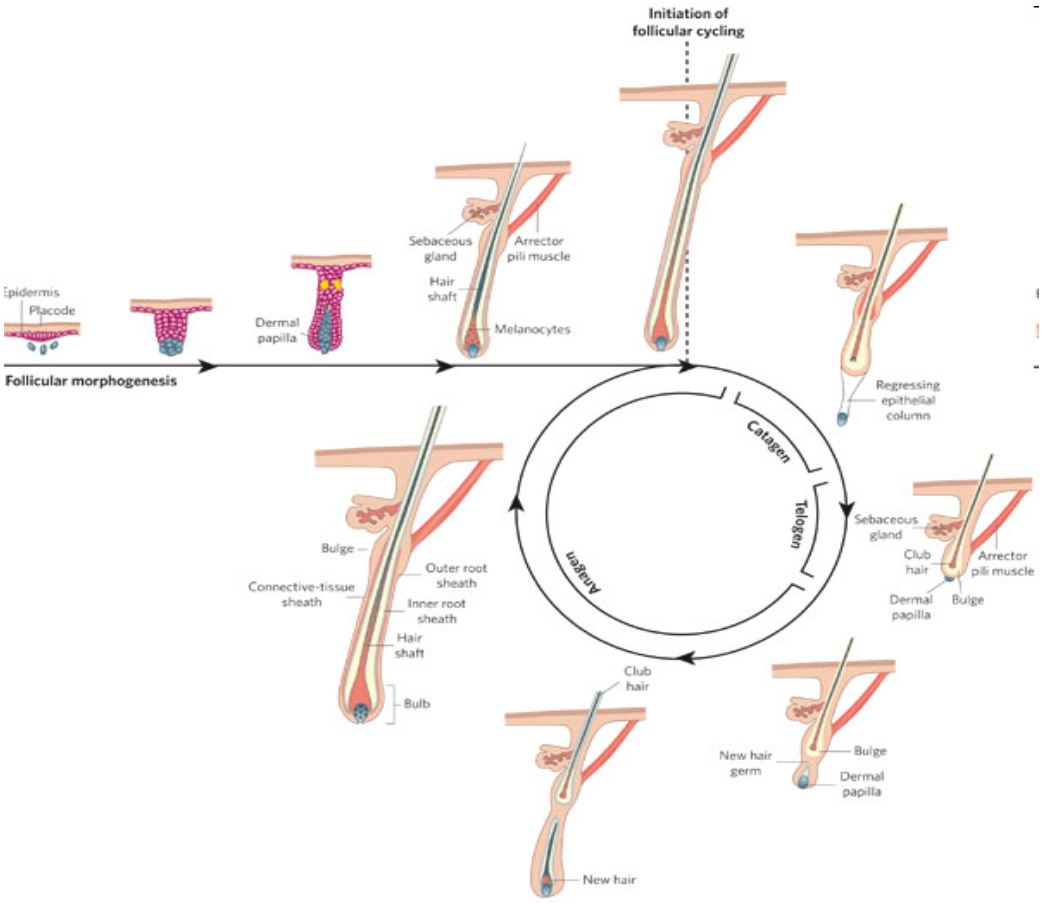

Fig.4: Schematic diagram displaying the regeneration and cycling events of human hair follicle.*During the entire life cycle, the hair follicle under go constant cycling involving stages of rapid proliferation (anagen), organ involution (catagen) and stage of quiescence (telogen). The anagen represents the longest phase of HF cycling and lasts about 1-6 years (Fuchs.E, 2007).*

1.3 Immune privilege and follicular immune privilege

1.3.1 Immune privilege

While being an integral component of the skin's immune system (Di Meglio et al 2011), the HF in man in mice also displays a number of special characteristics, the most important of which is its immunologically privileged nature (Paus et al. 1998, Paus et al. 2005; Conrad et al. 2005; Mayer et al. 2008; Gilhar et al. 2012)

The broad term of IP refers to the ability of some tissues to tolerate the growth of a distinct tissue within its vicinity without eliciting an immune response.Immune privilege was discovered when an allograft tissue from tumour cells and foetal cartilage could grow in the anterior chamber of the eye as well as in the brain

(Niederkorn 2003; Kinori et al. 2011,Breitkopf et al. 2013).This regeneration was attributed to the ability of the host tissue to sequester the alloantigens (Head and Billingham 1985). Over the years, this concept has further expanded to define suppressive mechanisms that offer protection to tissue compartments from the potentially deleterious immunological processes (Paus et al. 2003, Ito et al. 2008).

The organs/tissues that are recognized to show relative IP include the brain, eye, placenta, testis, anagen hair follicles, the proximal nail matrix, and the hamster cheek pouch (Head and Billingham 1985; Streilein 1993, Paus et al. 2005, Ito et al. 2005b). In this regard, the collapse of IP seems to be causally linked to common autoimmune disease like multiple sclerosis (Bruno et al. 2002), mumps orchitis (Filippini et al. 2001), chronic autoimmune hepatitis (Lobo-Yeo et al. 1990) and presumably some cicatricial alopecia's (Harries & Paus 2010). Cancer cells also exploit IP strategies in order to escape tumor immunosurveillance (Lu and Finn 2008, Topfer et al. 2011).

However, it is important to note that IP is always relative and a dynamic state, rather than a stable, fixed condition (Ito et al. 2008). IP operates through various mechanisms in different tissues. Some of the key mechanisms include absence of lymphatics (e.g. in the brain and the skin epithelium), presence of specific extracellular matrix barriers (e.g. blood-brain barrier, blood-retinal barrier, CTS of the HF), down regulation of (autoantigen-presenting) MHC class I molecules, expression of Human Leukocyte Antigen-G and Qa-2 in humans respectively mice, or expressing HLA-E, a ligand for natural killer cells inhibitory receptor (CD94/Natural Killer G2A) (Braud et al. 1998; Gao et al. 1997; Paus et al. 2005; Tripathi et al. 2006).Other mechanisms include, for example, downregulation of MHC class II class expression , secretion of TGF-β1, TGFβ2, α-MSH, macrophage inhibitory factor (MIF), CGRP, somatostatin, and expression of FAS ligand for bringing about apoptosis of autoreactive T cells (; Streilein 1993,Niederkorn 2002; Paus et al. 2005;Mellor and Munn 2008, Kinori et al. 2012, Pi et al. 2013,Breitkopf et al. 2013).

1.3.2 Hair follicle immune privilege

In autoimmune diseases like alopecia areata, melanocyte associated autoantigens are thought be one of the key players in the induction of the disease (Ito et al. 2008, Gilhar et al. 2012). Like the brain, foetus, and eye the HF also shares a perceived level of immune privilege (Paus et al. 2005). The functional key evidence in this regard comes from the work of Billingham(1971) who showed that transplantation of pigmented skin onto the back of white guinea pigs eventually led to the development of pigmented hair shafts, while the rest of the transplanted epidermis was rejected (Billingham 1971). These experiments show the ability of hair follicle melanocytes to survive and repigment the hair follicle despite expressing alloantigens. However, although there are multiple indications that strongly point towards to establishment of relative IP also in the HFs of mice and humans (see below) it remains to be formally proven that this is functional in human and murine HFs.

Some well-recognized individual components of the IP of murine and human HFs are summarized below (**Fig. 5**).

1.3.3. Components of immune privilege in the hair follicle

In the following individual key components recognized to be important for HF IP are introduced (**Fig.5** a, b) (Paus et al. 2005, Meyer et al. 2008).

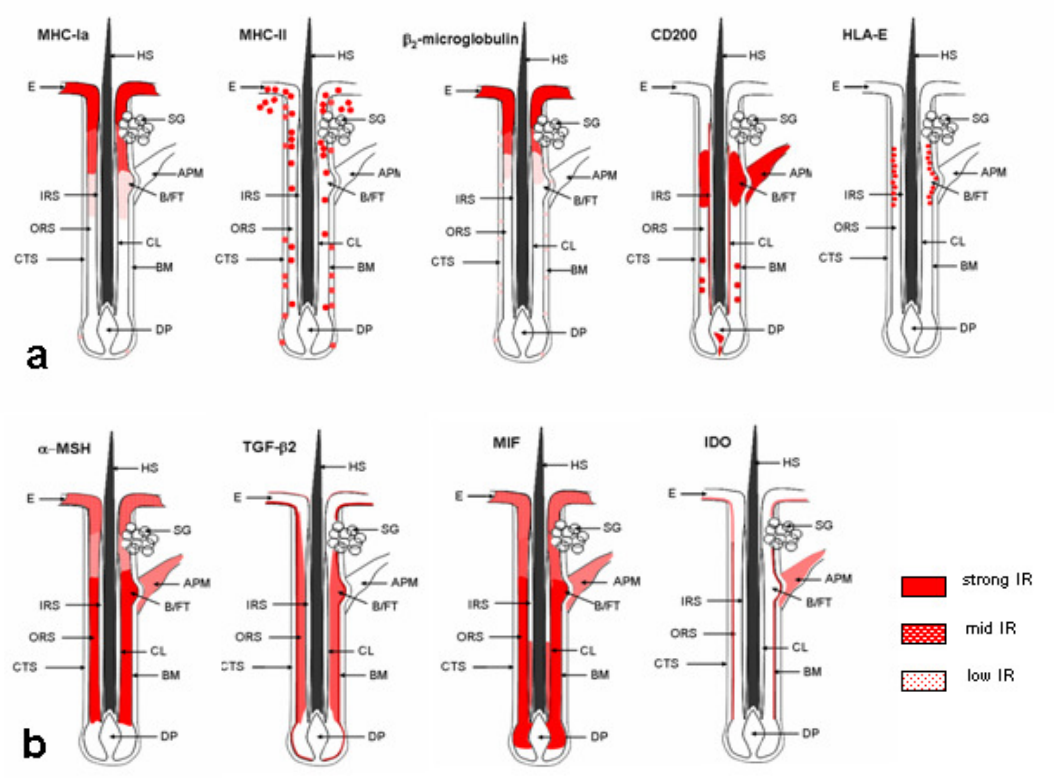

Fig.5: Schematic diagram displaying the quantitative expression of various markers which contribute to immune privilege of the human hair follicle.*Expression of MHCI, MHCII, CD200, ß2-microglobulin and HLA-E protein within the hair follicle (a).Expression of α-MSH, TGF ß2 B2, MIF and IDO with in the anagen VI hair follicle (b)., APM-arrector pili muscle, B-bulge, BM-basement membrane, CL-cuticle, CTS-cutaneous tissue sheath, DP-dermal papilla, FT-follicular trochanter, IRS-inner root sheath, ORS-outer root sheath and SG-sebaceous gland(modified from Meyer et al. 2008)*

1.3.3.1 Limited expression of MHC class I

While the anagen VI hair follicle expresses normal amounts of MHC class I in the infundibulum, the DP, and the CTS the proximal HF epithelium includes. the proximal ORS, IRS and the hair matrix of anagen HFs constitutively expresses low or minimal amounts of MHC I (Paus et al.1994c)(**Fig.5** a). This is also seen in human HFs (Harrist et al. 1983, Christoph et al. 2000, Mayer et al. 2008) and cultured human ORS keratinocytes (Limat et al. 1994). This distinct down

regulation of MHC I in the proximal ORS below the infundibulum is also seen in other mammals (Harrist et al. 1983; Paus et al. 1998; Christoph et al. 2000; Paus et al. 2005, Kinori et al. 2012).

1.3.3.2 Expression of CD200

The evolutionarily highly conserved immunoinhibitory molecule CD200 is also expressed on cells of nonhematopoietic origin and is thought to send a "non-danger signal" by interacting with CD200L that is expressed e.g. on T lymphocytes and dendritic cells, which are thereby inactivated (Bukovsky et al. 1983; Clark et al. 2003; Wright et al. 2003; Rosenblum et al. 2006; Norde et al. 2012, Rygiel and Meyaard L. 2012) The human HF also expresses CD200 throughout its central ORS(**Fig.5**a), including the bulge and proximal isthmus(Mayer et al. 2008).

This molecule has also been used to isolate bulge stem cells from both mice and humans, after gene expression profiling of stem cells revealed these progenitor cells to prominently express CD200 (Ohyama et al. 2006; Tumbar et al. 2004, Tiede et al. 2009). Skin-specific knockdown of CD200 in mice resulted in massive perifollicular skin inflammation, hair loss, and CA when transplanted onto the back skin of wild-type mice (Rosenblum et al. 2004). Furthermore, our own findings of decreased expression of CD200 in CA patients suggest an important role for CD200 in CA pathobiology (Harries et al. 2009a, Harries & Paus 2010; Harries et al. 2013)**.**

1.3.3.3 TGF-β (Tumor growth factor β)

TGF β exerts multiple different and at times opposing effects in cells and tissues of various types. To date, three isoforms have been discovered (TGF-β1, TGF-β2, and TGF-β3). TGF- β is stipulated to play a key role in establishing niches of relative IP by sending overall immunoinhibitory signals and by recruiting immune cells that are suppressive in nature (Cobbold et al. 2006; Wahl et al. 2006 Khalil 1999; Streilein 1993; Derynck et al. 1988;). For instance decreased expression of TGF-β2 in the brain facilitates inflammation by astrocyte or microglial cell interaction (Streilein 2003), and the presence of TGF–β in higher levels in the

anterior eye chamber inhibits natural killer cell- mediated cytolysis (Niederkorn 1999).

Apart from the above functions, TGF-β also influences APC for promoting the differentiation of Tregs (CD4+ CD25+ FoxP3). TGF-β1 and TGF-β2 likely play an important role also in HF IP immune privilege (Paus et al. 2005, Cobbold et al. 2006; Waldmann 2006). TGF β protein is found in the HF epithelium and is expressed widely in late anagen HFs and at catagen onset in ORS keratinocytes (Hibino and Nishiyama 2004). Immunoreactivity of cognate receptors (TGF-βRII) is found in the proximal and central regions of the outer root sheath keratinocytes during late anagen and catagen in mice (**Fig.5**b) (Paus et al. 1997, Foitzik et al. 2000) (**Fig.5**b). Additionally, TGF-β was shown to reverse the immune privilege collapse after induction with INF-γ and induces catagen *in vivo* in mice and in vitro in human scalp HFs (Philpott et al. 1994, Paus et al. 1999b, Foitzik et al. 2000; Ito et al. 2004; Paus et al. 2005; Schneider et al. 2009).

1.3.3.4 α-MSH

α-MSH is a proopiomelanocortin-derived peptide hormone with potent anti-inflammatory effects. Other derivatives include ACTH and the endogenous opioid, β- endorphin (Eipper and Mains 1980; Luger and Brzoska 2007). In mice, intracutaneous and intrafollicular POMC transcription and translation are controlled by the hair cycle (Slominski et al. 1998; Paus et al. 1999a; Slominski et al. 2000). It is secreted by the pituitary glands and tissues like the skin, especially in the hair follicle (Paus et al. 1999a, Bohm and Luger 2000; Ito et al. 2005a, Paus 2011) (**Fig.5**b). α-MSH induces melanogenesis and exerts it effect on both central and peripheral immune cells that express melanocortin receptors- family of G-coupled receptors(Cone et al. 1996).

As an immune regulator, α-MSH has many facets of controlling inflammation via activation of NFκB, increased expression of selected adhesion molecules, cytokine receptors, as well as by regulating cytokine release, anti oxidative enzymes and apoptosis (Luger et al. 2003, Brozoska et al. 2008). α MSH is also strkingly expressed in IP sites like the aqueous humor of the eye and the brain In

the eye, it induces Tregs to suppress T cell-dependent delayed type hypersensitivity and blocks IFN-γ production by T cells (Taylor and Lee 2011, Bohm et al 2012). In the brain, α-MSH suppresses the production of tumour necrosis factor-alpha by microglial cells via the Melanocortin receptor-1 (Wong et al. 1997, Luger and Brzoska 2007). α-MSH was also shown to prevent experimental induced encephalomyelitis (EAE) in mice, thus underscoring its potential to re-establish immune tolerance in the CNS and in anagen hair bulb (Taylor and Kitaichi 2008). Indeed, α-MSH can both prevent and restore IFN-g-induced HF IP collapse in organ-cultured human scalp HFs (Ito et al. 2004).

1.3.3.5 MIF

The conserved, ubiquitously expressed secreted protein MIF plays a key role during skin regeneration (Gilliver et al. 2011). Upon discovery, MIF was primarily thought to inhibit macrophage migration (hence its historical name), and was later shown to regulate macrophage and lymphocyte functions via cytokine production, cell activation and phagocytosis (Bernhagen et al. 1996). MIF has been implicated to have a role in delayed type hypersensitivity, sepsis (Bozza et al. 1999) and rheumatoid arthritis (Leech et al. 1999).

The skin expresses MIF in the basal layer of the epidermis, in endothelial cells and ductal and myoepithelial cells of the eccrine glands (Shimizu 2005a, Gilliver et al. 2011). IP sites that express MIF include the fetal trophoblast, brain (Galat et al. 1993) and the anagen hair follicle (Ito et al. 2008). MIF immunoreactivity is seen in the proximal part in the hair follicle epithelium and IRS (**Fig.5**b). MIF inhibits natural killer cells attack on MHCI negative cells by preventing the release of perforin as in many of the immune privileged tissues (Apte et al. 1998; Niederkorn 2006, Ito et al. 2008). Lesional skin from alopecia areata patients showed total absence or diminished expression of MIF in the ORS of the follicle compared to healthy patients (Ito et al. 2008), thus potentially contributing to the inability of the IP-compromised hair follicles to curtail attacks by natural killer cells (Gilhar et al. 2012).

1.3.3.6 IDO

Indoleamine-pyrrole 2, 3-dioxygenase (IDO) is an iron-containing enzyme that converts tryptophan into NAD+ via toxic intermediates like hydroxyanthranilic acid (Hainz, 2007) and plays a key role in IP tissues by suppressing T cell functions. IDO released by antigen presenting cells quenches tryptophan from interacting T cells, thereby facilitating FAS-mediated apoptosis of T cells (Fruci et al. 2013). IDO is expressed in various cells like dendritic cells, tumour cells, bone marrow matrix cells, astrocytes, and endothelial cells and in IP sites such as the fetus, aqueous humour of the eye,islet cells and the human HF epithelium(Mellor and Munn 2000,Chen et al. 2007; Jalili et al. 2007,Hainz et al 2007,Forouzandeh et al. 2008; Meyer et al. 2008)(**Fig.5**b) .

1.3.3.7 MHC class II

MHC class II (MHCII) is a transmembrane protein that primarily presents processed foreign antigens to CD4 T cells (Sompayrac 2012, Murphy 2011).It is present on antigen presenting cells like macrophages and dendritic cells and on B cells, while under pro-inflammatory conditions also some non-professional antigen-presenting cells such as keratinocytes can begin to express MHC class II (Yeoman et al. 1989,Fan et al. 2003,Pollack et al. 2013). MHC II expression is prominently down-regulated in IP sites, thus effectively abrogating the antigen-presenting functions of dendritic cells (Niederkorn, 2002; Paus, 2005). This is also seen in human HF (Christoph et al. 2000, Ito et al. 2004).

In the eye, TGF-ß down-regulates MHC II expression by antigen-presenting cells and affects the production TH1 type cytokines like IL10 (Niederkorn 2003). In the skin, HLA-DR+ Langerhans cells (LC) surround the immunoprivileged anagen hair follicle epithelium, and enter it exclusively above the bulge (Fig.5a), (Christoph et al. 2000, Meyer et al. 2008). The isthmus and proximal ORS show reduced number of MHCII + cells with the follicle bulb being devoid of any MHC II+ cells. In the CTS, the MHC II+ positive cells are distributed rarely in the isthmus but present in large numbers in the proximal CTS and around the hair bulb (Christoph et al. 2000). In similarity to human HFs, murine anagen follicles lack MHC II+ LC's in the hair bulb (Paus et al. 1998).

1.3.3.8 CD4+ and CD8+ T cells

CD4+ T cells mainly are activated by interacting with MHC II+ professional antigen- presenting cells such as Langerhans cells and macrophages (Sompayrac 2013). This interaction leads to creating a cytokine milieu that recruits cells of a destructive or regulatory nature(Konig et al 2002, Ramlingam et al.2012) In the human skin CD4+ cells are distributed mostly around the vessels.very few number are present in the inter dermal spaces and the region of infundibellum surrounding the hair follicle(Bos et al. 1987,Christoph et al. 2000).

Cytotoxic T cells and NK T lymphocytes express CD8 that mainly interact with MHC class I+ cells. In this manner,they function to attack tumour cells and virally infected cells that express tumour or viral antigens via MHC I, and participate in autoimmune disease like alopecia areata where MHC I-presented follicular autoantigens are thought to be recognized and attacked by CD8+ T cells (Kaufman et al 2010, Gilhar 2012). CD8+ T cells are rarely seen at the level of the hair follicle but some times very few numbers can be found at the distal part of the hair follicle (Christoph et al. 2000). ´

γδ-T cells are a primitive type of T lymphocytes that are predominantly found in the epidermis and distal HF epithelium; most recently, it was shown that these cells could actively regulate murine HF cycling (Klopper et al. 2013).

1.3.3.9 Mast cells

Mast cells are derived from hematopoietic progenitor cells and reside mainly in peripheral tissues and mucosal membranes (Botchkarev et al. 1997; Weber et al. 2003, Sugawara et al. 2012). Mast cells contain different types of granules loaded with tryptase, histamine, prostaglandins, and cytokines (Christy and Brown 2007; Metz et al. 2008). They play an important role in organ specific autoimmune disorders like arthritis, bullous pemphigoid, asthma and multiple sclerosis (Harvima and Nilsson; Sayed et al. 2008, Stelekati et al. 2009). For example, mast cells are involved in delayed-type hypersensitivity by activation of FcεR by IgE and activation of alternate pathways via the receptors for complement, various

cytokines, neuropeptides, hormones and TLR (bacteria) (Gilfillan and Tkaczyk 2006; Sayed et al. 2008). Mast cells influence the behavior of dendritic and T cells by expressing regulatory cytokines such as tumor necrosis factor alpha (Metz and Maurer 2007; Suto et al. 2006). Moreover, these cells also can act as antigen presenting cells by expressing MHCII and T cell receptors.

Mast cells might also aid in protecting from autoimmune diseases (Christy and Brown 2007; Sayed et al. 2011; Walker et al. 2012, Mayo et al. 2012, Christy et al. 2012, Brown & Hatfield 2012) and and play an important role in wound healing (Weller et al. 2006). For example, progression of systemic lupus erythematous in mast cell-deficient mice indicated their protective role against autoimmune multi organ failure (Christy and Brown 2007). In the anagen hair follicle mast cells are located in the CTS and are thus distributed all around the follicular epithelium in mice and man (Paus et al. 1994d, Christoph et al. 2000, Kumamoto et al. 2003, Ito N et al. 2010, Sugawara et al. 2012). Furthermore, mast cell numbers and activities show striking hair cycle-dependent changes both in rats and mice, and mast cells contribute to murine hair cycle regulation (Moretti et al. 1966; Paus et al. 1994d, Maurer et al. 1997, Botchkarev et al. 1995, Botchkarev et al. 1997).

1.3.3.10 ICAM-1 expression

Intercellular adhesion molecules (ICAM) are cell surface ligands belonging to immunoglobulin superfamily, which are mainly expressed on lymphocytes, endothelial cells etc. that adhere to integrins on leukocytes (e.g. ICAM-1 binds to LFA1 expressing cells) (Cameli et al. 1994). Endothelial cells prone to infection express increased levels of ICAM-1 that facilitates migration of lymphocytes to the site of inflammation (Munro 1993). Normal and immune privileged tissues sometimes express ICAM1 that would endanger their privilege, ICAM1 immunoreactivity in the hair follicle was found on the cutaneous tissue sheath (CTS), dermal fibroblast and perifollicular immune cells (Jaworsky et al. 1992, Müller-Röver et al. 2000).

For example, in healthy mice, perifollicular infiltrates can be seen surrounding the infundibular HF epithelium that expresses ICAM1 (Muller-Rover et al. 2000). In

non-inflamed skin some healthy anagen and catagen pelage follicles express ICAM1 below the level of SG and may thereby attract perifollicular infiltrates at the level of the bulge, thus potentially participating in a process that has been coined “programmed organ deletion” of the HF (Eichmuller et al. 1998). Furthermore, it was suggested that mice HF recruits perifollicular lymphocytes at the level of the bulge by expressing ICAM1 during stress (Arck et al. 2001, Joachim et al. 2008). Interestingly it was observed that the HF keratinocytes adjacent to perifollicular immunocytes show increased ICAM1-immunoreactivity (Christoph et al. 2000, Joachim et al. 2008). The role of ICAM1+ cells for carrying out such programmed organ deletion in CA remains to be clarified. (Eichmüller et al. 1998, Harries and Paus 2010, Harries et al. 2013)

1.3.4 Cicatricial alopecia and immune privilege

Gene and protein analysis of ORS keratinocytes (including bulge epithelial stem cells) from transgenic mice with keratin 15-promoter-driven green fluorescent protein show a down-regulation of many histocompatibility genes(H2-Q8, H-2K2, H-2D) (Morris et al. 2004). In other independent experiments involving mice or human follicles, it was shown that label retaining cells and ORS keratinocytes express TGF-β2 mRNA (Ohyama et al. 2006; Tumbar et al. 2004), a molecule that promotes IP in the eye (Niederkorn 2003, Kinori et al. 2012).

The state of bulge IP is compromised in human CA, especially in diseases such as lichen planopilaris or CDLE (Harries et al. 2009, Harries and Paus 2010). This is strongly supported by evidence that there is increased expression of MHC class I, β2 macroglobulin, and MHC class II in the bulge region of lesional CA skin (Harries et al. 2009a; Harries et al. 2009b; Harries and Paus 2010, Harries et al. 2013). Additionally, staining intensity for the stem cell marker keratin 15 in the bulge was comparatively decreased in lesional CA skin (Al-Refu and Goodfield 2009; Pozdnyakova and Mahalingam 2008, Harries et al. 2013).

These results suggest that the bulge undergoes both, collapse of IP and loss of epithelial stem cells in human CA (Harries and paus 2010, Al-Refu 2012). However, it remains to be understood whether this also occurs in EBA; which

steps lead to the loss of bulge IP; and whether IP restoration/protection strategies can be envisioned that restore the lost IP.

1.4 Cicatricial alopecia

In the following, some background information on CA is provided.

1.4.1. What is cicatricial alopecia

CA represents a complex group of hair follicle disorders that are subdivided into various categories based on the type of inflammatory infiltrates and disease phenotype (see table 1). Inflammation in many of the disease types mainly contributes to permanent replacement of the hair follicle by scar tissue. CA lesions in patients range from a diffuse pattern of hair loss to that of larger, often confluent alopecic patches. Patients sometimes complain of pain, itching sensation along with erythematous lesions, and pustules in severe cases of the disease (**Fig.6**) (Harries et al.2009). The prolonged nature of the disease and many other factors that contribute to its comeback make it a difficult disease to treat. Inflammation though considered one of the major contributors is only seen during the late and acute phases (Somani and Bergfeld 2008; Stefanato 2010).

The occurrence of CA is very rare when compared to the major autoimmune hair loss disorder, alopecia areata (Gilhar et al. 2012), and the epidemiological data on the occurrence of various forms of CA are very limited (Somani and Bergfeld 2008, Harries et al. 2009, Summers et al. 2011, Griffin et al. 2012, Rongioletti & Christana 2012).

1.4.2 Classification

The clinical picture presents a challenge to classify CA because of unknown etiology behind the disease process and due to the large amount of time taken for disease progression. The North American Hair Research society (NAHRS) has broadly classified the diseases into four major groups. a) Lymphocytic b) Neutrophilic c) Mixed d) Nonspecific. the lymphocytic group mainly consists of chronic cutaneous lupus erythematosus (CCLE), lichen planopilaris (LPP), frontal fibrosing alopecia, Graham Little syndrome, classic pseudopelade (Brocq), central centrifugal cicatricial alopecia (CCCA), alopecia mucinosa, keratosis follicularis

spinulosa decalvans. The neutrophil group consisted of folliculitis decalvans, dissecting cellulitis/folliculitis. The mixed group consists of Folliculitis (acne) keloidalis, Folliculitis (acne) necrotica, and erosive pustular dermatosis (Olsen et al. 2003, Harries et al. 2009, Rongioletti & Christana 2012).

1.4.3 Histopathology of cicatricial alopecia

The common histopathological evidence of CA includes the presence of various types of inflammatory infiltrates and lamellar fibrosis surrounding hair follicles. Histopathology of individual diseases shows distinct or even similar features with different type of infiltrates. The lymphocytic type of CA is characterised by hyperkeratosis, folliculitis and deep lymphoid cell infiltrates, often with some plasma cells. The late stages of the disease show lamellar fibrosis with basement membrane thickening (**Fig. 6**) (Stefano et al 2010, Harries et al 2009, Rongioletti and Christana 2012)

For example, early stages of lichen planopilaris display vacuolar changes with moderate lymphocytic infiltrates at the level of the infundibulum and isthmus. In the advanced stages, the disease is characterised by concentric lamellar fibrosis with decreased lymphocyte infiltrates (Hordinsky 2008).

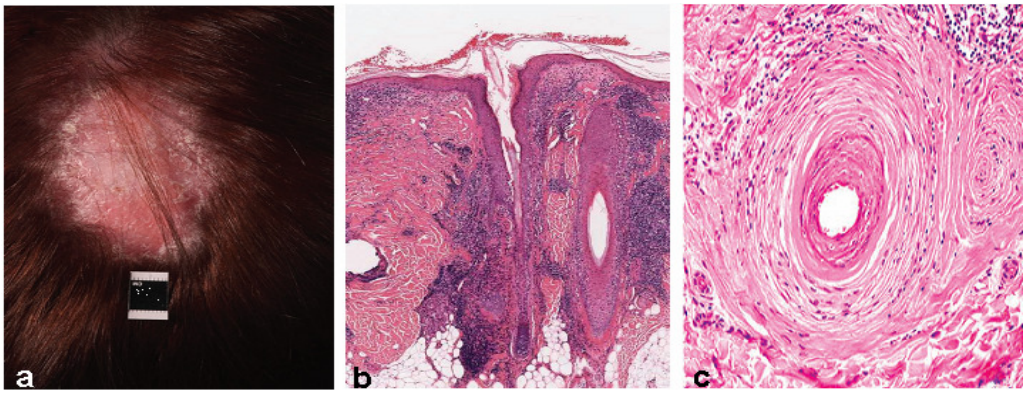

Fig.6: Disease phenotype and histopathology of cicatricial alopecia.*Lesion of cicatricial alopecia on the back head of a patient with CCCA (a).Pathological section stained with HE depicts dense inflammatory infiltrates surrounding the follicles (b, arrows).Concentric lamellar fibrosis with infiltrates (c, arrows) seen around the hair follicle in a pathological section stained with HE (fig a, b from Harries et al., 2009; c from Stefanato, 2010).*

CCCA is another progressive form of lymphocytic CA with early lesions showing pustules, crusting and erythema with bacterial infections. Histopathology shows concentric fibrosis with peri follicular and vesicular lymphoid infiltrates. (Somani and Bergfeld 2008) The pseudopelade of brocq is a distinct group that displays concentric lamellar fibrosis, loss of sebaceous glands, and follicular units may represent the end-stage of various types of CA (Sperling et al. 2000,Sperling and Whiting 2003).

In the classical neutrophil type of CA, folliculitis decalvans, histopathology in early lesions shows acute dense perifollicular and intrafollicular neutrophils, lymphocytes, histiocytes and plasma cells. Late stages like many other diseases show perifollicular fibrosis and interstitial fibrosis (Whiting 2001,Castano-Saurez et al. 2012).In the mixed-type of folliculitis (acne) keloidalis early stage lesions show follicular dilatation with neutrophils, and follicular rupture with peri follicular abscesses. The late stages display lymphocytic and plasma cell infiltrates with hypertrophic scar tissue and hyalinised keloidal bundles. Nonspecific CAs mostly represent end stages of other CAs dominated by lamellar fibrosis and scar tissue formation (Somani and Bergfeld 2008; Stefanato 2010).

1.4.4 Cicatricial alopecia as a secondary event in other skin diseases

CA can present as a secondary event in other dermatological diseases like EBA, bullous pemphigoid, psoriasis, mastocytosis, and organoid nevus. It is also seen in diseases like hepatitis and inflammation induced tissue damage (Al-Zaid et al. 2011; Bardazzi et al. 1999; Jackson and Callen 1998; Miteva et al. 2011; Severs et al. 2008; Xu et al. 2003).

In the acquired forms of bullous disease like epidermolysis bullosa acquisita (EBA) and bullous pemphigoid (BP), infiltrates target structural proteins in the basement membrane leading to blister formation after binding of pathogenic IgG. Primary lesions include blisters with infiltrates at the basement membrane zone and secondary scarring together compromise hair follicle regeneration (Miteva et al. 2011; Tosti et al. 2010). However, the pathobiology of these secondary forms of CA, and the mechanisms and extent of stem cell damage and/or IP collapse in the

bulge in EBA remain completely obscure. This is to be further examined in the current thesis, using EBA mouse models.

1.4.5 Pathobiology of CA

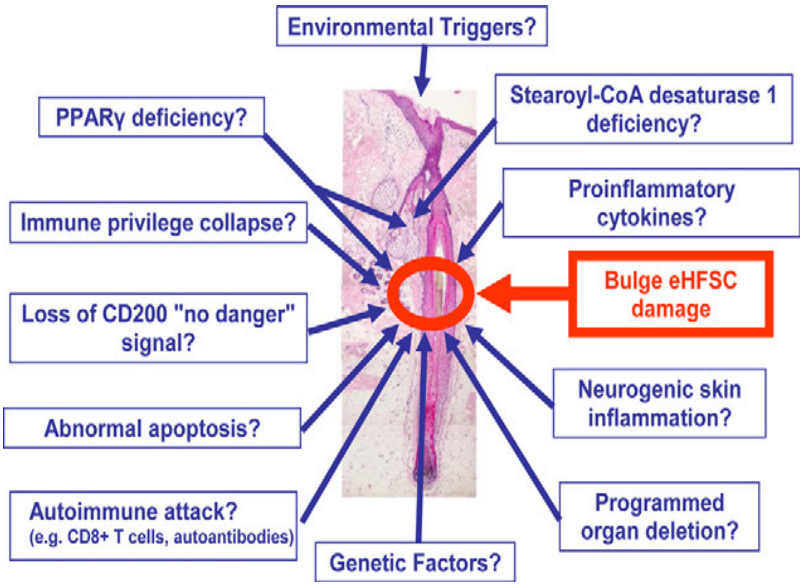

Fig.7:. Various factors that are thought to contribute to the disease process of CA (from Harries et al., 2010).

The actual triggers for recruitment of inflammatory infiltrates at the hair follicle are not well characterised. Not much is known about the type of events in the hair follicle keratinocytes, SG or epidermal keratinocytes that directly contribute to the pathobiology of the various forms of CA. Many theories underlying in the pathogenesis of CA have been proposed over the years (**Fig.7**) (Stenn et al. 2006,McElwee 2008,Harries and Paus 2010, Karnik and Stenn 2012). CA is hypothesised to occur due to irreversible inflammatory damage of epithelial HF stem cells. In fact patient samples showed decreased expression of keratin 15 in the lesional skin of CA patients(Pozdnyakova et al. 2008).Evidence in this regard also comes from the ablation of stem cells from the hair follicle by introduction of suicide gene in mice using keratin 15 promoter (Ito, 2005). These mice lost hair

follicle and sebaceous glands further indicating that destruction these cells may play a vital role in the generation of cicatricial alopecia (Paus and Czarnetzki 1994a, b, Mobini et al. 2005, Cotsarelis 2006, Tiede et al. 2007a, Harries et al. 2013).

The constitutive expression of MHC I below the level of the sebaceous gland in the normal follicle is either low or minimal. The HF ORS also expresses CD200 a marker mainly seen on bone marrow stem cells (Rosenblum et al. 2004). Furthermore, the anagen follicles secretes substances like α- MSH and TGFß that act as anti-inflammatory ”guardians of HF IP” (Paus et al. 2005). Equally important, the absence of fully functional LC’s and the scarcity of intraepithelial T lymphocytes in and below the bulge suggest that these cells are actively suppressed and/or discouraged from entering the bulge epithelium (Paus et al. 2005).

Recent evidence suggests that the bulge -a major target of inflammatory infiltrates in CA that is devoid of MHC I expression in the normal state (Ito T et al. AJP 2004, Meyer et al. 2008) becomes prominently MHC I+ only in the diseased state, e.g. in lichen planopilaris (Harries et al. 2013,Harries et al. 2010, Harries and Paus 2010)

The role of PPAR-γ in development of lichen planopilaris remains to be one of the most intriguing findings in the field of CA research. Karnik et al (2009) found that targeting PPRG specifically in the stem cells under the influence of keratin 15 promoter lead to phenotype that is similar to lichen planopilaris with alopecia and inflammatory infiltrates in mice.

Recently it was shown that Ahr (Aryl hydrocarbon receptor) signalling contributes to CA. Transgenic mice lacking Ahr showed progressive alopecia and histopathology showed perifollicular inflammation, follicular cysts, loss of sebaceous glands, and follicular scarring in the advanced stages.These results add more complexity to our current understanding of CA and points to the role of the yet unexplored area of lipid metabolism in causing cicatricial alopecias (Karnik et al. 2009,Harries et al. 2009,Sten and Karnik 2010,Paniker et al 2012)

The sebaceous gland is loaded with complex lipids that aid the hair follicle egress through the hair canal (Stenn et al. 1999). The detection of complex lipids in the

diseased skin samples and the presence of excessive inflammatory infiltrates surrounding the hair follicle in asebia mice (which show both CA and degeneration of their SGs along with a massive increase in the number of skin mast cell) further support a role for the sebaceous gland in preventing follicular inflammation (Sundberg et al.2000, Stenn, 2001). Also the accumulation of complex lipids in the hair canal and the desquamation of the IRS does not allow the hair shaft to be shed and thereby pushing the hair shaft into the DP, thus supposedly causing rupture and accumulation of infiltrates (Rongioletti and Christana 2012,). These findings invited the hypothesis that the sebaceous gland plays a key role in CA pathogenesis (Stenn 2001, Al-Zaid et al. 2011).

When sections of CA patients with lichen planopilaris were examined, most of the follicles were surrounded by macrophages. Further, the pattern of gene expression from array analysis indicated immediate macrophage response. 85 % of the upregulated genes depended on the p53 pathway, indicating a possible role of p53 in CA pathogenesis (Karnik et al. 2009). Further, the frequent presence of S.aureus infections in patients within the neutrophil subtype of CA raises the question whether infections may trigger an inflammatory response that destroys the hair follicle (Jackow et al. 1997; Powell et al. 1999). This pathogenesis concept was further supported by the observation that the serum of patients with lupus erythematosus cross-reacts with Epstein Barr virus epitomes (Poole et al.2009, Draborg et al 2012).

1.4.6 Treatment

Entitty	Clinical features	D.scopy	Histopathology	Management
Lichenplanopilaris(LPP)	Single or multiple, patchy or extensive scarring alopecia on the vertex and parietal areas with perifollicular erythematous papules and hyperkeratosis at the margins of the expanding area. Predominant activity at the periphery of alopecic patches. Lichen planus may coexist. Frontal fibrosing alopecia and Lassueur-Graham-Little-Piccardi syndrome pattern	Follicular plugging, hair casts, peripilar white dots, interfollicular simple red loops, and arborizing red lines with absent follicular openings	Predominantly perifollicular lymphocytic infiltrate with interface lichenoid pattern and minimal perivascular involvement. Destroyed root sheaths with replacement of the follicles by extensive perifollicular lamellar fibrosis. DIF: colloid bodies with IgM, C3, and IgG along the upper hair follicle with a band of fibrinogen at the basal membrane zone	**First line**: Topical corticosteroids (clobetasol,betamethasone), intralesionalcorticosteroids (triamcinolone acetonide 3–10mg/mL), topical tacrolimus,antimalarials(hydroxychloroquine 200mg bid) **Second line**:Prednisone, oral cyclosporine, oralretinoids, **Third line**:Mycophenolate mofetil, griseofulvin, Tetracyclines
Discoid (chronic cutaneous) lupus erythematosus(DLE)	Erythematous and scaling plaques progressing to atrophy, telangiectasia with central hypopigmentation, and peripheral hyperpigmentation on the vertex of young women. Predominant activity in the centre of alopecia patch. Non-scalp lesionsmay coexist on the face	Absent follicular openings, follicular keratin plugs, erythema around follicles, arborizing red lines, and pigmentary changes	Superficial and deep perivascular and periadnexal lymphocytic infiltrate with follicular and epidermal interface changes. Thickened basement membrane. Mucin. Fibrosis. Broad dermal loss of elastic fibers including the elastic follicular sheath. DIF: granular or linear pattern of IgG, IgM, and C3 along the dermal-epidermal junction	**First line**: Topical corticosteroids (clobetasol,betamethasone), intralesionalcorticosteroids (triamcinolone acetonide 3–10mg/mL), antimalarials(hydroxychloroquine), sunscreen **Second line**: Oral prednisone, topical tacrolimus **Third line**: Thalidomide, isotretinoin, dapsone,methotrexate
Pseudopelade of Brocq	Small, smooth, and slightly depressed alopecic atrophic patches resembling "footprints in the snow" with irregular outlines over the vertex of middle-aged Caucasian women. No visible inflammation	Absent follicular openings	No inflammatory infiltrate at the dermo-epidermal junction or follicular plugging. Fibrous hyalinized tracts, thickened elastic fibers in hyalinized dermis. DIF: negative	First line: Topical corticosteroids (clobetasol, betamethasone), intralesional corticosteroids (triamcinolone acetonide 3–10mg/mL), antimalarials (hydroxychloroquine200mg bid) Second line: Prednisone, oral retinoids, topical tacrolimus

Table I: Clinical, dermatoscopic, and histopathologic features of the main primary cicatricial alopecia entities and their therapeutic management, D.Scope=Dermatoscopy. (*modified from Rongioletti and Christana 2012****).*** **Continued on next page**

Table I: Continued

Entitty	Clinical features	D.scopy	Histopathology	Management
Central centrifugal cicatricial alopecia (CCCA)	Progressive alopecia at the vertex or mid- top of the scalp in young to middle-aged women of African-American descent that gradually spreads centrifugally without evident inflammation. Single or grouped hairsmay survivewithin zones of scarring	Not well defined	Eccentric atrophy of the outer root sheath epithelium, concentric lamellar fibroplasias. Hair fiber granulomas within fibrous tract remnants. Perifollicular lymphocytic infiltrate	First line: Avoid physical and chemical traumas, topical corticosteroids, topical tacrolimus, intralesional corticosteroids, antibacterials (tetracyclines), oral corticosteroids
Folliculitis decalvans (FD)	Single focus of alopecic patch characterized by an advancing border studded with pustules and perifollicular papules on the vertex of young and adult men resulting in central scarring. 'Tufted hair folliculitis' in the evolving disease	Low hair density and loss of follicular ostia, with thinned shafts of the remaining hairs	In the early phase, perifollicular andintrafollicular neutrophils, replaced by lymphocytes and plasma cells. Abscess in the absence of sinus tract formation. Gram-positive bacteria in the infundibulum. Dermal fibrosis and follicular remnants (late stage)	First line: Oral and topical antibacterial–corticosteroid Second line: Isotretinoin, zinc sulfate, dapsone
Dissecting cellulitis of the scalp (DCS)	iPainful, fluctuant nodules, abscesses, and interconnecting sinus tracts that begin on the occiput or vertex in young African American men aged between 20 and 40 years. Acne conglobata and hidradenitis suppurativa	Not well defined	Early perifollicular and interfollicular neutrophilic infiltrate becoming mixed later. Follicular occlusion. Neutrophilic accumulation around squamous epithelium (sinus tract) and granulomatous reaction due to follicular rupture with fibrosis. DIF: negative	First line: Oral isotretinoin–oral and topical antibacterials, intralesional corticosteroids Second line: Oral prednisone, zinc sulfate, dapsone

1.4.7 Cicatricial alopecia associated with bullous disease

CA is a major hallmark of both genetic and acquired forms of bullous dermatoses (**Fig.8**). In bullous disorders like BP and EBA, lesions of alopecia along with associated scar tissue are noticed on the scalp. The underlying pathogenesis is unknown. Over the years, the presence of infiltrates in tissue section leads to the speculation that inflammatory events lead to permanent loss of stem cells (Tosti et al. 2010, Miteva et al.2011). The access to stem cells occur due to destruction of basement membrane (BM) proteins associated with outer root sheath keratinocytes of the hair follicle bulge region by surrounding infiltrates

The proteins plectin, alpha 6 beta 4 integrins, collagens -type VII collagen, type IV, hemi desmosomes –desmoglein 3, desmoglein 1, plakophilin, plakoglobin, Laminins- laminin 311, laminin 322, laminin 5 are expressed on the cell membrane that anchors the basal layer ORS keratinocytes and epithelial stem cells to the basement membrane (Chan 1997, Joubeh et al.2003, Watt and Fujiwara et al. 2011). The follicular distribution of these proteins, which are also found in the bulge and isthmus region, is thought to be similar to that of the epidermis (Tosti et al. 2010, Miteva et al.2011). The distribution pattern of some of these proteins (e.g. laminin 322 and α6β4 integrins) is non-homogeneous .On the other hand, desmoglein 3 is widely distributed throughout the basal layer of the ORS in the infundibulum and isthmus region. Type VII collagen is uniformly distributed all along the basement membrane of the follicle in both humans and mice (Tosti et al. 2010).

In autoimmune bullous dermatoses, autoantibodies against BM-associated autoantigens are generated in great quantities by plasma cells and thus compromise the structural integrity of the dermoepidermal junction, eventually causing blistering. These autoantibody-induced blisters can also occur along the BM of the HF (Tosti et al. 2010) and can thus potentially damage the HF and its vital stem cell zone, the bulge. One of these autoimmune bullous dermatoses is EBA, a member of the group of epidermolysis bullosa disorders (see below). Autoimmune CA is also seen in pemphigus vulgaris, in which autoantibodies bind to desmoglein 3, leading to blisters with acantholysis (Veraitch et al. 2013, Kurzejet al. 2012). Mice with spontaneous mutations in desmoglein 3 show hair

loss and separation of inner and outer layers of the follicular ORS is prominent in these models (Holm et al. 2010). Cicatricial pemphigoid is due to the production of antibodies towards BP230 and/or laminin 332. However, CA occurs in only seven percent of the total patients suffering from cicatricial pemphigoid (Miteva et al. 2011).

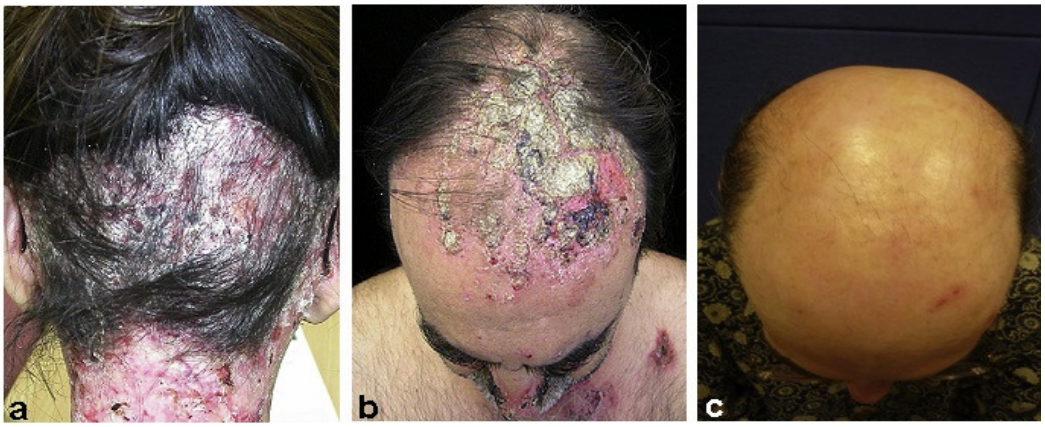

Fig.8: Cicatricial alopecia in bullous diseases.*Lesions of alopecia in the occipital skin of a patient with hereditary form of bullous disorder-recessive dystrophic EB (a). b) Lesions of cicatricial alopecia on the forehead from a patient with pemphigus vulgaris (b, arrows). Scarring alopecia with scalp involvement in a patient with epidermolysis bullosa acquisita (c) (a from Miteva et al., 2011; b, c from Tosti et al., 2010*)

Epidermolysis bullosa disorders are classified mainly into two individual types a) genetic b) acquired forms. Familial forms include epidermolysis bullosa simplex, junctional epidermolysis bullosa, dystrophic epidermolysis bullosa, and Kindler syndrome (Gonzalez et al. 2013,Fine et al. 2008). Hair loss in epidermolysis bullosa simplex can occur due to mutations in keratin 5 or keratin 14 genes, desmoplakin gene or due to deficiency of plakophilin 1. Hair loss of scarring phenotype can be seen in diseases with mutations in desmoplakin and due to deficiency of plakophilin (Varki et al 2006). Junctional epidermolysis bullosa occurs due to defects in laminin (Non-Herlitz), deficiency and mutations in type XVII collagen, and reduction of integrin β4 (pyloric atresia) (Pfender and Lucky 2008). Severe to moderate alopecia with scarring phenotype also occurs in patients having junctional epidermolysis bullosa (Varki et al. 2007). A mouse

model was also generated in order to study genetic influences in non-Herlitz junctional epidermolysis (Bubier et al. 2010, Fine 2010,Intong et al. 2012)

Dystrophic epidermolysis bullosa occurs due to mutations in collagen7A1 (recessive dystrophic epidermolysis bullosa) Scalp lesions show folliculitis-like lesions, follicular papules and pustules. Histopathology shows scar tissue formation along with infiltrates (Fine and Mellirio 2009a, b, Tosti et al. 2010, and Murauer et al. 2011). Kindler syndrome occurs due to defect in kindlin or fermitin family homolog1. Patients with Kindler syndrome do not have alopecia, as the HF does not express these proteins (Gonzalez 2013, Intong et al. 2012, and Tosti et al. 2010).

1.5 EBA (Epidermolysis bullosa acquisita)

In the acquired form of epidermolysis bullosa (EBA), hair loss of the scarring phenotype occurs due to production of autoantibodies against type VII collagen of the lamina densa (Miteva et al. 2011). Lesions involve blisters with scar and milia formation in mechanically stressed areas. Additionally, mice models for EBA show blisters with inflammatory associated hair loss (Sitaru et al.2005,Bieber et al.2010,Ludwig et al 2011).

1.5.1 What is EBA?

EBA is an autoimmune skin disease of blistering phenotype. Mostly,EBA occurs in aged individuals with only very few cases of children being reported (Schmidt et al 2002, Mayazumi et al. 2006). The frequency of EBA in the normal population is comparatively low and widely varies between countries-Scotland (49 cases per million births), States (54 cases per million births), Japan (7.8 cases per million births) and in Croatia (9.6 cases per million births) (Horn et al.2008, Fine et al.1999).

The disease extends from a mild to severe cases of blisters typically seen on the skin, oral cavity and mucosal surfaces such as gut and vagina (**Fig.9**).

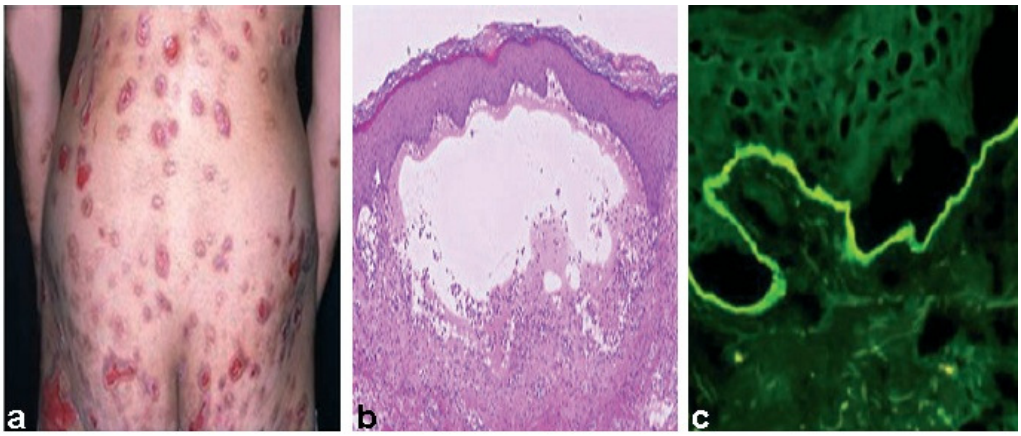

Fig.9: Phenotype and histology of bullosa acquisita.*Bullous lesions seen on the buttocks of a patient with epidermolysis bullosa acquisita (a).HE staining showing dermal epidermal separation with infiltrates (b). c) Immunofluorescence staining depicting the binding of pathogenic antibodies to type VII collagen in the basement membrane(c). (modified from Ishii et al., 2011).*

Formation of blisters is chiefly due to the immune triggers like complement fixation,neutrophil activation and T cell responses after the binding of pathogenic autoantibodies to type VII collagen (Hashimoto et al 2012,Chen et al. 2012 ; Ludwig and Zillikens 2011)**.** Treatment of EBA involves using topical, oral and intravenous immunosuppressants, intravenous immunoglobulins and/or other immunomodulatory therapies (Meurer et al. 2012)

Type VII collagen constitutes the major part of anchoring fibrils in the basement membrane of the skin. Pathogenic anti type VII collagen antibodies belong to IgG subclasses that predominantly belong to 2a, b isotypes. Most of the time, isolated antibodies from serum samples bind mostly to N terminal of the noncollagenous domain one (NC1). Furthermore, antibody is binding to non collagenous domain two (NC2) were isolated as well (Recke et al 2009, Ludwig et al 2011, Saleh et al 2011, Licarete et al 2012.). Recently, mouse models have been developed for studying or analysing the pathogenic mechanisms and the disease phenotype in these mice closely resembles the human disease, with visible blisters and associated CA lesions (Gupta et al. 2012, Ishii et al. 2011; Remington et al. 2008).

1.5.2 Animal models of EBA

Given the relative rarity of EBA, it has become imperative to develop mouse models, which closely resemble the human disease in order to dissect the

pathobiology of EBA. In this regard, different strategies based on the use of animal models have been developed in the recent past.

The first EBA model was produced by directly injecting purified anti type VII collagen IgG from sera of patients into hair less immune competent mice. These mice developed blisters, crusts and loss of nails (Woodley et al. 2006). Another passive EBA model was developed by injecting polyclonal IgG antibodies raised in rabbits against the non collagenous (NC1) domain of type VII collagen into C57BL6 mice (Sitaru et al. 2005,Samavedam et al. 2013). In this model, like in its human counterpart, mice develop crusts, blisters and erosions within four to six days after antibody injection. CA is found on both ventral and dorsal skin, most prominently around the eyes, snout, head, neck, and back skin after 12 days (**Fig.10**) (Sitaru et al. 2005, Samavedam et al. 2013).

An active model of EBA was produced by injecting a recombinant protein (GST-mCOL7C) and titermax® as an adjuvant over a period of four weeks into SJL mice. These mice developed blisters on the head, flanks, abdomen and tail (Bieber et al. 2010, Ludwig 2012, Sesarman and Sitaru 2013; Hashimoto et al. 2012). Over the year's mouse models representing hereditary types were also developed. Mouse model that resembled DEB have also been generated by conditionally inactivating COL7 expression, these mice developed mucocutaneous blistering, nail dystrophy, and deformities of the extremities (Fritsch et al. 2008). Mouse model with autosomal recessive mutation as result of insertion of long terminal repeat of murine leukemia into laminin 2 gene resulted in generalized non-Herlitz JEB as in humans These mice developed blistering along with bone demineralization (Bubier et al. 2010)

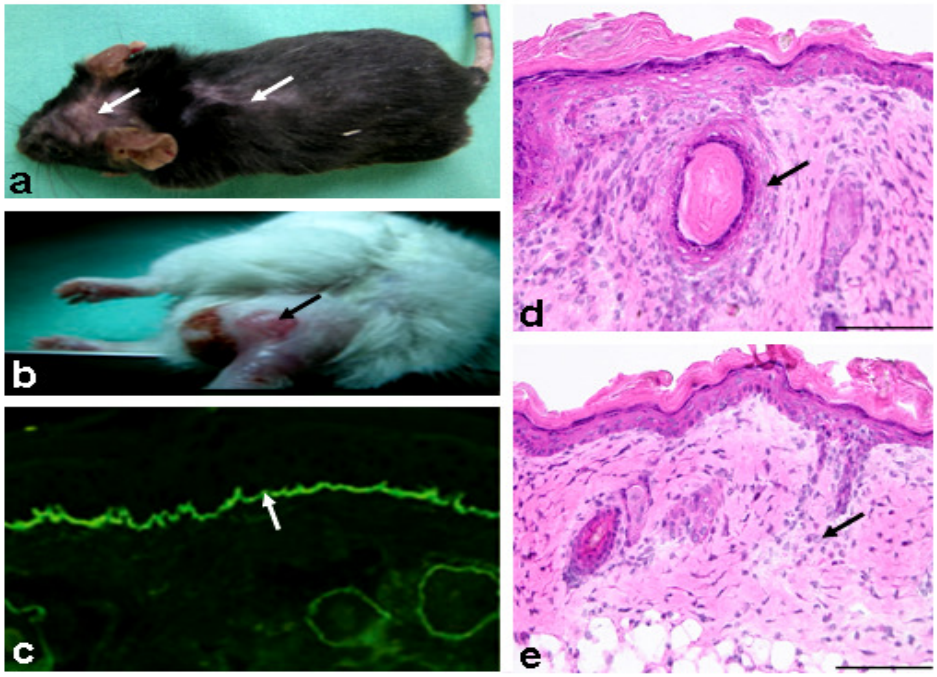

Fig.10: Mice models of epidermolysis bullosa acquisita.*Passive mouse model of epidermolysis bullosa with scarring phenotype (C57BL6/6J), arrows indicating lesions of alopecia on the head and back skin (a).Active mouse model of epidermolysis bullosa with blisters (SJL), arrows indicating blister and associated alopecia on the back skin (b). pathogenic IgG deposition at the dermal epidermal junction, as shown by immunofluorescent imaging in the active mouse model of EBA (c).(from Hanna et al.).H&E staining depicting lamellar fibrosis (arrow) along with surrounding infiltrates in the peri lesional skin of passive mouse model of EBA (d).H&E staining depicting a deceased hair follicle (arrow) in the peri lesional skin of active mice model of EBA (e). (Fig b, c from Sitaru et al., 2006.)*

1.5.3 Structure and function of type VII collagen

In order to understand the molecular target of EBA, the role of collagen type VII in skin biology shall be briefly reviewed.

Epidermis and dermis are separated by a BM and are held together by an intricately composed anchoring complex, consisting of hemidesmosomes, anchoring filaments and anchoring fibrils (**Fig.11**a-b) (Bruckner-Tuderman et al.1999, Gelse et al.2003.).

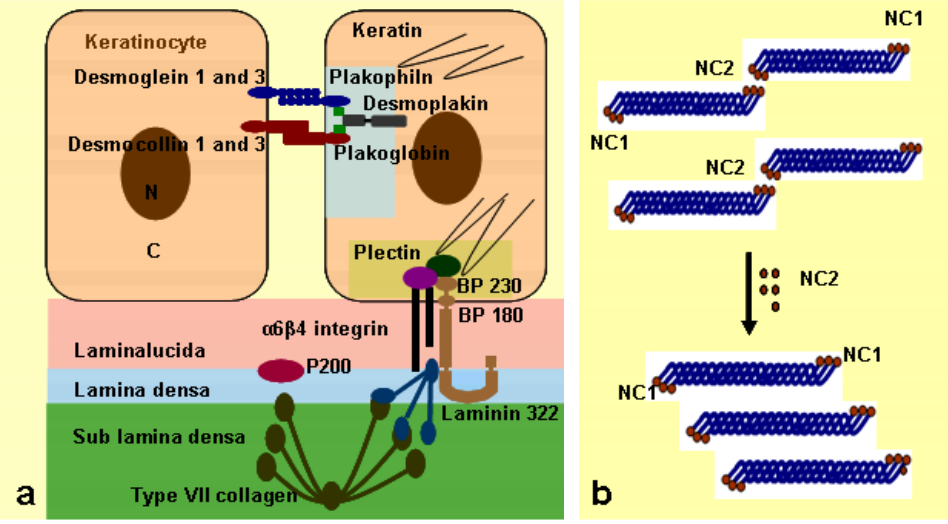

Fig.11: Schematic diagram showing various structural proteins of the epidermal keratinocytes and basement membrane.*Desmosomes are skin structural proteins having complexes of desmoglein-desmoplakins that hold adjacent keratinocytes together. Hemidesmosome are group of integrin proteins that form a link between the basement membrane and keratins of the keratinocytes via the non covalent plectin plaques. Integrins moreover connect the under laying collagen filaments (type VII and type IV) in the lamina densa via laminin 5/ laminin 322 (modified from Bieber et al., 2010(a).Diagram depicts the processing of the pro-peptide to form individual molecules of type VII collagen(modified from (Bruckner-Tuderman et al.1999) that criss cross the lamina densa or end up in anchoring plaques.BP-bullous pemphigoid antigen, C-cytoplasm, N-nucleus, NC1-noncollagenous domain 1, and NC2-non collagenous domain 2(b).*

The anchoring filaments help in connecting the lamina lucida to the lamina densa. These fibrils extend from one point to another point within the lamina densa and even end up as anchoring plaques. These fibrils act as molecular nets that collect other collagens like laminin 5 and type VII collagen. Laminin 5 appears to aid in the attachment of α6 β4 integrin to type VII collagen (Rousselle et al. 1997, Bruckner-Tuderman et al.1999,). Type VII collagen is a ~ 1000 KD macromolecule consisting of repeated dimers of NC1 domain and collagenous triple helix. Type VII collagen precursor protein consists of fragments of non collagenous (NC1-145 KD) domain at the N terminal end, a collagenous triple helix (145-kD), and a noncollagenous domain (NC2-30-kD) at the C terminal end (Gelse et al.2003, Burgeson et al. 2006).

Subsequently after secretion of the pro-peptide into the matrix via the golgi, collagen fibers bind laterally in a non stacked fashion (anti parallel arrangement) with the help of a disulfide bond formed between the carboxyl terminals of NC2 domains. Further, the NC2 pro peptide is cleaved to form a continuous anchoring fibril. The collagenous domain consists of repeats of GLY-X-Y- sequences, which fold into a triple helix and interrupted by non-collagenous sequences constituting 19 - 36 amino acids. This structured arrangement renders it more flexibility (Bruckner-Tuderman et al.1999).

1.5.4 Anti-collagen type VII autoantibodies in EBA

As stated, EBA occurs due to the presence of anti type VII collagen antibodies in the blood of patients. Mostly the pathogenic autoantibodies belong to to the immunoglobulin G (IgG) class (Sitaru et al. 2007, Ludwig and Zillikins et al 2011). It was reported that a subgroup of EBA patients also shows secretory IgA autoantibodies against type VII collagen (Wozniak et al. 2013). Moreover EBA can also occurs as secondary event as anti-type VII antibodies are also found in the serum of patients with other autoimmune diseases, such as systemic lupus erythematosus and inflammatory bowel disease (Licarete et al. 2012).

Most of the autoantibodies in EBA patient serum are detected by direct immunofluorescence or ELISA (Saleh et al 2011, Marzano et al. 2012). The IgG seen in EBA patients spans from G1 to G4 subclasses with IgG 1 seen more frequently in almost all patients (Sitaru et al. 2007). Interestingly, each of these antibodies have different modulatory capabilities: IgG 1 and IgG 3 activate all type of Fc receptors and activate the C1 of the complementary system. IgG4 activates Fc receptor in a subdued manner and does not activate C1q. IgG2 shows restricted activation of Fc receptors and C1q (Mihai et al. 2007) The inflammatory events leading to blister formation mainly involve activation of complement and granulocytes (Csorba et al.2012). Recently it was shown that increased galactosylation of IgG1 antibodies to type VII collagen prevents EBA in the passive model (Karsten et al. 2012).

1.5.5 Pathobiology of EBA:

EBA represents a rare skin disorder wherein the binding of autoantibodies to type VII collagen in sub lamina densa leads to a blister formation (**Fig.12**a-b). This phenomenon is observed mostly on the skin (especially areas of mechanical stress), mucosal membranes of the pharynx, gut and vagina (Ludwig and Zillikins et al. 2012, Hashimoto et al. 2012). Autoantibodies to type VII collagen belongs IgG subclass that predominantly consists of either IgG 1, IgG 2a or 2b subtypes. These antibodies bind mostly to the NC1 domain of type VII collagen with varying affinities. However, pathogenic antibodies that bind to different regions (NC2) have also been isolated from the serum of patients (Chen et al. 2012). Histological examination of lesional skin showed dense neutrophil accumulation at the affected site (Chiriac et al. 2007). Efforts are underway to understand disease mechanisms as to how the blister formation occurs by focusing on individual components.

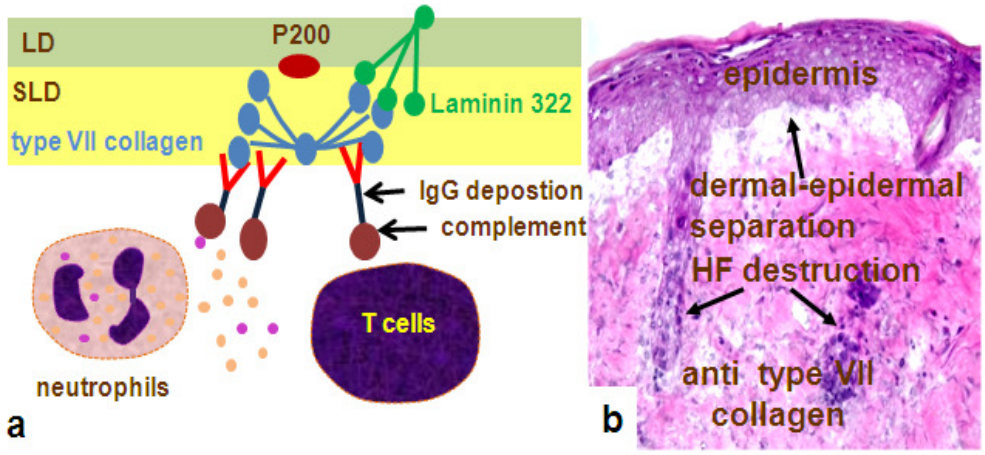

Fig.12: Schematic diagram showing the pathogenesis of EBA*. a) The deposition of autoantibodies to type VII collagen in the basement membrane leads to complement deposition, this complex recruit neutrophils that further bring tissue destruction (a) Histology showing dermal epidermal separation and hair follicle destruction in EBA mice (b).LD-Lamina densa, SLD-sub lamina densa.*

In this regard, it was proved that pathogenic antibodies were shown to fix complement upon binding to type VII collagen in the BM (Mihai et al. 2007). Follow up experiments on factor B deficient mice reveal no disease process upon induction with pathogenic IgG but lead to blister formation upon injecting neutrophils. Furthermore, blocking C5a also diminished antibody-mediated disease. These experiments strongly support a direct role of complement and

neutrophils in causing antibody-mediated blistering in the employed murine EBA models (Chiriac et al. 2007, Ludwig and Zillikins 2011).

Neutrophils are considered as major participants of tissue injury in both human and mouse models of EBA. They bring about tissue injury by dislodging various proteases and ROS at the dermal epidermal junction. Functionally blocking of neutrophils with anti-Gr1-antibody clone RB6-8C5 and using protease inhibitors lead to complete redemption of the disease even after repetitive injections of antitype VII collagen IgG (Chiriac et al. 2007)

Recently, T cells were suggested to be important partners in the disease process by aiding antibody production in the EBA mice model (Sitaru et al. 2010). Injection of T cells sensitized with fragments of GST-mCOL7C into nude SJL mice potentiates a full-blown disease. Recently it was found that EBA susceptibility in SJL mice is dependent on the H2s haplotype within the MHC and is due to a specific cytokine gene profile (IL 4/IFN) in the draining lymph nodes that promotes Th1 responses (Hammers et al.2011). Clinically it was also shown that patients treated with both immunoadsorption and rituximab maintained intermediate levels of IgG for about 25 months. Further, ex vivo experiments with mononuclear cells derived from patients revealed a decrease in T cell (Th1,Th2 and IL-10+ T cells) responses to Col VII-NC1.This experiment shows a clear nexus between proactive T cells/Tregs and B cell clones in causing EBA in patients (Muller et al. 2010,Ludwig and Zillikins et al.2011,Uiije and Shimizu 2012). However, it was shown that induction of disease by injecting GST-mCOL7C into the SKH1 strains depleted of regulatory T cells (hairless mice) did have a normal disease (Chen et al. 2006) and this might indicate that T regs might not participate in disease induction.

The association of MHC with autoimmune inflammatory diseases is widely studied.(Nepom and Erlich 1991,Wucherpfenning and Strominger 1995,Fernando et al. 2008). In a cohort of small group of patients with EBA, an association of the disease with HLA DR2 has been noticed. Similarly, in murine models, H2s was strongly associated with disease since inbred SJL mice injected with recombinant mCOL7 and Titermax® showing highest EBA susceptibility (Ludwig et al 2011, Ludwig and Zillikins et al. 2011). The role of cytokines in causing EBA of the

inflammatory phenotype remains under exploration. Recent evidence points to a role for IL 6 in protecting mice from EBA via the down-regulation of IL1ra (Samavedem et al. 2012). As the role of using intravenous IgG therapies comes into prominence so are the role Fc receptors in inducing blistering in EBA (Ludwig and Zillikins et al. 2011). Through genetic studies in EBA patients and functional studies in animal models Kasperkiewicz et al (2012) recently showed the role of both activating FcγRIV and inhibitory FcγRIIB in the pathogenesis of EBA. The mechanisms that lead to tolerance break down against COL7, however, remain largely unclear (Kasperkiewicz et al. 2011, Kasperkiewicz et al. 2012, Hashimoto et al. 2012)

1.5.6 General questions on how CA occurs in EBA

It is undisputed that CA in EBA occurs due to binding of autoantibodies to type VII collagen in the HF basement membrane. This causes blisters and alopecia as the HF basement membrane is rich in type VII collagen (Miteva et al. 2011). However, this alone does not explain the occurrence of scarring alopecia , a hallmark of both passive and active mouse models of EBA(Ludwig and Zillikens 2011), since CA likely occurs as a result of irreversible epithelial HF stem cell damage in the bulge zone of the HF's ORS (Paus and Cotsarelis 1999, Cotsarelis 2006, Harries & Paus 2010, Harries et al. 2013). Therefore, it remains an important challenge of EBA research to clarify how such HF stem cell damage as a result of antibody-induced blistering is initiated. The current thesis project attempts to make a first step towards elucidating the likely complex underlying bulge pathobiology in EBA.

1.6 Aims of the thesis, working hypothesis, and specific questions addressed

The major goal of the current study was to better understand the key mechanisms behind the irreversible destruction of epithelial HF stem cells in EBA -associated CA.

The underlying **working hypothesis** that guided this study was that anti-collagen type VII antibody-induced perifollicular inflammation may endanger the bulge IP, which would make bulge stem cells more susceptible to further inflammatory damage, thus eventually inducing excessive HF stem cells death, depletion of the

bulge stem cell niche, and ultimately exhausting the HF's capacity to regenerate. Clinically, this would result in CA.To probe this working hypothesis; the following **specific questions** were addressed**.**

1. **Is there is immunophenomenological evidence that the bulge region of murine telogen HFs is immunoprivileged?**
2. **What important inflammatory cell infiltrates bring about hair follicle destruction?, Moreover, does the presence of infiltrates correlate with a) bulge IP collapse b) key cytokine changes and c) HF epithelial stem cell damage in passive and active mouse models of EBA?**
3. **Does injecting Anti FcγRIV antibody reverse the process of inflammation mediated follicular damage. by restoring bulge immune privilege?**

1.7 Experimental design

To examine whether IP exists in the bulge of murine telogen follicles (which had not been documented before), key IP markers, i.e. MHC class I, CD200 and TGF-β1 were assessed by immunohistology.

To study the phenomenology of HF stem cell destruction we employed both the passive and active mouse models of EBA. Passive model mice (C57/BL6) were injected with pathogenic anti COL7 IgG , while in active EBA model mice a recombinant protein (GST-mCOL7c) was repetitively injected withTitermax®. Only back skin devoid of visible skin lesions and alopecia was examined so as to obtain early lesional HFs that were under the attack of anti-collagen VII antibodies and/or inflammatory cell infiltrates, but had not yet been destroyed.

Besides follicular IP parameters such as MHC I and CD200, various inflammatory infiltrate markers such as CD4+ cells, MHC II+ cells, mast cells, CD54+ cells and CD8+ cells, and selected cytokines (IL-15, TGFß1 [Mirghomizadeh et al. 2009,Foitzik et al. 2000]) were analyzed by quantitative immunohistomorphometry. This was complemented with analyzing the expression of CD34, a sensitive murine bulge stem cell marker CD34 (Lander et al.2012.) to ascertain the extent of damage to the bulge stem cell compartment.

Finally, to test the hypothesis that FcγRIV receptor also plays a key role in mediating the HF damage that leads to EBA-associated CA, BALB/C mice were simultaneously injected with anti-COL7 IgG and anti FcγRIV antibodies (Nimmerjahn et al.2011, Kasperkiewicz et al. 2012, Hashimoto et al.2012). Subsequently skin sections of non-alopecic skin were stained and quantified for changes in important bulge IP markers such as MHC I and CD200, and the distribution of various relevant immunocytes like CD4+ cells, MHC II+ cells and mast cells in the ORS, CTS and dermis was determined by quantitative immunohistomorphometry

1.8 Limitations of the study

The present study has several limitations, this study is limited by small sample size, which also limits us to perform the required statistics. Further, an increase of at least one or two mice per group in all the experiments would have markedly improved the data both qualitatively and quantitatively. In case of the active model which has a longer incubation time, the experimental data has been derived from one single experiment and requires one additional experiment for making improvedconclusions.

2.0 Materials and Methods

2.1 Mice, animal license and mice experiments

Murine back skin was taken after EBA induction in a collaborative effort with the laboratory of Prof. R. Ludwig and Prof. D. Zillikens, Department of Dermatology, University of Lübeck, Germany. For the passive model, active model and treatment with anti FcγRIV experiments female C57BL/6J mice, SJL mice and BALB/c aged between seven to eight weeks old were purchased from Charles River. All animal experiments were caged and treated in the animal facility of the University Hospital Schleswig-Holstein under protocols approved by the respective governmental authorities (09/37f, Ministerium für Landwirtschaft, Kiel, Germany) and the guidelines of state authority for animal research conduct (LaGetSi, Berlin, Germany).

2 to 4 mice were examined for each group and placed into cages with normal day/night cycles. To obtain reproducible results, two independent repeats of each experiment were performed (in the case of the passive EBA model and treatment with anti FcγRIV). Owing to time constraints, only a single independent experiment could be performed for the active model mice.

2.1.1 EBA mouse models employed: Passive versus active mouse models

For passive EBA model mice, 6 week old C57BL6 mice were used and injected with purified anti type VII collagen IgG (Samavedem et al.2012).In the active EBA mouse model, the disease was induced in six week old SJL mice by injection of purified GST-mCOL7C + Titermax®(Hammers et al. 2011, Hirose et al. 2012). Details are listed below.

2.1.2 Antibody purification

Pathogenic IgG antibodies inducing EBA were affinity-purified using previously established protocols (Sitaru, 2005). Briefly, IgG antibodies were isolated using protein G sepharose column (Amersham Biosciences, Heidelberg, Germany) from serum of rabbits immunized with recombinant fragments located within the murine

NC1 domain of COL7. Columns were washed with intermittent changes of PBS, glycine buffer, sodium chloride and PBS. Latter 50 ml of serum was poured onto the column and put on a rocking platform at 4^{o}C for 1 hour. The flow through was discarded and latter washed with PBS. Column was then eluted with 0.1M glycine buffer and the pH of eluate was adjusted between 7.0-7.3 using Tris Base. This was then concentrated by concomitant cycles of filtration and centrifugation at 4000 rotations per minute for 60 minutes in a cold room. The filtered protein was washed with PBS to remove contaminants and measured in the photometer to get the desired quantity of protein. Reactivity of isolated polyclonal IgG was tested by performing immunofluorescence (IF) microscopy on murine skin (Mihai et al. 2007, Kasperkiewicz et al.2011, Hirose et al.2012).

2.1.3 Production and purification of COL7 fragment

The NC1 domain (amino acids spanning from 757–967) of mCOL7C epitope was produced as a GST (glutathione-s-transferase) fused protein after cloning the required sequence into a prokaryotic expression system. The expression system was then transformed into bacteria and then transferred onto plates with antibiotic for selection. Positive colonies were then picked and grown in liquid broth for obtaining the desired OD value. Starter culture was transferred to large conical flasks containing 500 ml of medium and stored at 37^{o}C for 2.5 hrs at 220 rotations per minute to get desired optical density. Then isopropyl-beta-D-thiogalactopyranoside (0.5mM) was added to the flask and incubated for 3 hours at 37^{o}C. To get the desired protein bacteria were spun down and lysed. The supernatants were collected after centrifugation and glutathione-affinity chromatography was performed and the protein of interest was eluted later (Sesarman, 2008; Hammers et al., 2011).

2.1.4 Passive transfer studies, evaluation and blocking of FcγRIV

EBA was induced as described before (Sitaru, 2005, Hirose et al 2012). In brief, 2-4 mice per group were subcutaneously injected with 7.5 mg/kg of anti-COL7 rabbit IgG. Untreated mice and mice treated with normal rabbit IgG (NR-IgG) were used as parallel controls. Mice were clinically evaluated prior to the first IgG injection and at 4, 8 and 12 days thereafter. For blocking FcγRIV receptor, anti FcγRIV

receptor isotype 9E9 produced in hamster (clone A19-3, BD Biosciences) (Nimmerjahn et al. 2005) was subcutaneously injected into mice 3 times (day 0, 4, 8) at a dose of 10 mg/kg body weight followed by six alternate injections of anti-COL7 pathogenic IgG 7.5mg/kg over a period of 12 days. At day 0, 4, 8 and 12, mice were scored over the body surface for alopecia, erythematous lesions, crust and erosions and sacrificed at day 12 (Kasperkiewicz et al 2012,).

2.1.5 Active transfer studies and evaluation

In case of the active EBA model, a concoction was prepared by mixing 60 ug of column purified GST-mCOL7C with 60µl adjuvant Titermax® (Alexis, Lörrach, Germany). The concoction was subcutaneously injected once per week into 2-4 SJL/J (H-2s) (Charles River Laboratories Sulzfeld, Germany) mice per group for up to four weeks. Parallel controls were maintained by injecting either titermax or PBS Disease was measured as percentage of distribution of blisters crusts and alopecia seen on total body surface and assigned a range of scores from 0%, 1-5%, 5-10%, 10-15% and 15-20% and > 20% (Hammers et al.2011).

2.1.6 Mouse skin sampling and processing

Non-lesional back skin biopsies for the passive model were obtained from both diseased and control groups 12 days after IgG injection, and 9 weeks after injection of GST-mCOL7C for the active model. Skin biopsies were embedded using previously described cryoembedding techniques for murine hair research purposes (Paus et al, 1998) and immediately snap frozen using liquid nitrogen. For immunohistology, two corresponding 6µm sections containing longitudinal HFs were restored onto a single slide and stored at -80°C until further use. The same procedure was also followed for obtaining the back skin from the active mice models (Hoi et al. 2010).

2.2 Histochemistry

2.2.1 HE (Haematoxylin Eosin)

In order to visualize skin morphology HE histochemistry was performed. Slides were dried at room temperature for 10 minutes, fixed in acetone for 10 minutes and later rinsed in TBS buffer pH 7.6, slides were then put for ten minutes in

Mayer's hemalaun (Carl Roth GMBH, Germany), and rinsed under tap water for 15 minutes. Counterstaining was done for 1 minute in .1% eosin (Sigma, E4382) and differentiated in alcohol ranging from 70% to 100%. Slides were dipped in xylene for 10 minutes and mounted with Eukitt synthetic resin (O.Kindler & Co, Freiburg, Germany) as previously described (Kloepper et al. 2008,Tiede et al. 2009b,Knuver et al. 2012, Langan et al. 2013).

2.2.2 PAS (Periodic Acid Schiff staining)

This histochemical stain is used for identifying muco-polysaccharides and nicely demarcates the BM. This staining was done to detect BM destruction of the HF ORS by pathogenic anti-COL7 IgG after injection of antigenic recombinant protein.

Briefly, cryosections of skin from EBA mice and controls were dried for ten minutes at room temperature (RT) and acetone fixed for 10 minutes at -20°C. Slides were rinsed and were then incubated in 0.5% periodic acid (Fluka, Germany) at RT for 10 min. After a brief rinsing in distilled water, slides were incubated for 15 min in Schiff's reagent (Merck, Darmstadt, Germany), rinsed with distilled water and counterstained in haematoxylin (Carl Roth GMBH, Germany). Further the slides were incubated in xylene and mounted with Eukitt synthetic resin (O.Kindler & Co, Freiburg, Germany) (Torres Alvarez et al. 2011, Sarita et al 2012)

2.2.3 Toluidine blue staining

TB staining is used to detect the distribution of mast cells in various tissue compartments. TB binds to metachromatic granules of mast cells that give them their specific red purple colour. Slides were dried for 10 minutes at room temperature, washed for 2 minutes in distilled water and dipped in a cuvette containing 1% toluidine blue- (TB) (MERCK, Germany) (pH 8.9) for 1 min. Slides were rinsed in distilled water and differentiated in alcohol ranging from 70 to 100%, cleared in xylene for 10 minutes and mounted using synthetic Eukitt resin (O.Kindler & Co, Freiburg, Germany). Mast cells (MC) were counted at various locations and latter grouped into either non-degranulated or non degranulated groups when no metachromatic granules found or at least five granules were found surrounding the mast cells (Ito et al. 2010, Sugawara et al. 2012)

2.2.4 Leder esterase staining

Leder esterase staining uses the enzyme chloroacetate esterase as marker for cells of monocytic lineage (neutrophils, macrophages and mast cells) (Sugawara et al. 2012). When stained these cells display pinkish orange cytoplasm staining with green or blue nuclei (haematoxylin).Dried slides were fixed in 1% paraformaldehyde for 10 min, incubated in a pinkish flocculate dye made with a combination of naphthol AS-D chloroacetate in dimethylformamide and pararosaniline hydrochloride in veronal acetate solution for 60 min, and counterstained with haematoxylin for 1 min. MCs were detected post fixation as deeply stained pinkish-red granules and classified as degranulated when five or more red-purple granules could be found outside the cell membrane (Ito et al. 2010, Sugawara et al. 2012)

2.2.5 Immunohistochemistry: avidin-biotin-*peroxidase* complex (*ABC*)

Individual cryosections each measuring 6µm was put on to slides from snap frozen skin stored at -800C. Slides were later air dried at room temperature for ten minutes and fixed in ice cold acetone at -200C for 10 minutes. Slides were then rinsed 3 times for five minutes in TBS (pH-7.6-see appendix) and preincubated for 15 minutes with 3% peroxide in TBS to quench endogenous peroxidase. Slides were rinsed 3 times for 5 minutes in TBS and correspondingly sections were encircled with fat pen. Slides were preincubated with 10% serum (Dako, Glostrup, Denmark) for 20 minutes at room temperature in moist conditions. Serum was dropped onto a tissue section and loaded with corresponding primary antibody (**Table 1**) diluted in TBS containing 2% serum and placed at 40C overnight. Next morning slides were rinsed three times for five minutes in TBS and loaded with biotinylated secondary antibodies in a dilution of 1:200 for 45 minutes at room temperature, Goat anti rat or Goat anti rabbit secondary antibodies were either from Jackson Immunoresearch,Cambridgeshire,UK, Slides were washed 3 times for 5 minutes in TBS and loaded with ABC-Peroxidase (Vector Labs, Burlingame,CA,USA) for 30 minutes, this solution was prepared 30 mins in advance following the instructions on the datasheet. Slides were rinsed 3 times for five minutes in TBS and DAB substrate (Vector Labs, Burlingame, CA, USA) for 2-10 minutes. Solution was prepared by adding one drop of buffer solution combined

with two drops of 3, 3'-Diaminobenzidine (DAB) and 1 drop of hydrogen peroxide in 2.5ml of distilled water. To keep the solution homogeneous the tube was vortexed after adding each component. For negative controls, the corresponding primary antibody was omitted on the sections and loaded with TBS buffer. After loading DAB substrate slides were washed in TBS and counterstained in haematoxylin and was under running tap water for 5 minutes and mounted with Aqueous Mounting Medium (Dako Faramount, Dako,Glostrup, Denmark) (Muller-Rover et al.2000,Tiede et al.2009a,Holub et al. 2012).

2.2.6 Immunofluorescence microscopy

Immunofluorescence (IF) microscopy was done employing secondary IgG antibodies conjugated to either fluorescein isothiocyanate or rhodamine. All antibodies(goat anti rat, goat anti rabbit antibodies was purchased from Jackson Immunoresearch,Cambridgeshire, UK and correspondingly diluted in the ratio of 1:200 in TBS for further use. IF was similarly to the immunohistochemistry protocol described above, except that the peroxide quenching steps and incubation with substrates after second antibodies was omitted.The fluorescent dye 4,6 diamidino-2-phenylindole-dihydrochloride (DAPI) that binds specifically to DNA was used for counterstaining. After counterstaining slides were washed 3 times for five minutes in TBS and mounted with fluoromount G before coverslipping (Southern Biotechnologies, Birmingham,USA) (Kinori et al. 2012a,Ramot et al.2011).

For the tyramide signal amplification technique (TSA), which greatly enhances the immunofluorescence signal (Meier et al. 2012, Gaspar et al. 2011).), slides containing 6µm sections were air-dried at room temperature for 10 minutes and fixed in ice cold acetone at -20^{o} C for 15 minutes. Slides were rinsed 3 times for five minutes in TNT (see appendix) and peroxidase was quenched by incubating in 3% hydrogen peroxide in PBS (see appendix). Slides were washed and loaded with primary antibody and kept overnight in a moist chamber at 40^{o}C. Next morning slides were rinsed 3 times for 5 minutes in TNT and biotinylated secondary antibody was loaded onto the sections at a corresponding dilution of 1:200 in TNT and incubated for 45 minutes at room temperature. Slides were then rinsed 3 times for 5 minutes in TNT and incubated with horseradish peroxidase (TSA kit, Perkin-Elmer, Boston, USA) at dilution of 1:100 in TNT for 30 minutes.

Latter slides were rinsed 3 times for 5 minutes and loaded with either FITC or tetramethyl rhodamine dye diluted in a corresponding ratio of 1:50 in amplification diluent (TSA kit, Perkin-Elmer, Boston, USA) for 1-5 minutes. Slides were then washed 3 times in TNT, counterstained with DAPI and mounted with fluoromount G (Southern. biotechnologies, Birmingham, USA).

2.3 Quantitative (Immuno-) Histomorphometry

To access the role of other cells in causing EBA induced CA we counted the distribution of mast cells, MHC II+, CD4+ and CD8+ cells surrounding the bulge. In doing so we have specifically demarcated the zones of ORS, CTS and up to 50µm from CTS into the dermis surrounding the bulge and counted the cells in each individual field at 400X magnification. To get a definitive value we measured at least five individual follicles for each individual experiment at various locations of the section (Kinori et al. 2012).

Interestingly all the samples of passive EBA models displayed telogen follicles and since it is difficult to pinpoint exactly the location of the bulge we first demarcated the area of bulge from the lower portion of the sebaceous glands to the insertion of arrector pili muscle and also based on the previous evidence that some of K15-green fluorescent protein positive cells were found to reside in this area.we also demarcated this area to measure the staining intensity of some of the important parameters that controlled hair follicle immune privilege . In the active model however the untreated group displayed anagen follicles with a prominent bulge therefore a comparatively similar area of reference was demarcated in the telogen follicles of the active mice model and measurements of staining intensity were made in this area accordingly.

Staining intensity was measured using NIH ImageJ software. This imaging software distinguishes the light from dark areas and averages the intensity in a specified given area. The software was legally downloaded from the following website http://rsb.info.nih.gov/ij/. The data were obtained from the bulge region by averaging the mean intensities of the staining on both sides of the follicle. We used this method to get the staining intensities of MHCI, CD200, CD34, IL15, NGF and TGFβ-1. (Knuever et al.2012, Petukhova et al. 2010).

2.4 Statistical analysis

The data was analyzed using Graphpad prism version 4 for windows (Graphpad software,San Diego, California,USA). The results were expressed as standard error of the mean (mean±SEM) (Meyer et al.2008).

2.5 List of antibodies used

Antibody	Type	Company	Species	Dilution	Method	Reference
MHC I	IgG2a (ER-HR52)	BMA biomedical (T-2105)	Rat anti mouse monoclonal	1:50	ABC	Paus et al. 1994cPaus et al.1998 Rueckert et al.1998
MHC II	IgG2b (ER-TR3)	BMA biomedical (T-2106)	Rat anti mouse monoclonal	1:750	ABC	Paus et al.1998 Christoph et al.2000
CD200	IgG2a (OX-90)	AbD serotech (MCA 1958)	Rat anti mouse monoclonal	1:250	ABC	Rosenblum et al.2000 Wright et al. 2003
CMD4	IgG2a,κ	BD Pharmingen (550280)	Rat anti mouse monoclonal	1:200	ABC	Paus et al. 1998
CD8	IgG2b (YTS169.4)	AbD serotech (MCA1768)	Rat anti mouse monoclonal	1:175	ABC	Eichmuller et al.1999 Hennino et al.2007
CD34	IgG2a,κ (MEC14.7)	Abcam (ab8158)	Rat anti mouse monoclonal	1:250	ABC	Trempus et al.2004 et
CD54	IgG1,κ 3E2	BD Pharmingen (553251)	Hamster anti mousemono clonal	1:50	ABC	Muller-Rover et al. 2000
IL-15	IgG	R&D Systems (AF447)	Goat anti mouse polyclonal	1:500	ABC	Mohamadzad eh et al.2001 Orinska et al 2007
TGF-β1	IgG	Santa Cruz Bio-technology (sc-146)	Rabbit anti mouse polyclonal	1:350	ABC	Paus et al. 1997

Table 2: List of primary antibodies used. *The table shows the corresponding name, isotype, source, animal source from which the antibody is derived, dilution and detection method used with corresponding references. ABC =Avidin Biotin complex.*

3. RESULTS

3.1. Murine telogen follicles display immune privilege in the follicular bulge region.

The primary focus of this chapter was a) to search for phenomenological indicators of IP in the bulge region of mouse telogen follicles and b) to study the basic distribution pattern of inflammatory cells around normal telogen follicles, thus complementing previous work, whose results served as immunohistological quality control (Paus et al. 1994 c, d, Paus et al. 1998)

3.1.1 MHCI, CD200 and TGFβ-1 are expressed in varying levels in themurine telogen follicles

The goal was to find whether normal mouse telogen follicles (derived from control animals) expressed markers of IP in their bulge region and infundibulum, as they are present in anagen VI follicles of humans (Paus et al. 1994 c, b, Paus et al. 1998, Paus 2005). Routine IHC was performed on frozen back skin sections taken from 6 weeks old normal C57/BL6 mice to specifically stain for MHC class I (MHCI), CD200 and TGFβ-1.

This revealed reduced MHC I expression in the bulge region and infundibulum. (**Fig.13**a,d).The expression was higher in the infundibulum in comparison to the bulge. Instead, the expression of CD200 antigen was very high throughout the epithelium of telogen HFs and differed between bulge infundibulum, but was virtually absent from the epidermis (**Fig.13** b,d). However, in contrast anagen HFs, only very subtle, if any TGF-β1 immunoreactivity (IR) (**Fig.13** c, d) was seen in the bulge region (curved bracket) of murine telogen follicles, bulge IR for TGFß1 was slightly, but higher than in the infundibulum.

This indicates that the bulge region of murine telogen HFs, like its counterpart in human anagen VI follicles (Meyer et al. 2008) displays relative IP, as indicated by the sharply reduced MHC I and the massive CD200 expression.

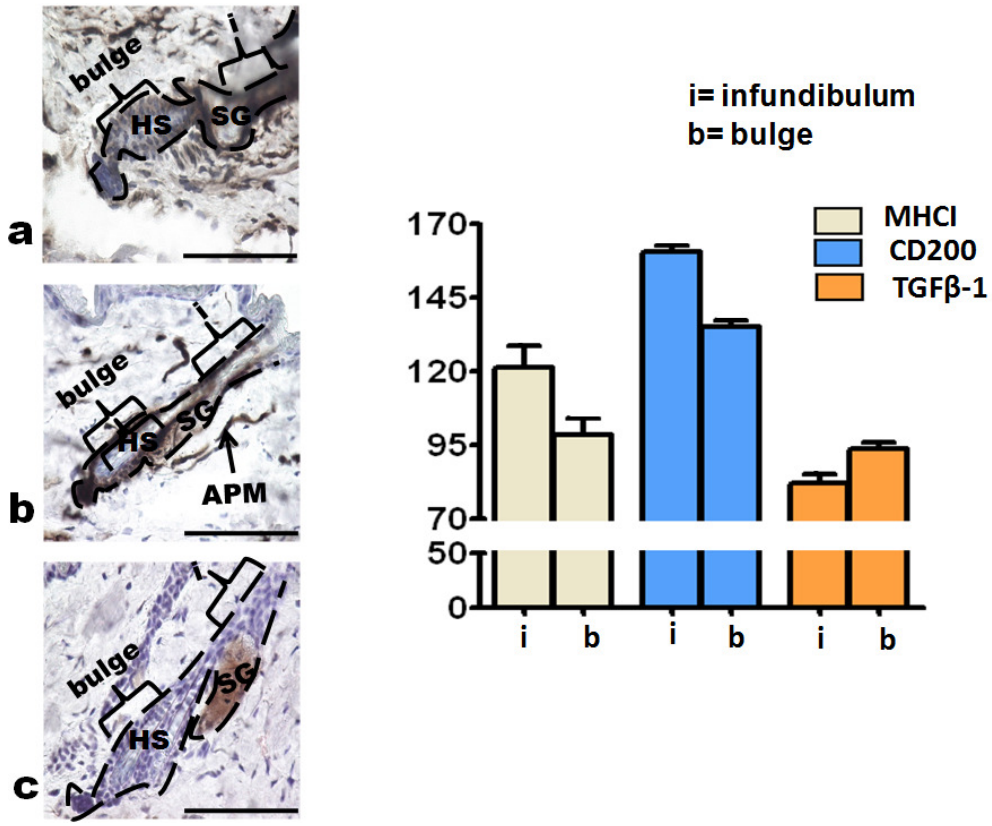

Fig.13: Indications of immune privilege in the bulge and infundibular region of a normal telogen hair follicle of C57BL/6 mice. *Representative picture showing MHC I immunoreactivity (green) in the hair follicle bulge and infundibulum of normal telogen follicle of C57BL/6 mice (a).CD200 immunoreactivity (brown) in the hair follicle bulge and infundibulum of normal telogen follicle of C57BL/6 mice (b).TGF β1 immunoreactivity (brown) in the hair follicle bulge and infundibulum of normal telogen follicle of C57BL/6 mice(c). Quantitative analysis indicates varying levels of expression of MHC I, CD200 and TGF β1 in the bulge and infundibulum of a normal mice telogen follicles (d). ABC - peroxidase method, DAB, in brown, counterstaining using haematoxylin. (d) N=29-39 HFs/group, derived from 2-3 mice/group from two individual experiments, mean +/-SEM.APM, arrector pili muscle, HF, hair follicle, HS, hair shaft,I, infundibulum,SG, sebaceous gland, Scale bars=100 µm.*

3.1.2 Distrbution of MHCII, CD4+ and mast cells in the bulge region of murine telogen follicile

In order to be able to more comprehensively judge the immunological characteristics of normal telogen HFs before they are attacked by anti-collagen type VII autoantibodies, frozen sections from 6 week-old normal C57/BL6 control mice were also immunostained for MHC II+ cells, CD4+ cells and mast cells.

This showed that the HF epithelium (ORS), including the bulge region, is almost devoid of MHC II+ cells (=Langerhans cells) (Eichmüller et al. 1998, Paus et al. 1998). This suggests that MHC class II-dependent antigen presentation within the HF epithelium is severely compromised, in line with the concept that the telogen HF enjoys relative IP. Intramesenchymal MHC II+ cells (=mainly macrophages) are relatively rare in the HF mesenchyme (CTS), and are predominantly located in the perifollicular dermis. (Arrows, **Fig.14** a, b).

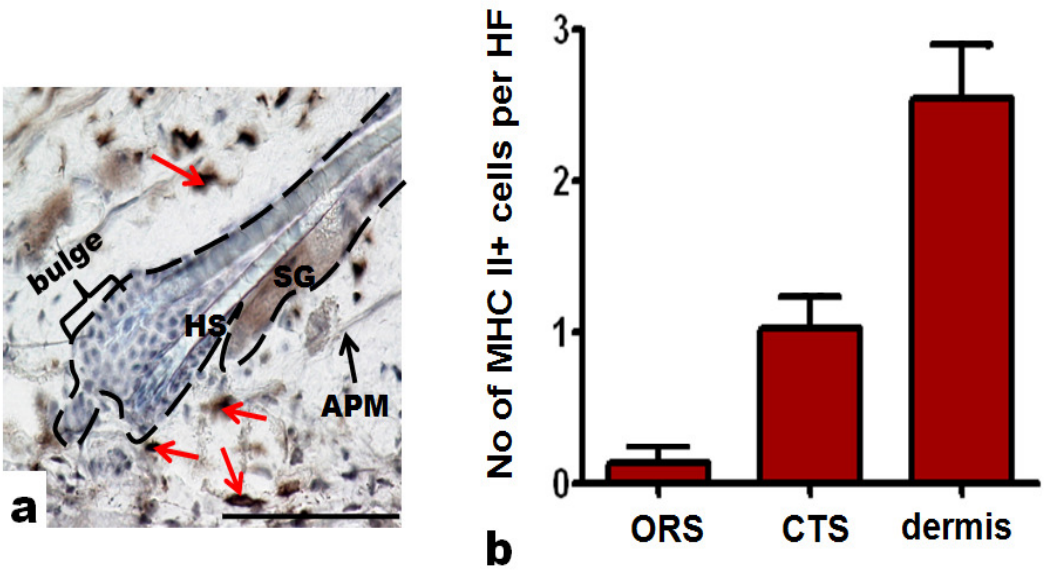

Fig.14: Distribution of MHC II+ cells within and around the bulge region of a normal murine telogen hair follicle. *MHC II+ immune cells (brown/red arrows) surrounding the hair follicle bulge region of normal telogen follicle of C57BL/6 mice (a).Quantitative analysis showing the distribution of MHC II+cells in both the CTS and dermis surrounding the bulge region of telogen hair follicles (b) ABC - peroxidase method, DAB, brown, counterstaining using haematoxylin. N=35 HF's, derived from 2-3 mice per group from two*

individual experiments, mean +/-SEM. APM, arrector pili muscle, CTS, connective tissue sheath, HF, hair follicle, HS, hair shaft, SG, sebaceous gland, Scale bars=100 µm.

No CD4+ cells were found in the infundibular epithelium of murine telogen HFs, including in the bulge zone. Like perifollicular MHC II+ macrophages, CD4+ T cells were only present in low numbers in the HF mesenchyme (CTS), and primarily resided in the perifollicular dermis (Arrows, **Fig.15**a,b).

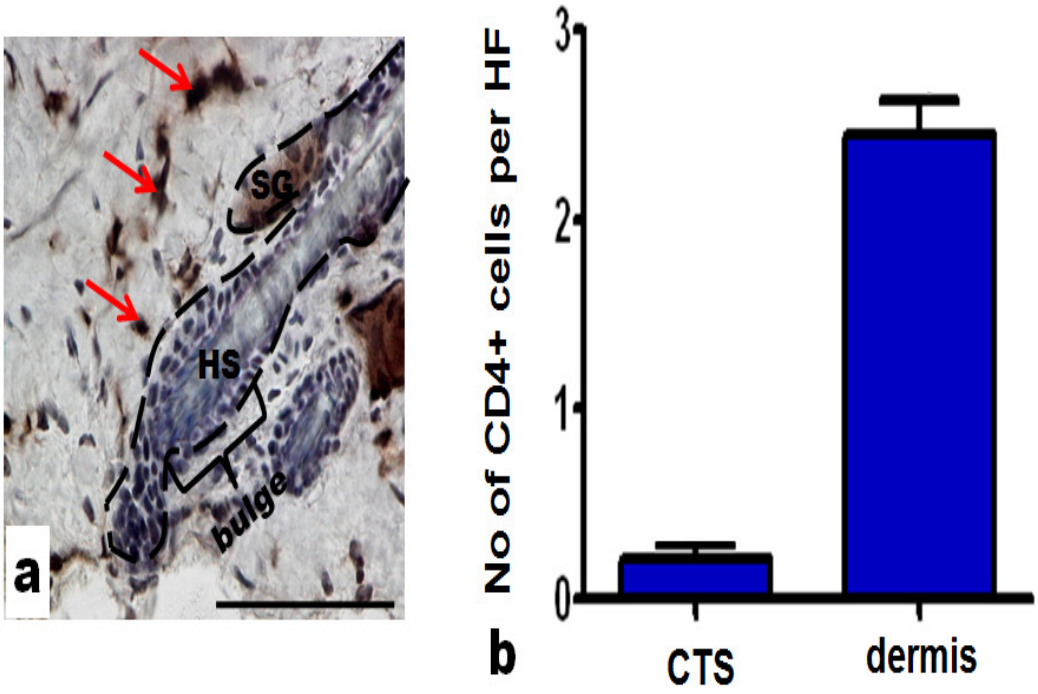

Fig.15: Distribution of CD4+ T cells around the bulge region of a normal murine telogen hair follicle. *CD4+ immune cells (red/yellow arrows) surrounding the hair follicle bulge region of normal telogen follicle of C57BL/6 mice (a).Quantitative analysis showing the distribution of CD4 +cells in both the CTS and dermis surrounding the bulge region of telogen hair follicles (b). ABC - peroxidase method, DAB, brown, counterstaining using haematoxylin N=42 HF's, derived from 2-3 mice per group from two individual experiments, , mean +/-SEM. APM, arrector pili muscle, CTS, connective tissue sheath, HF, hair follicle, HS, hair shaft, SG, sebaceous gland, Scale bars =100 µm*

Essentially the same was true for mast cells, whose total number was even lower than that of perifollicular macrophages and CD4+ T cells (Arrows, **Fig.16** a,b) . Most perifollicular mast cells that were detected around normal murine telogen HFs by Leder esterase (**Fig.16**a) or toluidine blue histochemistry (not shown) were activated, i.e. degranulated (**Fig.16a**).

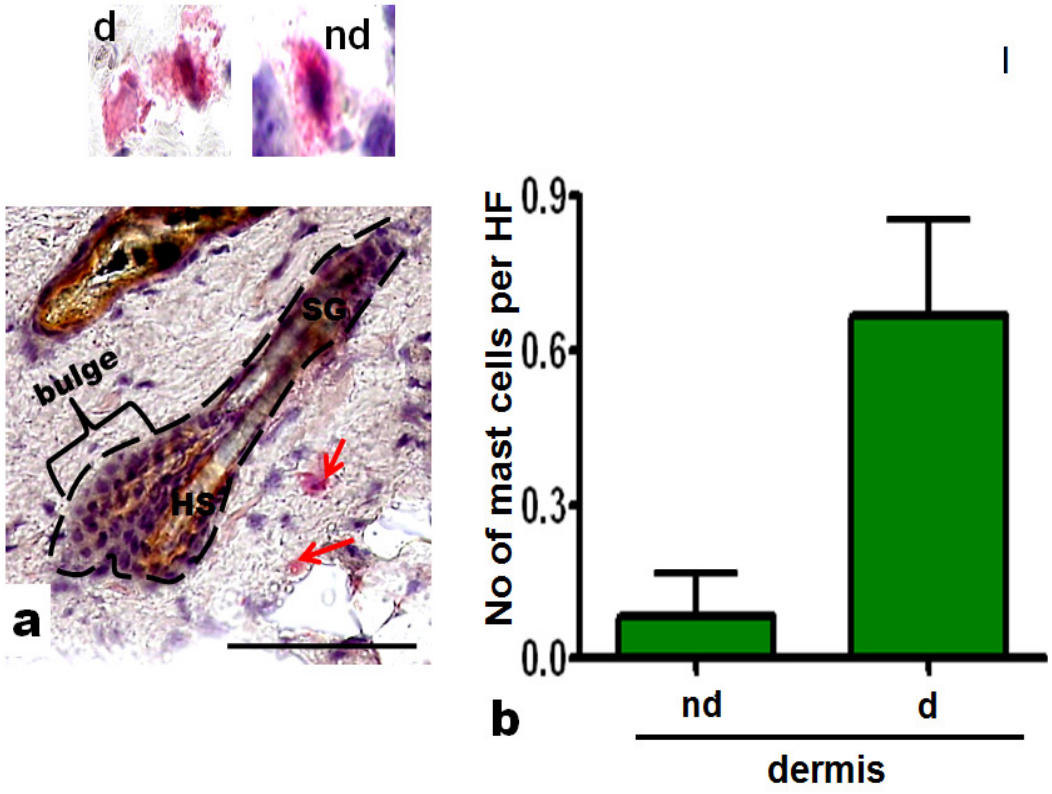

Fig.16: Distribution of mast cells around the bulge region of a normal telogen hair follicle. *Leder esterase staining showing mast cells (pink/red arrows) around the hair follicle bulge region of normal telogen follicle of C57BL/6 mice (a).Quantitative analysis showing the distribution of mast cells in both the CTS and dermis surrounding the bulge region of telogen hair follicles (b).Leder esterase staining in pink, counterstaining using haematoxylin. N=22 HFs, derived from 2-3 mice from two individual experiments, , mean +/-SEM. APM, arrector pili muscle, CTS-connective tissue sheath ,HF, hair follicle, HS, hair shaft, SG, sebaceous gland, Scale bars=100 µm*

3.2 Injecting anti COL7IgG antibodies into mice causes blisters associated with scarring alopecia

On this general background of murine telogen HF-associated skin immunophysiology, immunopathological changes were evaluated that developed after exogenous anti-collagen type VII antibodies were injected (passive EBA mouse model) (Sitaru et al.2005.). The purpose of the following experiments was to determine whether

a) Inflammation as a consequence of antiCOL7IgG injection resulted in a destruction of the HF basement membrane, namely in the bulge zone,

b) Indications of bulge IP collapse were present

c) To characterize alterations in the peri- and intrafollicular inflammatory and cytokine signaling milieu associated with antiCOL7IgG-induced damage of murine telogen HFs in the passive EBA mouse model.

3.2.1 Hair follicle damage occurs in anti COL7 IgG injected mice (passive EBA model)

Mice injected with anti COL7IgG showed blisters with hair loss on the snout,scalp and the thigh regions,further analysis of non lesional skin by routine histology showed infiltrates in the treated mice in comparison to control groups (**Fig.17** a,d,e)

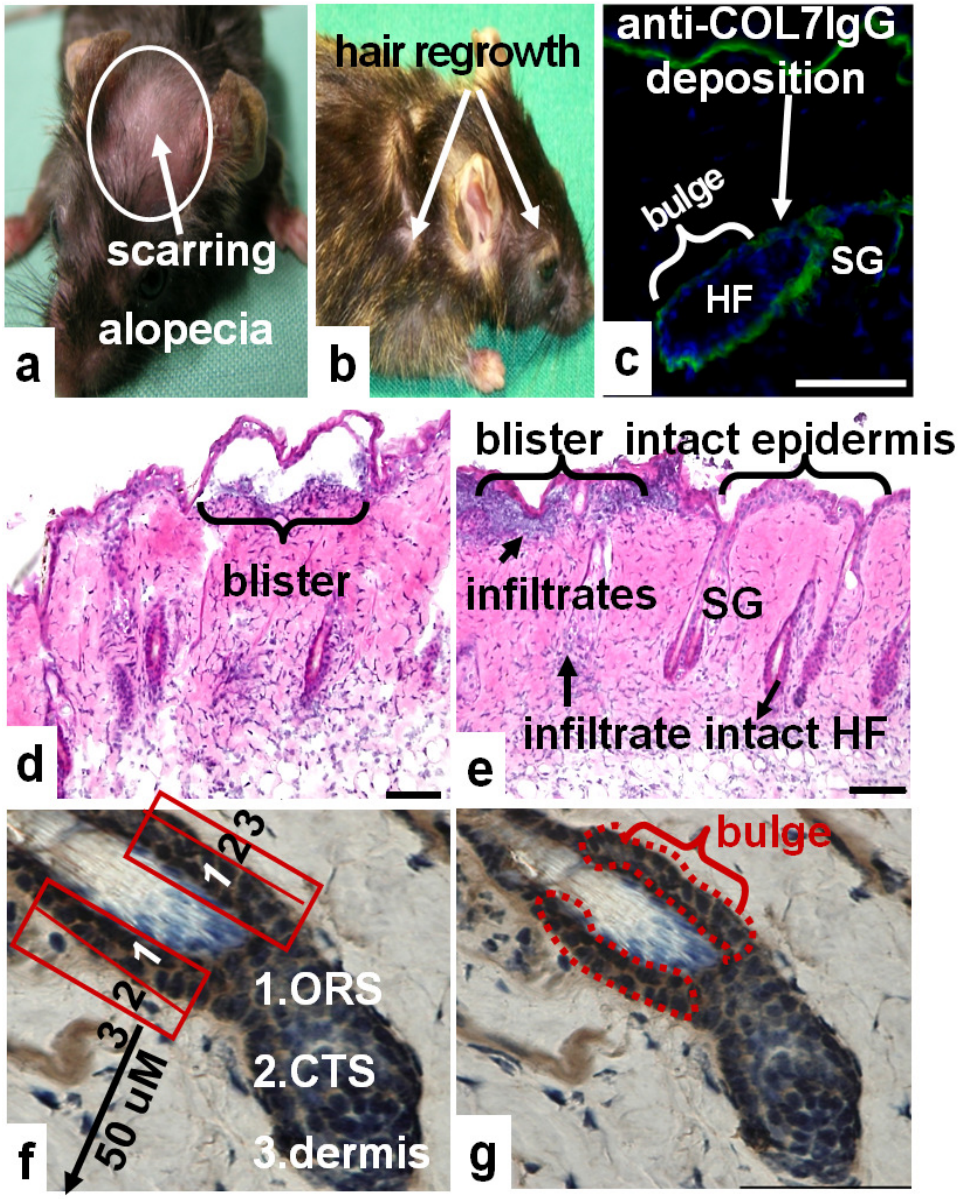

Fig.17: Disease phenotype and scarring alopecia seen in anti COL7 IgG injected mice. *Scarring alopecia (white circle) lesion on the scalp of mice injected with anti type VII collagen (a).Regrowth of hair follicles after sixty four days, after injecting anti COL7 IgG (b). Indirect immunofluorescent staining depicting the deposition of anti type VII antibodies along the basement membrane-FITC labeled antibodies in green (c). Beginning blister formation along with associated inflammatory cell infiltrates (d, e). Note that these infiltrates were essentially devoid of neutrophilic granulocytes, as examined by anti mouse* Ly-6G and Ly6c antibody *(data not shown).Photos depicting the defined reference areas that were assessed for quantitative immuno histomorphometry of telogen hair follicles,with emphasis on the bulge region(f), where individual cells and IR patterns were counted (g). Bulge compartment (red line) in which quantitative*

immune morphometry was done using NIH image J. APM, arrector pili muscle, HF, hair follicle, HS, hair shaft, SG, sebaceous gland, scale bars =100µm.

BM damage is one hallmark of bullous autoimmune dermatoses (Ludwig and Zillilkins 2012, Hashimoto et al.2012), and an intact BM and CTS appear to form a functionally important component of normal HF IP (Paus et al. 2005, Reynolds et al. 1999, Ito et al. 2008).

3.2.2 Basement membrane is destroyed in in antiCOL7 IgG injected mice

Therefore, it was next determined by PAS histochemistry whether BM damage is seen after the injection of pathogenic antibodies. As shown in (**Fig.18**), this was indeed the case, since the PAS staining intensity, which reflects the amount of mucopolysaccharides available for reaction with the staining reagent (Sarita et al.2012), was markedly reduced in non-alopecic skin of test EBA mice that had received the pathogenic antibody compared to vehicle-treated control mice.

However, follicular BM damage generally was much less severe than that seen at the dermoepidermal junction in passive EBA mice (Biber et al.2010,Kasperkiewicz et al.2012,Samavedam et al.2012), and overt blistering, i.e. separation of the ORS from its underlying follicular BM and CTS, was never seen in non-alopecic skin of antiCOL7IgG-injected mice.

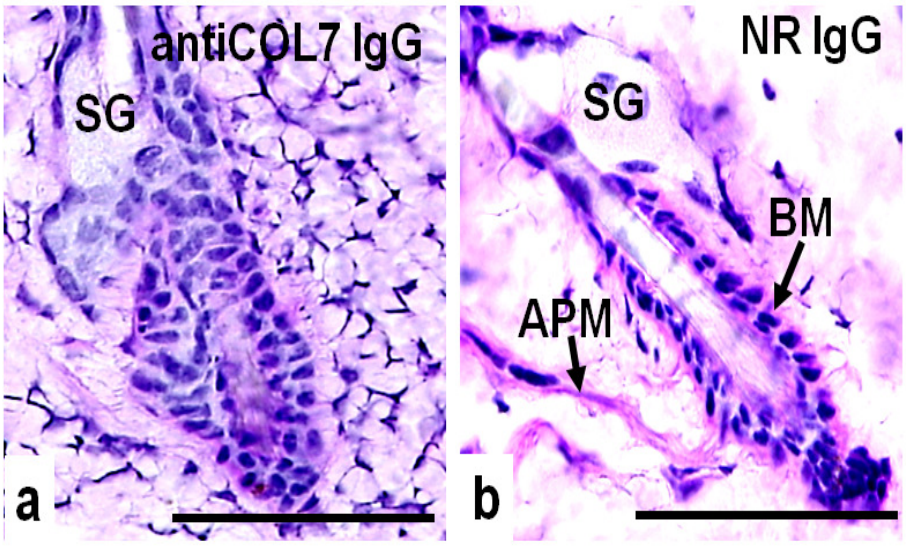

***Fig.18**: **Basement membrane destruction in antiCOL7 IgG injected mice** .decreased intensity of PAS staining in antiCOL7 IgG injected mice with surrounding infiltrates(a) in comparison to control group(b. arrector pili muscle, BM, basement membrane,HF, hair follicle, HS, hair shaft, SG, sebaceous gland, scale bars =50µm.*

This suggests that the HF BM and its underlying CTS are considerably more resistant to the structural integrity-disrupting activities of antiCOL7IgG than the dermoepidermal BM.

In order to assess primarily early damage events, rather than late, secondary ones induced by massive inflammation, in the subsequent studies only *non-alopecic* back skin was examined from test and control mice. This was done under the assumption that bulge and overall HF damage within skin lesions that showed macroscopically appreciable alopecia had already progressed too far to be sufficiently instructive for the purpose of the current study (note that overt hair loss in mice routinely indicates the presence of a major degree of HF dystrophy, since undamaged murine HFs do not shed their hair shafts, even when major hair cycle abnormalities are present (Hendrix et al. 2005, Sundberg et al. 2005); therefore, the absence of alopecia in the skin of test and control mice raised the likelihood that major HF dystrophy had not yet developed in the examined skin regions).

3.3 Immune privilege is compromised in antiCOL7 IgG injected mice (passive model)

It is now widely thought that the bulk of the destruction in EBA occurs due to the action of neutrophils, as evidenced by dermato histopathological examination of EBA patient skin and several studies in experimental EBA models (Ludwig and Zillikins 2011,Chiriac et al.2007,Mihai et al. 2007). However, preparatory analyses for the current study had revealed almost no neutrophils at all (as assessed by HE and Ly-6G and Ly6c (RB6-8C5) antibody staining in the examined back skin (which was, as explained before, devoid of skin lesions and alopecia of either test or control EBA mice) (data not shown). Therefore, other mechanisms than neutrophil-induced HF damage had to be considered. Namely, the working hypothesis was pursued that anti-collagen type VII antibody-induced perifollicular inflammation may endanger the bulge IP. This was thought to make bulge stem cells more susceptible to further inflammatory damage, thus possibly triggering excessive bulge stem cell death. After depletion of the bulge stem cell niche, the HF's capacity to regenerate would be exhausted, resulting in CA. In consequence, we next searched for immunophenomenological evidence that the bulge IP is compromised after injection of pathogenic antiCOL7 IgG antibodies (Meyer et al. 2008). This hypothesis was mainly probed in the passive EBA mouse model, and was complemented with one pilot experiment in the active EBA model.

As expected from the literature (Kasperkiewicz et al.2012, Samavedam et al. 2012,Bieber et al.2010), alopecic skin lesions became prominent four to six days after injection of anti-COL7 IgG antibodies, generally with the clinical appearance of scarring alopecia, as indicated by skin scaling (hyperkeratosis), erythema, and erosions with crusting (**Fig.17**a). However, rather unusual for other forms of CA (see Introduction), several mice showed almost complete hair regrowth in previously alopecic lesions 2-3 months after antibody injection (**Fig.17**b).

Such spontaneous hair regrowth in untreated skin generally is a rare event in human CA and, by itself, already suggests a sufficient preservation of HF epithelial stem cells and retained HF regeneration capacity, despite substantial inflammatory HF damage, wherever hair regrowth occurred. This phenomenon occurred (**Fig.17**b) although dense perifollicular inflammatory cell infiltrates developed in passive EBM mice (**Fig.17**d,e).

Therefore, at least in this passive mouse model EBA, it is questionable whether EBA-associated alopecia represents a genuine form of classical cicatricial alopecia (CA), whose hallmark is the irreversibility of hair loss due to epithelial stem cell destruction in the bulge (Harries et al. 2009a, Harries et al.2009b, Harries and Paus 2010) (see also below: CD34 results).

3.3.1 MHC I IR increase in bulge of mice injected with anti COL7IgG

Next, the bulge IP status of passive EBA mice in non-alopecic skin was compared with that of normal telogen HFs (see above), using MHCI and CD200 as main read-out parameters. This revealed slightly, but marked increase in levels of MHC I IR in the bulge of telogen EBA hair follicles (**Fig.19** b,c) in comparison to NRIgG-treated control HFs (**Fig.19** a,c). Note that both experimental groups showed considerably higher baseline MHC I expression than untreated telogen mouse HFs (**Fig.13**a, see above). This indicates that the injection of excessive amounts of IgG, by itself, already endangers MHC I-based HF IP.

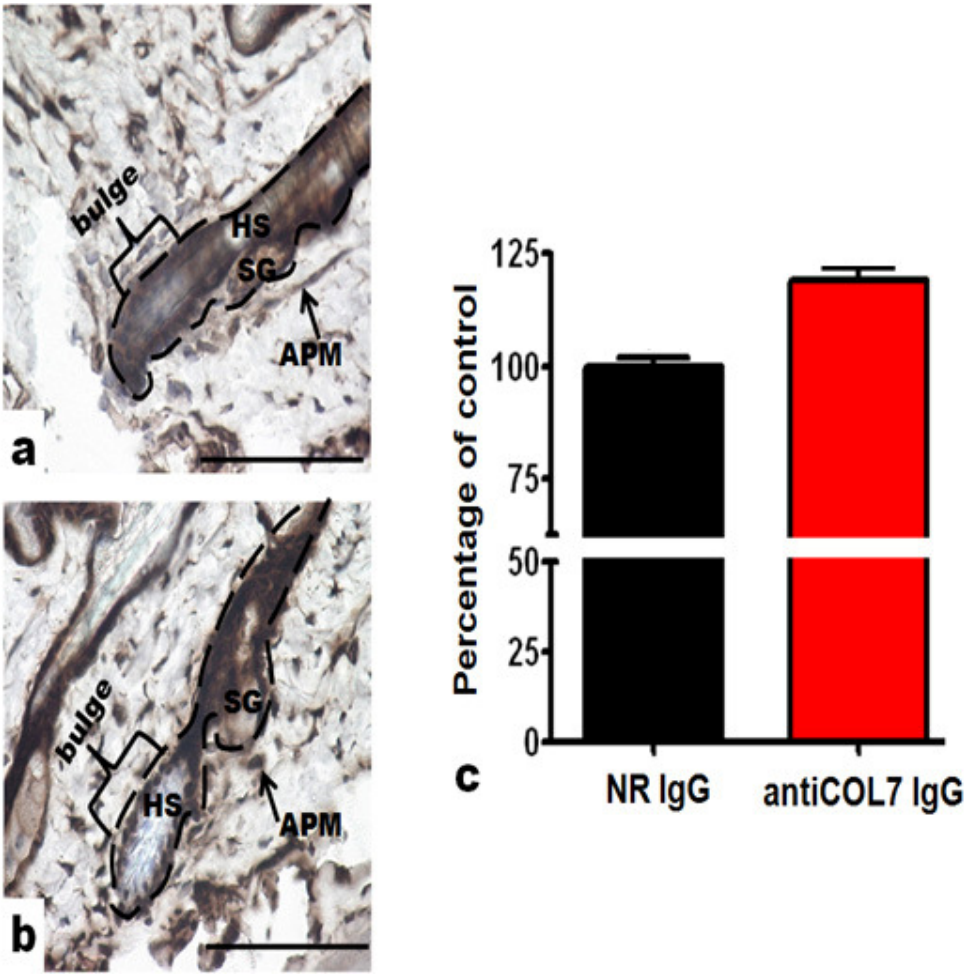

Fig.19 Increase in MHC I immunoreactivity in the telogen bulge of mice injected with anti type VII collagen antibody.*MHC I immunoreactivity (brown) in the bulge region of mice injected with NR IgG (control) (a). MHC I immunoreactivity (brown) in the bulge of mice injected with anti type VII collagen IgG (b). Quantitative analysis reveals increased levels of MHC I immunoreactivity at the level bulge in anti COL7 IgG injected mice in comparison to control group (c).ABC - peroxidase method, DAB, brown, counterstaining using haematoxylin. N=24-32 HF, derived from 2-3 mice/group from two individual experiments, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100µm.*

3.3.2. CD200 IR does not change in the bulge of mice injected with anti COL7IgG

CD200 expression in the bulge remained high after injection of either pathogenic or non-pathogenic IgG antibodies and was essentially unchanged when antiCOL7IgG- and NRIgG-treated HFs were compared with each other (**Fig.20** a, c*)*.No considerable change in baseline expression of CD200 was seen in test

mice in comparison to untreated controls Thus, CD200-based HF IP does not appear to be compromised by the injection of excessive amounts of anti COL7 IgG or control IgG. Taken together, these results suggest that injection of EBA-eliciting pathogenic antibodies primarily compromise the MHC I-based murine HF IP.

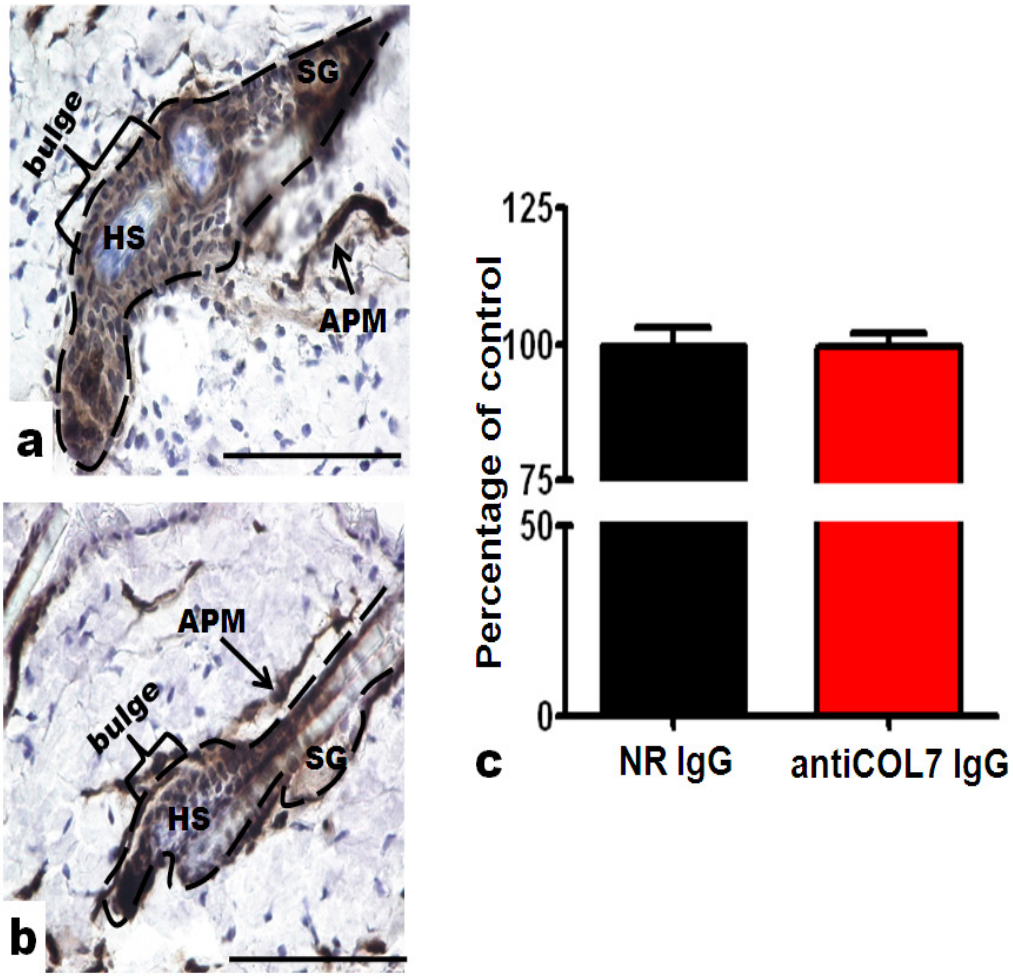

Fig.20: No change in CD200 immunoreactivity in the bulge of mice injected with pathogenic antibody. *CD200 immunoreactivity (brown) in the bulge region of mice injected with NR IgG (a). MHC I immunoreactivity (brown) in the hair follicle bulge of mice injected with anti type VII collagen IgG (b). Quantitative analysis reveals no decrease in the levels of CD200 immunoreactivity at the level bulge in anti COL7 IgG injected mice in comparison to control group (c).ABC - peroxidase method, DAB, brown, counterstaining using haematoxylin. N =35-47 HF's, derived from 2-3 mice/group from two individual experiments, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.*

3.4. Inflammatory infiltrates are increased in the bulge of mice injectedwith anti COL7 IgG antibodies.

To characterize the infiltrates that lead to BM damage and the associated IP collapse, cryosections were immunostained for selected infiltrate markers such as MHC II+,CD4+, or CD8+ cells and mast cells and were evaluated by quantitative immunohistomorphoemtry in defined reference areas (see **Fig.17** f).

3.4.1 CD8+ t cells are increased in/around the bulge of mice injected with anti COL7IgG antibodies

Since MHC I-presented follicular autoantigens would primarily be recognized by CD8+ T cells (Murphy 2011,Paul 2012, Sompayrac 2012), which also play a prominent role in other inflammatory hair loss disorders such as alopecia areata, CDLE, lichen planopilaris, and graft-versus-host disease (Weedon D 2009, Harries et al.2009, Harries and Paus 2010, Gilhar et al.2012), CD8+ T cells were examined first. This showed a slight, but not much increase in the number of detected CD8+ cells in the CTS of antiCOL7IgG-treated HFs, compared to the CTS of control IgG-treated HFs. This corresponded to a slight decline in the total number of CD8+ T cells in the interfollicular dermis (**Fig.21** c). If these trends can be confirmed (e.g. by analyzing a higher n of sections/mice), they might suggest that CD8+ T cells migrate from the dermis towards the HF.

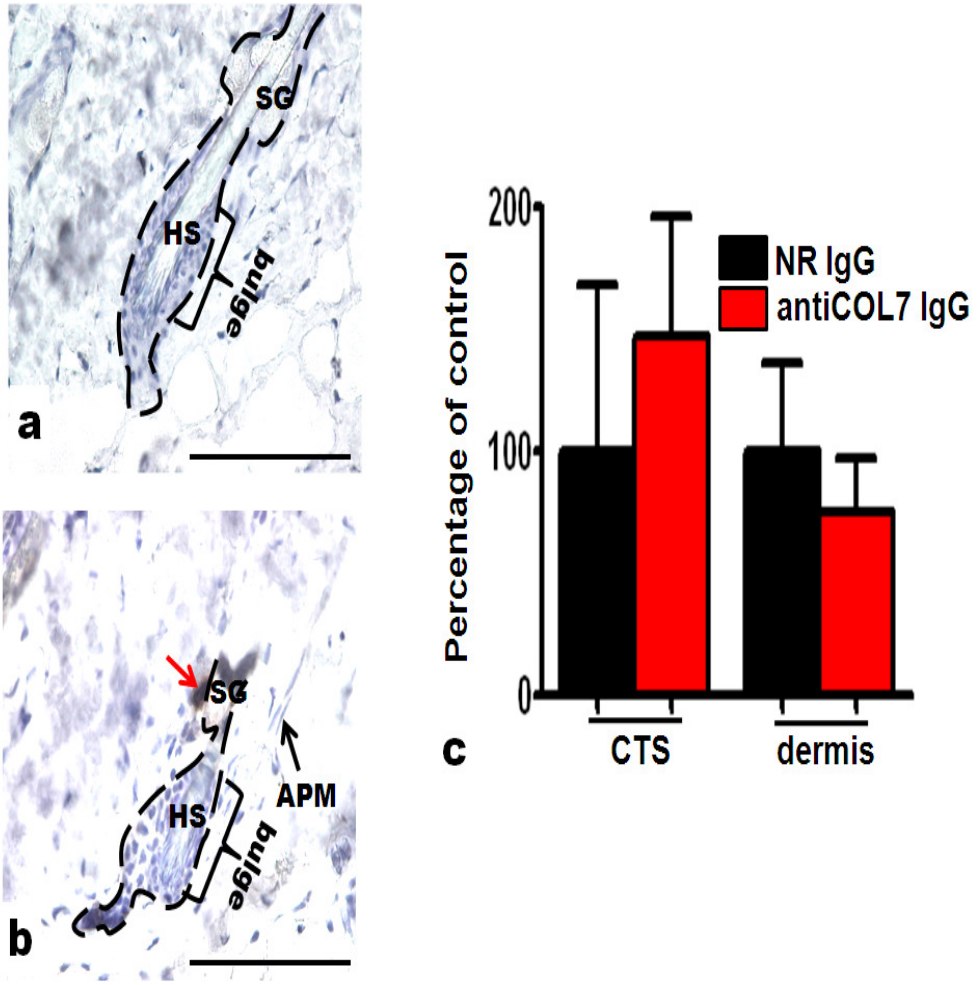

Fig.21: Simultaneous increase and decrease in CD8+ cell number in the perifollicular CTS and dermis of mice injected with anti type VII collagen antibody. *Histochemical staining shows absence of CD8+ cells surrounding the hair follicle bulge region of mice injected with NR IgG(a). CD8+ cells (red arrow) in the hair follicle bulge of mice injected with anti type VII collagen IgG (red arrows) (b). Quantitative analysis reveals slight increase and a decrease in the number of CD8+ cells in the perifollicular CTS and in the surrounding dermis of mice injected with anti COL7 IgG in comparison to control group(c).ABC-peroxidase method, DAB, brown, counterstaining using haematoxylin. N =13-31 HF's, derived from 2-3 mice/group derived from one experiment, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale Bars=100µm.*

3.4.2 CD4+ cells are increased in/around the bulge of mice injected with anti COL7IgG antibodies

Furthermore, the number of CD4+ cells was increased markedly in the CTS, but not the dermis around the bulge of mice treated with anti COL7IgG in comparison with NR IgG treated mice (see arrows, **Fig.22** a-c). This suggests that the massive deposition of anti-COL7-specific pathogenic antibodies at the BM zone of diseased HFs, but not just of IgG, induced specific changes (e.g. secretory activities?) in the CTS and/or ORS that attracted T helper lymphocytes to this critical HF compartment.

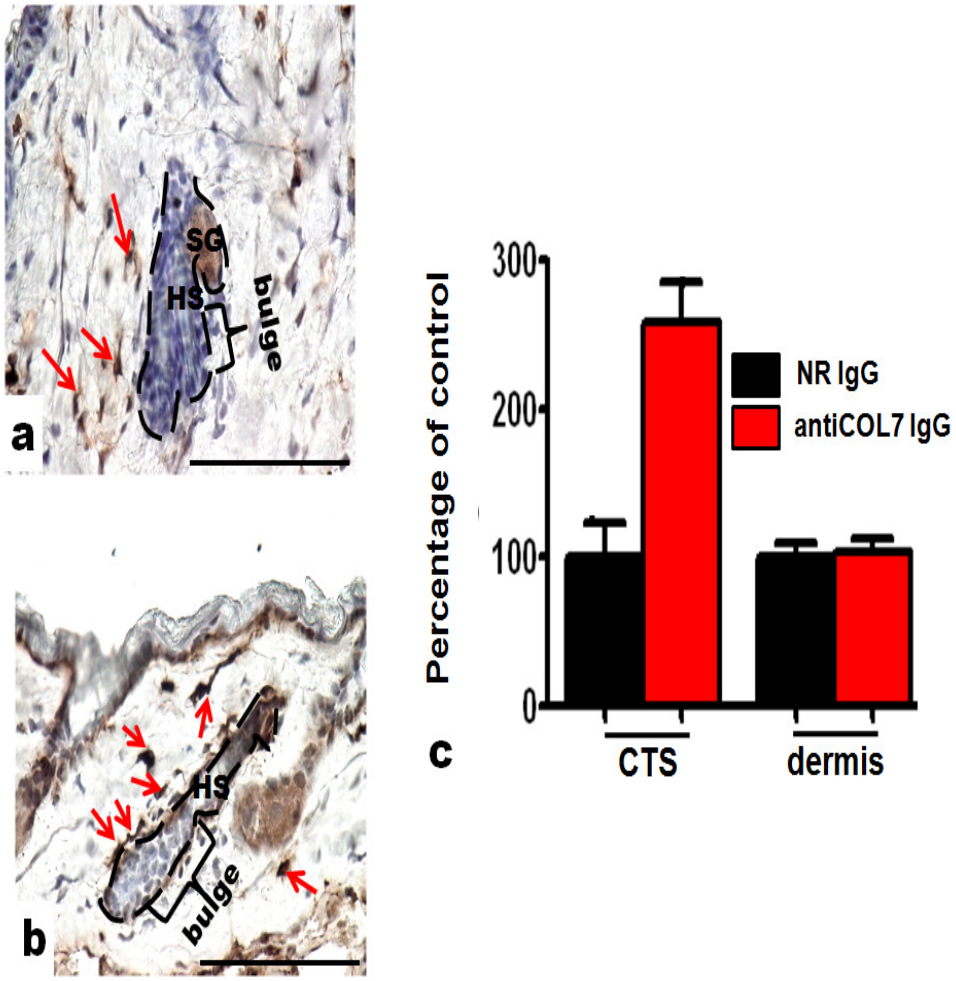

Fig.22: Increase in CD4+ cells around the bulge of mice injected with anti type VII collagen. *CD4 positive cells in the CTS surrounding the bulge region of mice injected with NR IgG (red arrows) (a). CD4 positive cells surrounding immunoreactivity (brown) in the bulge of mice injected with anti type VII cologne IgG (red arrows) (b). Quantitative analysis reveals increase in the number of CD4+ cells in the CTS surrounding the*

bulge in anti COL7 IgG injected mice in comparison to control group (c).ABC-peroxidase method, DAB, brown, counterstaining using haematoxylin. Note the non-specific IR in the SG. N =49-48 HF's, derived from 4-6 mice/group, derived from 2-3 mice/group from 2 different experiments, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm

3.4.3 MHC II+ cells are increased in/around the bulge of mice injected withanti COL7IgG antibodies

In addition, the number of MHC II+ cells (in the CTS (and tendentially also in the dermis) surrounding the bulge of telogen HF was slightly higher in anti COL7IgG-treated mice compared to NRIgG-injected controls. (Fig**23**a-c).

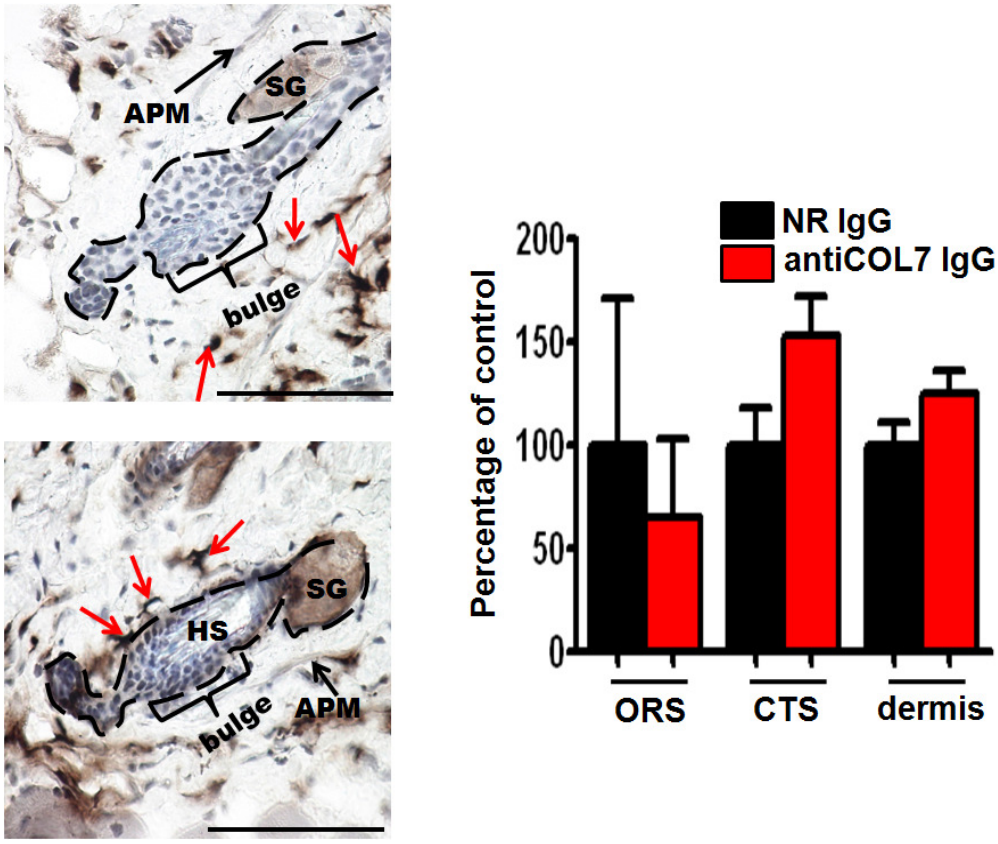

Fig.23: Increase in MHC II + cells around the hair follicle bulge of mice injected with anti type VII collagen. *MHC II positive cells in the CTS surrounding the bulge region of mice injected with NR IgG (red arrows) (a). MHC II positive cells surrounding immunoreactivity (brown) in the bulge of mice injected with anti type VII collagen IgG (red arrows) (b). Quantitative analysis reveals an increase in the number of MHC II positive cells in the CTS surrounding the bulge in anti COL7 IgG injected mice in comparison to control*

group (c).ABC-peroxidase method, DAB, brown, counterstaining using haematoxylin. Note the non-specific IR in the SG. N =32-38 HF's, derived from 2-3 mice/group from two individual experiments, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.

3.4.4 Mast cells are increased in/around the bulge of mice injected with anti COL7IgG antibodies

Finally, the number of both degranulated and non-degranulated mast cells was increased in the dermis of mice injected with antiCOL7 IgG in comparison to NRIgG-injected control mice (**Fig.24** a,c). Though, due to large inter-individual and inter-section differences in the number of histochemically detectable mast cells, this difference was much higher only for degranulated mast cells, their overall increase and enhanced activity (=degranulation) suggests the induction of a vigorous response of perifollicular mast cells to the deposition of pathogenic antibody.

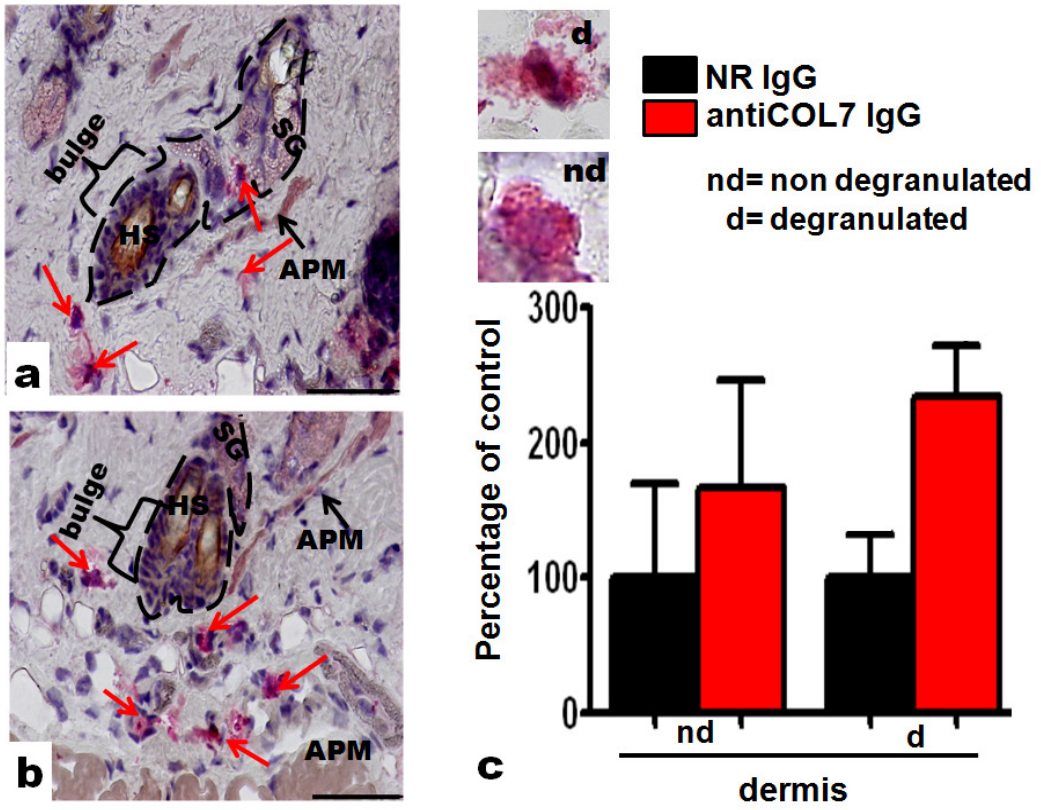

Fig.24: Increase in degranulated mast cells in the dermis of mice injected with anti type VII collagen. *Leader esterase staining shows mast cells (red arrows) surrounding the bulge region of mice injected with NR IgG (red arrows) (a). Mast cells (orange-pink) surrounding the bulge of mice injected with anti type VII cologne IgG (red arrows) (b). Quantitative analysis reveals an increase in the number of mast cells in the dermis surrounding the bulge in anti COL7 IgG injected mice in comparison to control group (c).leader esterase staining, orange-pink, counterstaining using haematoxylin. N =44-46 HF's, derived from 2-3 mice/group from two experiment. mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland,nd = non-degranulated mast cells (< 5 extracellular mast cell granules), d= degranulated mast cell (=5 or more extracellular mast cells) , scale bars=100µm.*

Taken together, these data suggest an important role of inflammatory cell infiltrates, such as MHC II+ cells, CD4+ cells and mast cells, in the initial process of basement membrane damage and bulge IP collapse after the injection of pathogenic anti collagen VII antibodies.

3.5 Injecting antiCOL7IgG antibodies into mice leads to change in expression of cytokines in the follicular bulge

3.5.1 IL15 IR increases in the bulge of mice injected with anti COL7IgG antibodies

This pathogenic antibody-induced increase in the perifollicular inflammatory cell infiltrates most likely driven by changes in the release of immunocyte-attracting secreted factors, such as cytokines, chemokines and growth factors. From the great number of candidates to be considered in this respect, we opted to further examine at least two, i.e. IL-15 and TGFß1, by quantitative immunohistomorphometry.

IL15 was selected, because it is not only a potent pro-inflammatory signal that activates many immunocyte populations, including mast cells, T cells, and macrophages (Rausch et al. 2006, Rückert et al. 2009, Di Sabatoni et al. 2011, Steel et al 2012, Berzofsky et al.2012) and exerts complex regulatory effects on murine mast cells (Orinska et al. 2007), but also operates as a major modulator of apoptosis suppressor, namely of T cells and mast cells (Bulfone-Paus et al. 1997, Berard et al. 2003, Di Sabatoni et al. 2011, Steel et al 2012). TGFß1 was

chosen since it is a key immunoinhibitory growth factor, namely in immune privileged tissues (Wahl et al. 2006, Niederkorn 2002), and is a main growth factor that drives tissue fibrosis and other scarring processes (Penn et al.2012).

These analyses showed a slight, but a considerable increase in the IL15 immunoreactivity in and around the telogen bulge region of antiCOL7 IgG-injected mice compared to IgG controls (**Fig.25** a-c)

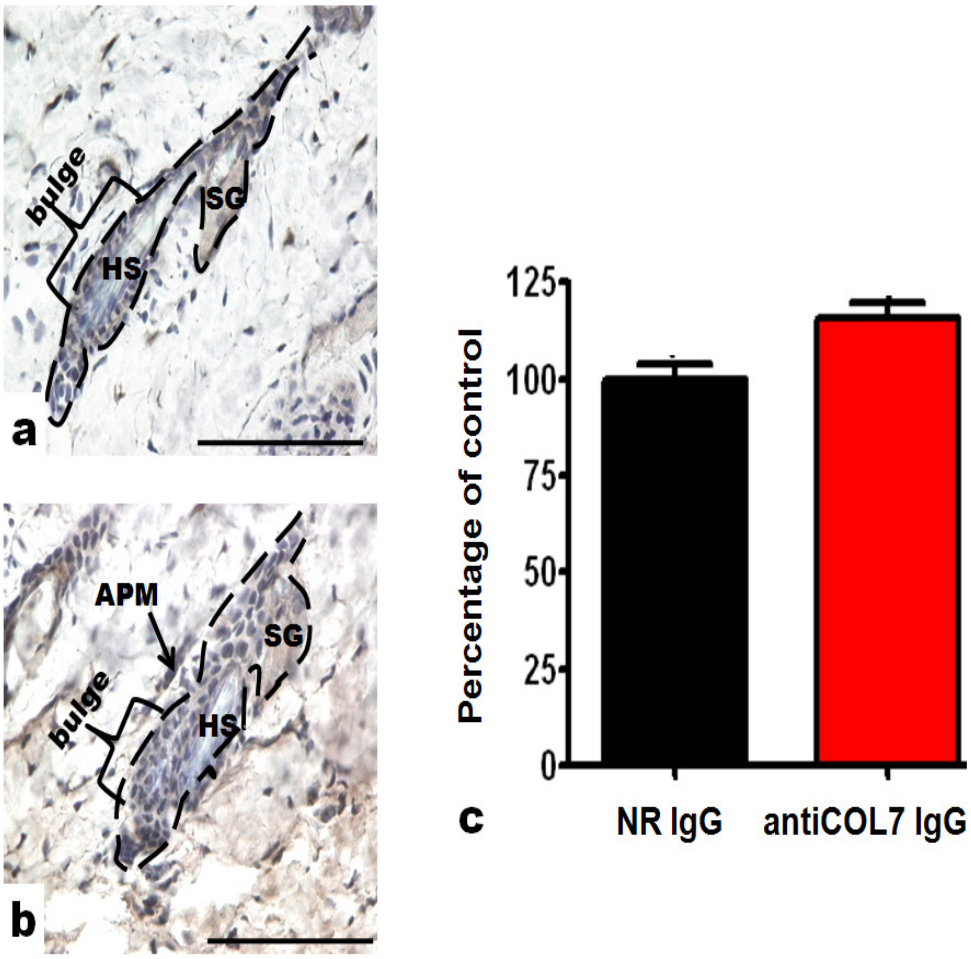

Fig.25:.IL15 immunoreactivity increases in the bulge of mice injected with anti type VII collagen antibody. *IL 15 immunoreactivity (brown) in the hair follicle bulge region of mice injected with NR IgG (a).IL 15 immunoreactivity (brown) in the bulge of mice injected with anti type VII collagen IgG (b). Quantitative analysis reveals partial increase in the levels of IL 15 immunoreactivity at the level bulge in anti COL7 IgG injected mice in comparison to control group (c).ABC - peroxidase method, DAB, brown, counterstaining*

using haematoxylin. N =22-34 HFs/group, derived from 2-3 mice/group from two individual experiments mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale Bars=100µm.

3.5.2 TGFβ-1 IR increases in the bulge of mice injected with anti COL7 IgG antibodies

In many telogen HFs, TGFβ-1 IR in the bulge epithelium of pathogenic antibody-injected was strikingly up-regulated compared to controls (see **Fig.26** a, b), when systematically evaluated by quantitative immunohistomorphometry (**Fig. 26**c). .

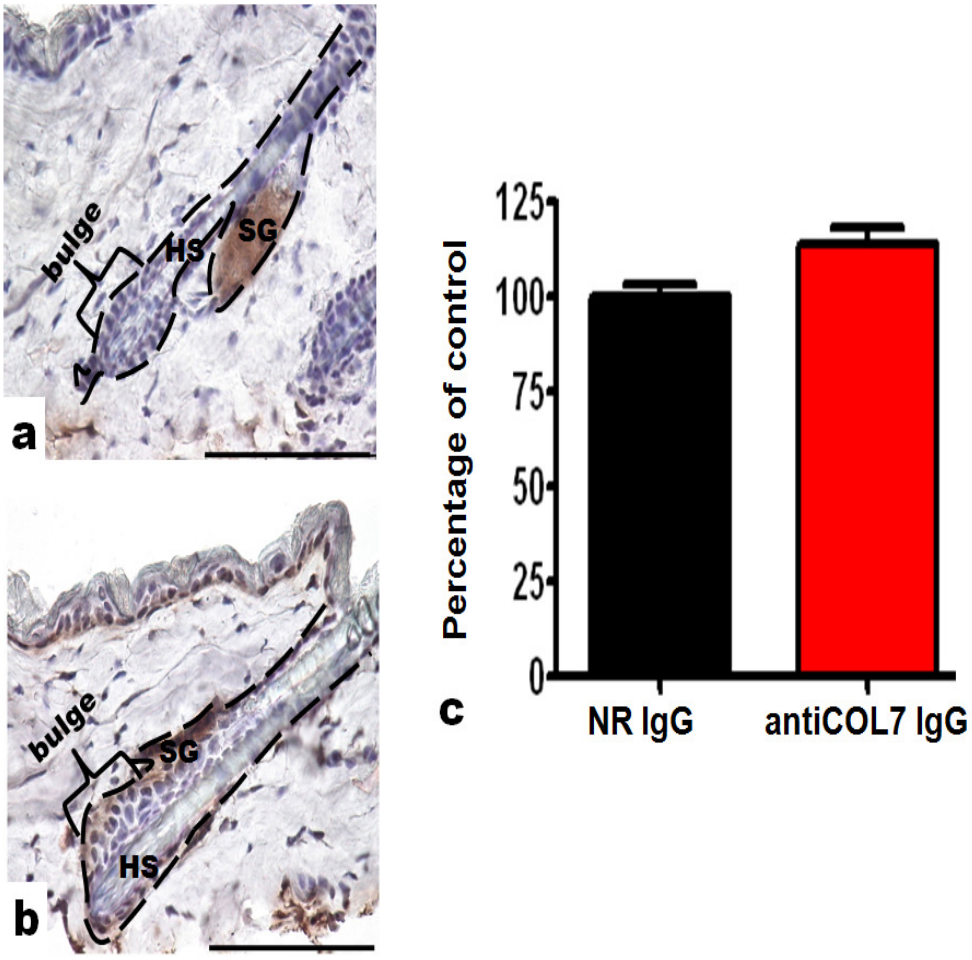

Fig.26: TGFβ1 immunoreactivity increases in the bulge epithelium of mice injected with anti type VII collagen antibody. *TGFβ1 immunoreactivity (brown) in the bulge region of mice injected with NR IgG (a). TGFβ1immune reactivity (brown) in the hair follicle bulge of mice injected with anti type VII collagen IgG (b). Quantitative analysis reveals a partial increase in the levels of TGFβ1 immunoreactivity at the level bulge in anti COL7 IgG injected mice in comparison to control group (c).ABC - peroxidase method, DAB, brown, counterstaining using haematoxylin.* Some nonspecific staining is seen in the sebaceous glands

due to the binding of secondary antibody to the secretions loaded in the sebaceous glands.*N =26-30 HF's, derived from 2-3 mice/group from two individual experiments, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100µm.*

These results suggest that the massive deposition of anti-collagen VII antibody at the follicular BM induces of complex responses in the HF epithelium and the perifollicular mesenchyme: on the one hand protein expression *in loco* of the pro-inflammatory and mainly anti-apoptotic cytokine, IL-15, is increased; on the other, the potent immunoinhibitory and fibrosis-promoting growth factor,TGFß1, is also up-regulated.

3.6 Injection of antiCOL7 IgG antibodies leads to damage of stem cells in the follicular bulge

3.6.1. CD34 IR shows subtle changes in the bulge of mice injected with antiCOL7IgG antibodies

Finally,it was examined by CD34 quantitative immunohistomorphometry to which extent the bulge is damaged in the passive murine EBA model. After extensive attempts to detect murine bulge epithelial stem cells with a the one standard marker, e.g. keratin 15 (Morris et al. 2004, Ito et al. 2005), had failed (data not shown), CD34 was selected because it is a very sensitive alternative marker of murine bulge epithelial stem cells (Trempus et al. 2003, Trempus et al.2007, Cotsarelis 2006, Nagao et al. 2012, Singh & Morris 2012a).

This demonstrated, only in some test mice, antiCOL7IgG injection led to a mild decrease in the staining intensity of the bulge epithelium for CD34; overall CD34 IR in the bulge was essentially indistinguishable between test and controls HFs (**Fig.27** a-c).

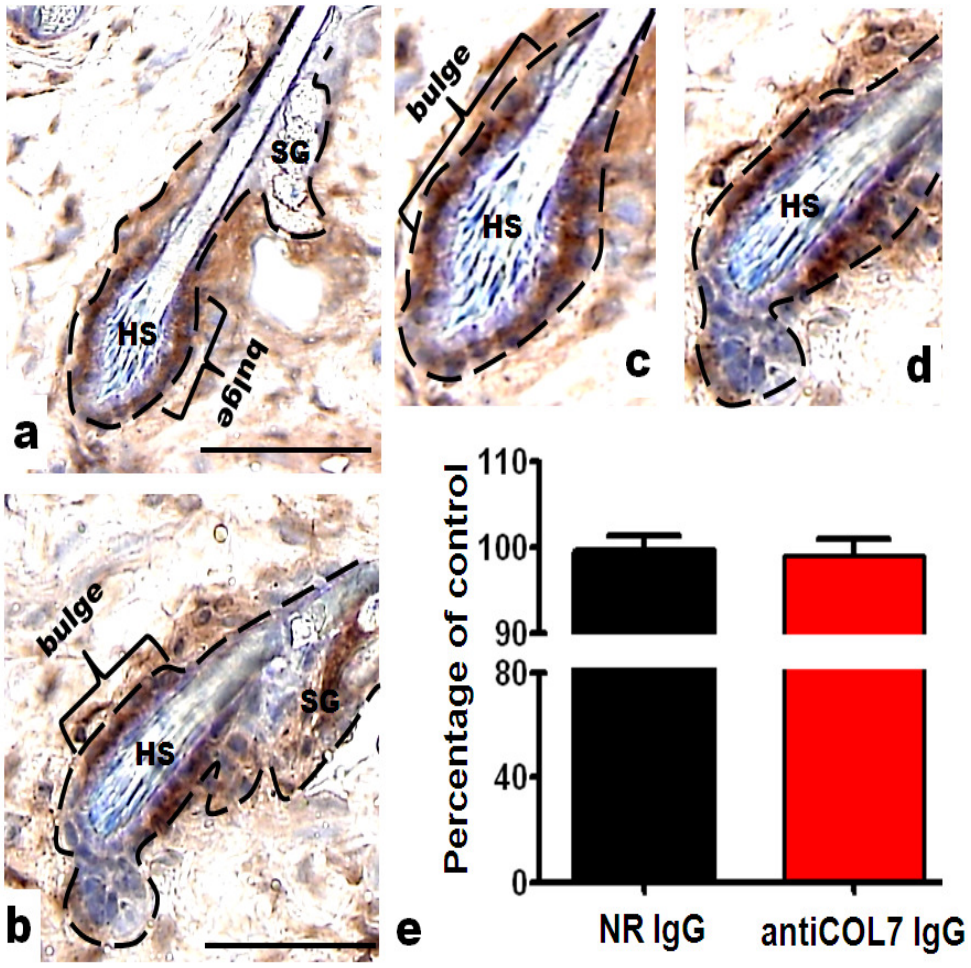

Fig.27: CD34 immunoreactivity showed slight change in the bulge of mice injected with anti type VII collagen antibody. *CD34 immunoreactivity (brown) in the bulge region of mice injected with NR IgG (a). CD34 immunoreactivity (brown) in the hair follicle bulge of mice injected with anti type VII collagen IgG (b). Quantitative analysis reveals no change in the levels of CD34 immunoreactivity at the level bulge in anti COL7 IgG injected mice in comparison to control group (c).ABC -peroxidase method, DAB, brown, counterstaining using haematoxylin. Higher magnification of CD34 staining intensity in the follicular bulge of both NRIgG (c) and antiCOL7 IgG (d) injected mice N = 32-46 HF's, derived from 2-3 mice/group from one individual experiments, mean +/-SEM APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale Bars=100 µm*

3.6.2 CD34+ cells are decreased in the bulge of mice injected with anti COL7IgG antibodies

To get more specific detail on whether the bulge cells are being destroyed, the individual number of CD34+ cells was also counted in the follicular bulge of each follicle in both control and test mice. This showed a more pronounced (**Fig.28**.a-

c) (though nonsignificant) decrease in follicular bulge in comparison to the very slightest decrease seen in CD34 staining intensity in antiCOL7 IgG injected mice. (**Fig.27** a-c). These trends let one suspect that counting of additional HFs and sections would reveal a decrease the number of CD34+ cells in the bulge.

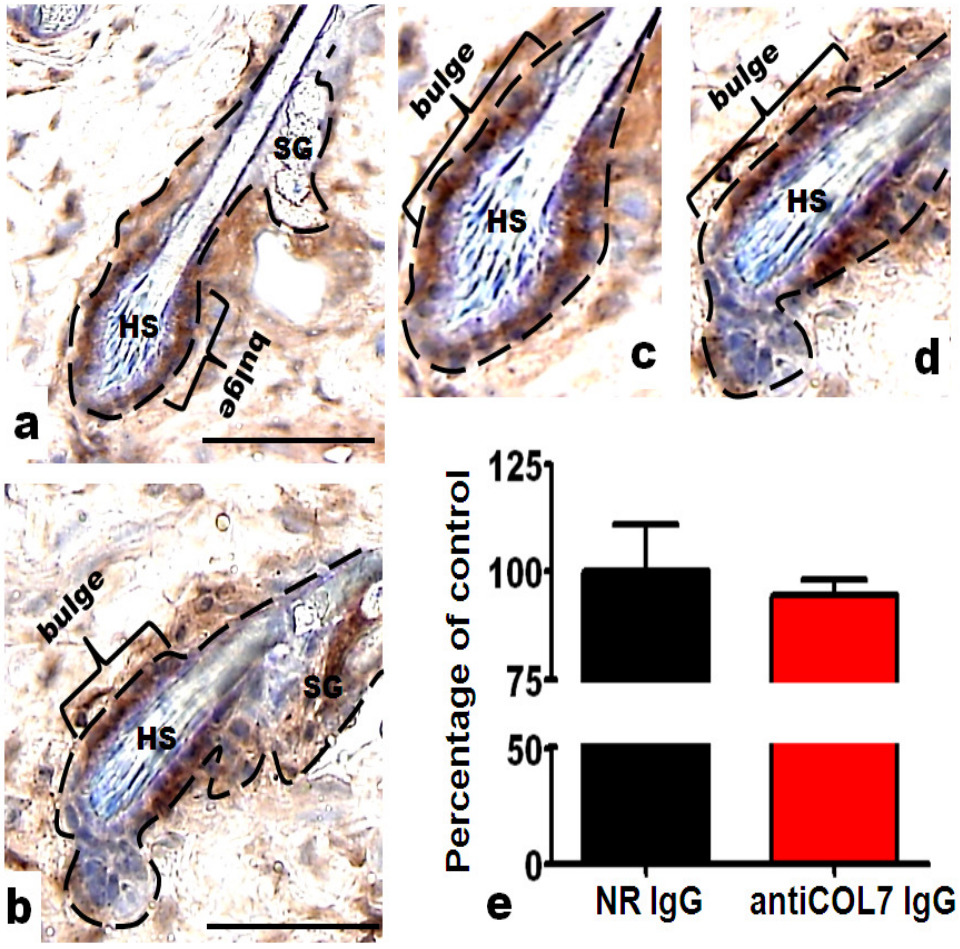

Fig.28: decrease in number of CD34+ cells in the follicular bulge mice injected with anti type VII collagen antibody. *CD34+ cells (brown) stained in the bulge region of mice injected with NR IgG (a). CD34+ cells stained (brown) in the hair follicle bulge of mice injected with anti type VII collagen IgG (b). Higer magnification of CD34+ cells in the follicular bulge of NRIgG (c) and antiCOL7 IgG (d) injected mice. Quantitative analysis reveals an overall decrease in the no of cells expressing CD34 at the level bulge in anti COL7 IgG injected mice in comparison to control group (e).ABC -peroxidase method, DAB, brown, counterstaining using haematoxylin. Higher magnification of CD34 staining intensity in the follicular bulge of both NRIgG (c) and antiCOL7 IgG (d) injected mice N = 27-51 HF's, derived from 2-3 mice/group from one individual experiments, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale Bars=100 µm*

However, these data show that epithelial HF stem cells in the bulge largely survived the massive deposition of pathogenic antibodies in the follicular BM and the subsequent inflammatory cell attack in early lesional HFs (i.e. in skin areas where no overt alopecia and skin damage was as yet macroscopically visible). This unexpected finding nicely explains why the initial macroscopic hair loss seen in passive model EBA mice (**Fig.16**.a) often was reversible within 2-3 months (**Fig.16** b) and underscores the concept that EBA-associated alopecia, at least in this murine EBA model, does not represent a genuine scarring alopecia.

3.7 Injecting GST-mCOL7C+Titermax® into SJL mice leads to blisters with scarring alopecia

Since CA is seen in active EBA mice, i.e. after injecting recombinant COL7, various immunopathological mechanisms were evaluated in the bulge region of telogen follicles.The rationale of these experiments was to determine whether

a) Basement membrane destruction occurs in follicular bulge region of GST-mCOL7C injected mice as a result of surrounding inflammation.

b) IP collapse occurs in the follicular bulge of mice injected with GST-mCOL7C

c) Changes in the protein expression of key cytokines and various immune cells can be detected in the GST-mCOL7C injected mice in the bulge that lead to destruction of the follicle.

3.7.1 Hair follicle damage occurs in GST-mCOL7C+Titermax®injected mice (active EBA model)

As expected, mice injected with GST-mCOL7C and Titermax® showed blisters with hair loos mainly on the snout and flanks, moreover deposition of pathogenic IgG was seen along the basement membrane (**Fig.28** a,b). Unlike in lesional skin, no progressive HF dystrophy was seen in the non-lesional skin of both control and diseased mice (**Fig.29** c-e)

3.7.2 The basement membrane is destroyed in GST mCOL7C+Titermax® injected mice

Therefore, PAS staining was employed to determine the extent of BM damage in non-lesional back skin of active EBA model mice. PAS staining showed visibly decreased mucoplysaccharide levels in the non-lesional back skin of mice injected with GST-mCOL7C and Titermax® in comparison to Titermax® injected control group (**Fig.28** i), suggesting BM damage. However, no visible separation of the follicular ORS from the BM was as seen. This indicates that the follicular BM is slightly more resistant to damage than the epidermis in the active mouse model of EBA. (Biber et al.2010,Kasperkiewicz et al.2012,Samavedam et al.2012).

Further analysis of non-lesional back skin by routine histology revealed the presence of infiltrates and the presence incipient blisters (**Fig.29**.f,g). Despite the previous evidence that neutrophils play a key role in the disease process (Chiriac et al. 2007,Ludwig and Zillikins 2011), non-lesional back skin,surprisingly, showed no or very few neutrophils by histology(data not shown).

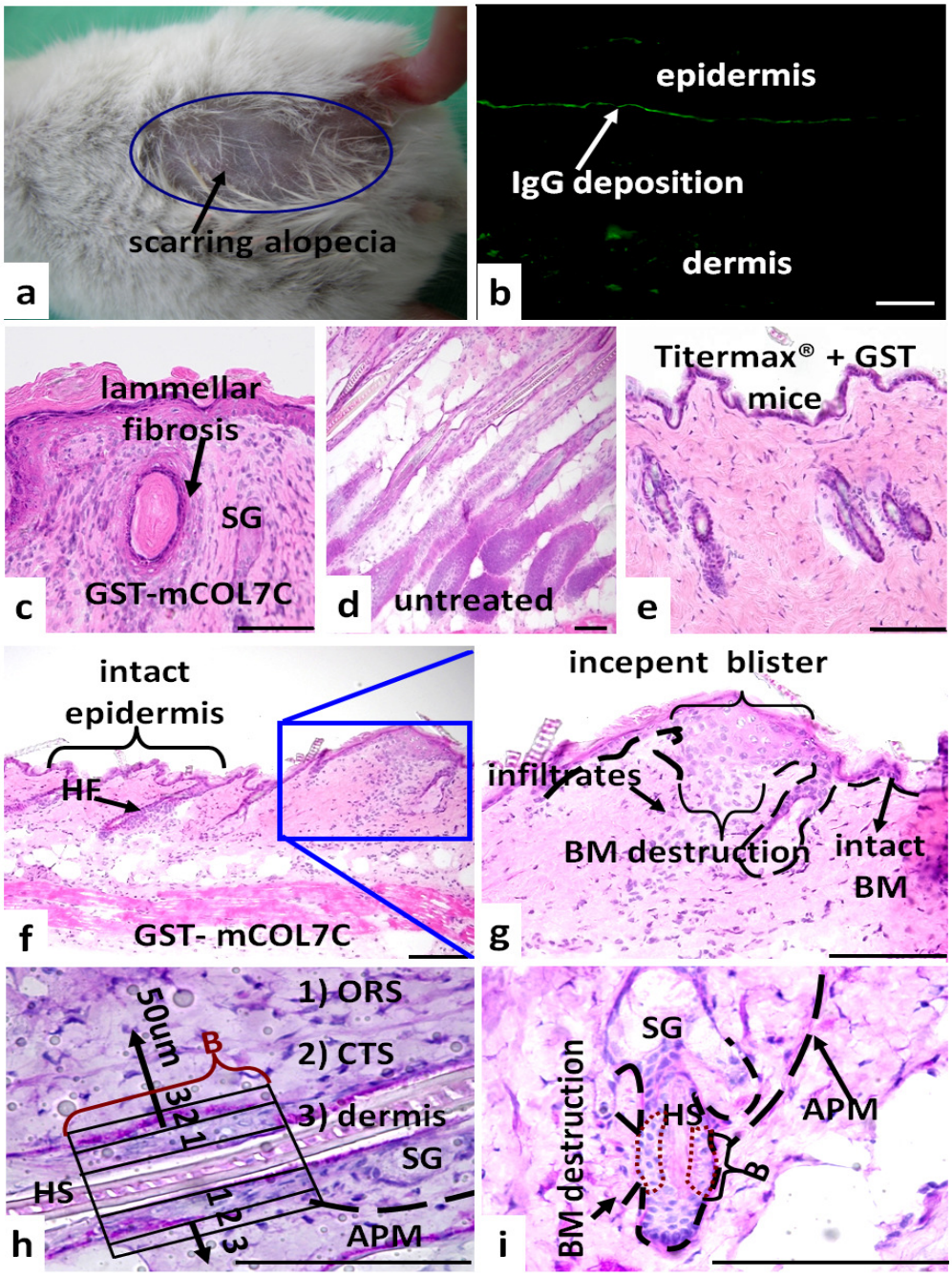

Fig.29: Disease phenotype and scarring alopecia seen in GST-mCOL 7 C + Titermax® injected mice. *Scarring alopecia (blue circle) seen in SJL mice injected with recombinant type VII collagen (a).Indirect immunofluorescent staining depicting the deposition of anti type VII along the basement membrane. FITC labeled antibodies are shown in green (b). Lamellar fibrosis seen in the back skin of mice injected with type VII collagen (d), in comparison with normal (e) and GST+Titermax® (f) injected mice. Hair follicle destruction in the back skin mice injected with recombinant type VII collagen (g, h). Diagram depicting*

compartmental areas where individual cells were counted around the bulge (i). PAS staining depicting destruction of basement membrane (dotted line) in mice treated with along with the bulge compartment (red line) in which quantitative immune morphometry was done using NIH image J. Scale bars =100 um

3.8 HF bulge IP is compromised in mice injected with mCOL7C and Titermax®

Next, it was examined, whether the bulge IP is also compromised in the active EBA mouse model.The bulge of humans and mice showed greatly decreased expression of MHCI (Paus et al. 1994c,Ito et al. 2004, Meyer et al. 2008). Quantittaive immunohistomorphoemtry was performed to check whetherinjection of mCOL7C and Titermax® altered MHC I expression in the bulge. This revealed only a minimal in the level of MHCI expression in the bulge (**Fig.30**a-c).

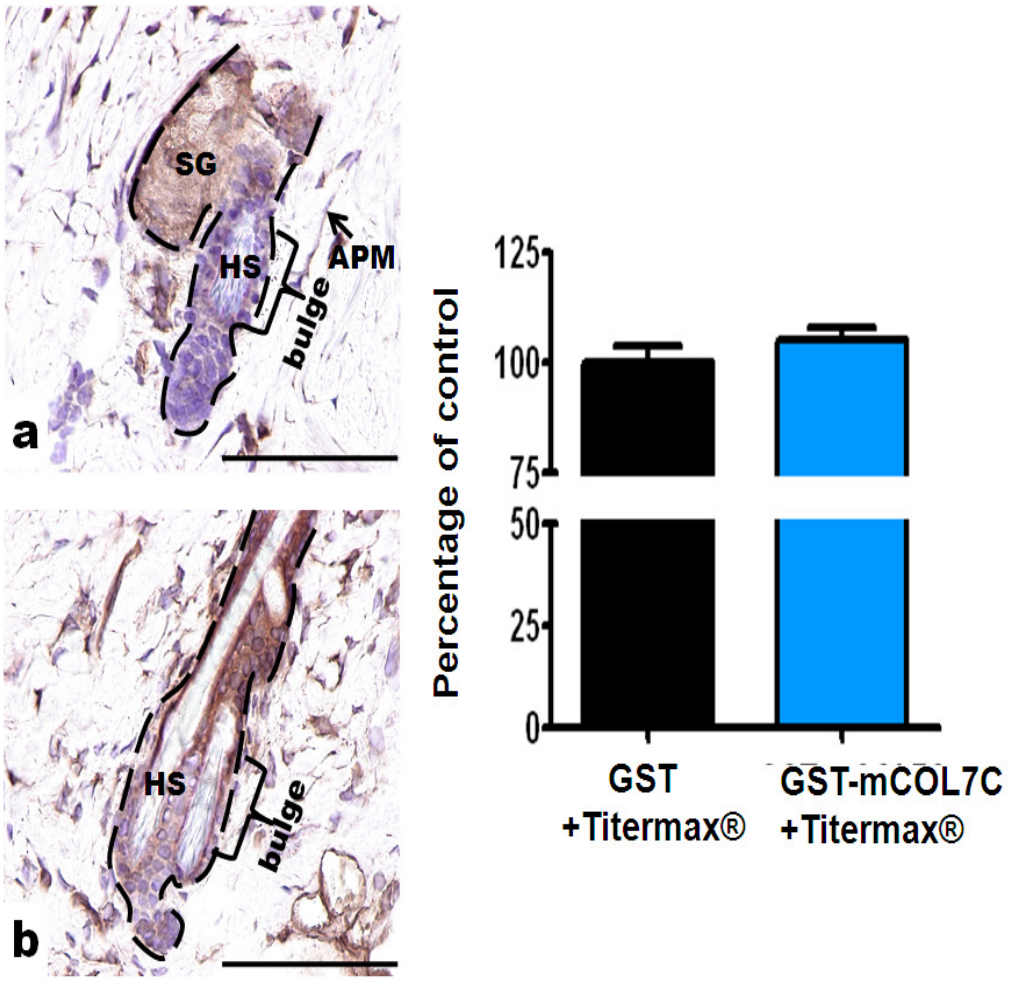

Fig.30: Slight increase in MHC I immunoreactivity in the bulge of mice injected with GST-mCOL7 C + Titermax®.*MHC I immunoreactivity (brown) in the bulge region of mice injected with GST-Titermax (a). MHC I immunoreactivity (brown) in the bulge of mice injected GST-mCOL7 C and Titermax (b) was often increased compared to that of vehicle controls (a). However, quantitative analysis did not reveal increased levels of MHC I immunoreactivity at the level bulge in GST-mCOL7 C and Titermax injected mice in comparison to the Titermax® control group (c).ABC - peroxidase method, DAB, brown, counterstaining using haematoxylin. N=6-9 HF's, derived from 2-3 mice/group from one experiment, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.*

Therefore, the "no danger" signal, CD200, was examined next. This showed a decrease in CD200 protein expression in the bulge compared to the titer max control (**Fig.31**a-c). This indicates that anti-CLO7 autoantibodies and infiltrate they attract in the active EBA mouse model may compromise bulge IP primarily by decreasing CD200 expression, just as this has most recently been seen in lichen planopilaris (Harries et al. 2013).

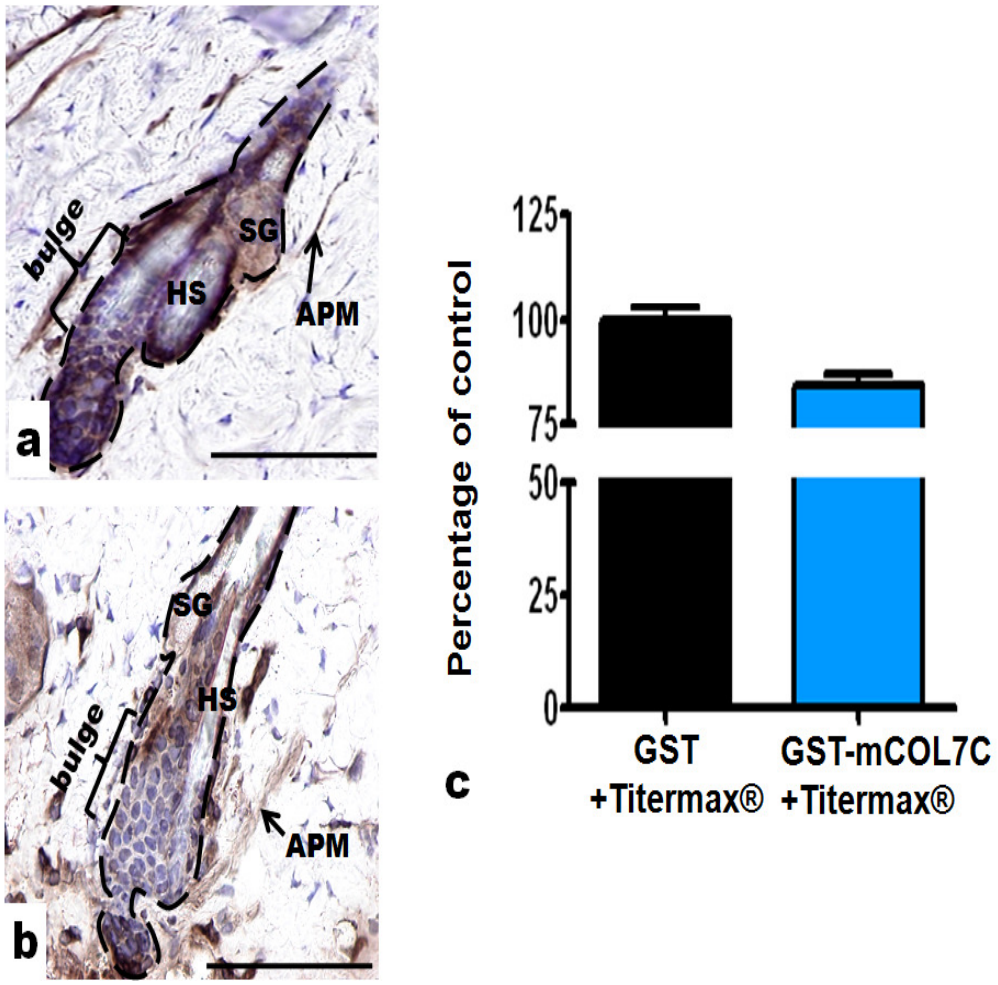

Fig.31: Decline in CD200 immunoreactivity in the bulge of mice injected with GST-m COL7 C + Titermax®. *CD200 immunoreactivity (brown) in the bulge region of mice injected with GST-Titermax (a). CD200 immunoreactivity (brown) in the bulge of mice injected with GST-mCOL7 C and Titermax (b). Quantitative analysis reveals a decline in the levels of CD200 immunoreactivity in the bulge epithelium in GST-mCOL7 C and Titermax injected mice in comparison to control group (c).ABC–peroxidase method, DAB, brown, counterstaining using haematoxylin. N = 5-7 HF's, derived from 2-3 mice/group from one individual experiment, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.*

3.9 Inflammatory Infiltrates are increased in the bulge of GST-mCOL7C and Titermax® injected mice

One major aim of this thesis project was to characterize various inflammatory infiltrates that may have contributed to IP collapse by aiding BM damage.For this.MHCII+,CD4+,CD8+cells and mast cells were immunohistomorphometerically quantified in defined reference areas (**Fig.27** h).

3.9.1 CD8+ T cells are minimally increased around the bulge of GST-mCOL7C+Titermax® injected mice

CD8+ cells were shown to play a key role in inflammatory hair loss disorders such as alopecia areata, CDLE, lichen planopilaris, and graft-versus-host disease (Weedon D 2009,Harries et al. 2009, Harries & Paus 2010, Gilhar et al. 2012, Harries et al. 2013). This revealed a minimal increase in the number of CD8+ cells in the dermis surrounding the bulge of mice injected with mCOL7C+Titermax® in comparison to the control group (**Fig.32**a-c).

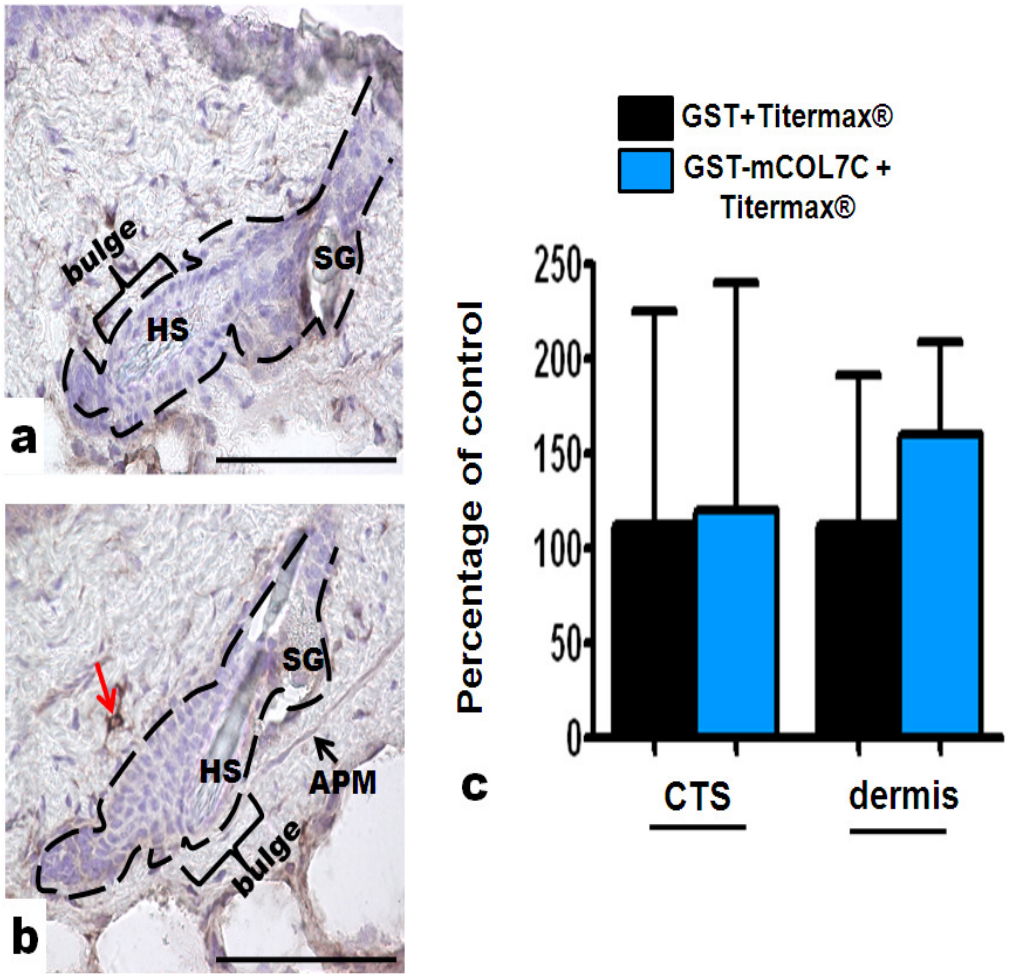

Fig.32: Minimal increase in number of perifollicular CD8 + cells in mice injected with GST-m COL7 C + Titermax®. *Histochemical staining shows very few numbers of perifollicular CD8+t cells in mice injected with GST + Titermax® (a), while increased number isolated perifollicular CD8+t cells (red arrow) are seen mice injected with GST-m COL7 C + Titermax® (red arrows) (b). Quantitative analysis reveals an increase in the number of CD8+ t cells in the dermis in mice injected with GST-m COL7 C + Titermax® in comparison to control group (c). ABC - peroxidase method, DAB, brown, counterstaining using haematoxylin.N=7-12 HF's, derived from 2-3 mice/group from one individual experiment,mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.*

3.9.2 CD4+ cells are increased in the bulge of GST-mCOL7C+Titermax® injected mice

Next, the number of CD4+ T cells was immunohistomorphometricaly quantified. This showed an increased number of CD4+ cells in all the measured compartments of ORS, CTS and dermis of test versus control HFs (**Fig.33**a-c). These results thus raise the possibility that the test HFs secreted chemokines that attract CD4+ cells into their vicinity in response to the deposition of antibodies after injection mCOL7C+ Titermax®.

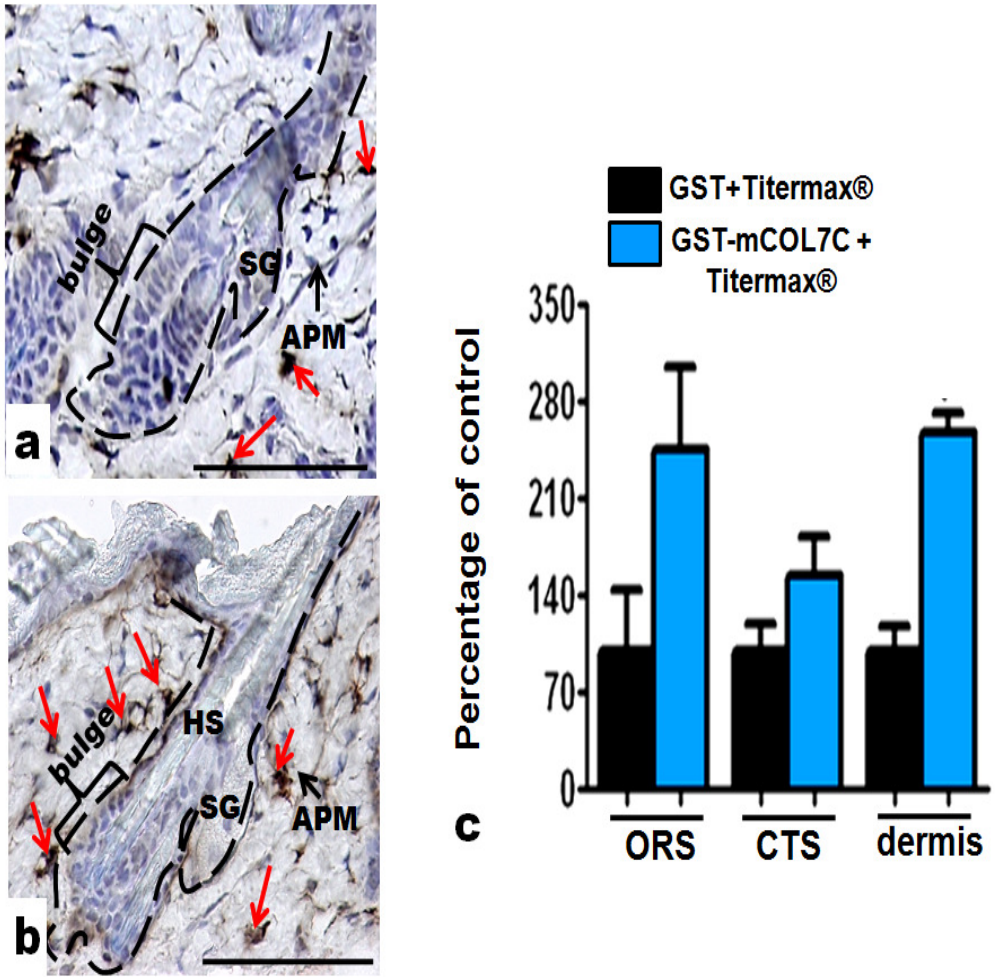

Fig.33: Increase in perifollicular CD4+t cells in mice injected with GST-m COL7 C + Titermax®. *Intradermal CD4 positive cells in the CTS surrounding the bulge region of mice injected with GST + Titermax (red arrows) (a). CD4 positive cells surrounding immunoreactivity (brown) in the hair follicle bulge of mice injected with GST-m COL7 C + Titermax® (red arrows) (b). Quantitative analysis reveals an increase in the number of CD4+ cells in the bulge ORS, CTS and dermis of GST-m COL7 C + Titermax® injected mice in*

comparison to control group. (c). ABC-peroxidase method, DAB, brown, counterstaining using haematoxylin N = 20 HFs/group, derived from 2-4 mice/group from one experimentmean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.

3.9.3 CD54+ cells are increased in the bulge of GST-mCOL7C+TitermaX® injected mice

Considering the increase in CD4+ cells, the number of CD54+ cells/ICAM-1 positive cells in various compartments such as ORS, CTS and dermis was also assessed (Muller-Rover et al. 2000). Although the number of CD54+ cells in the dermis of test mice was markedly higher than in controls. (**Fig.34**a-c). .

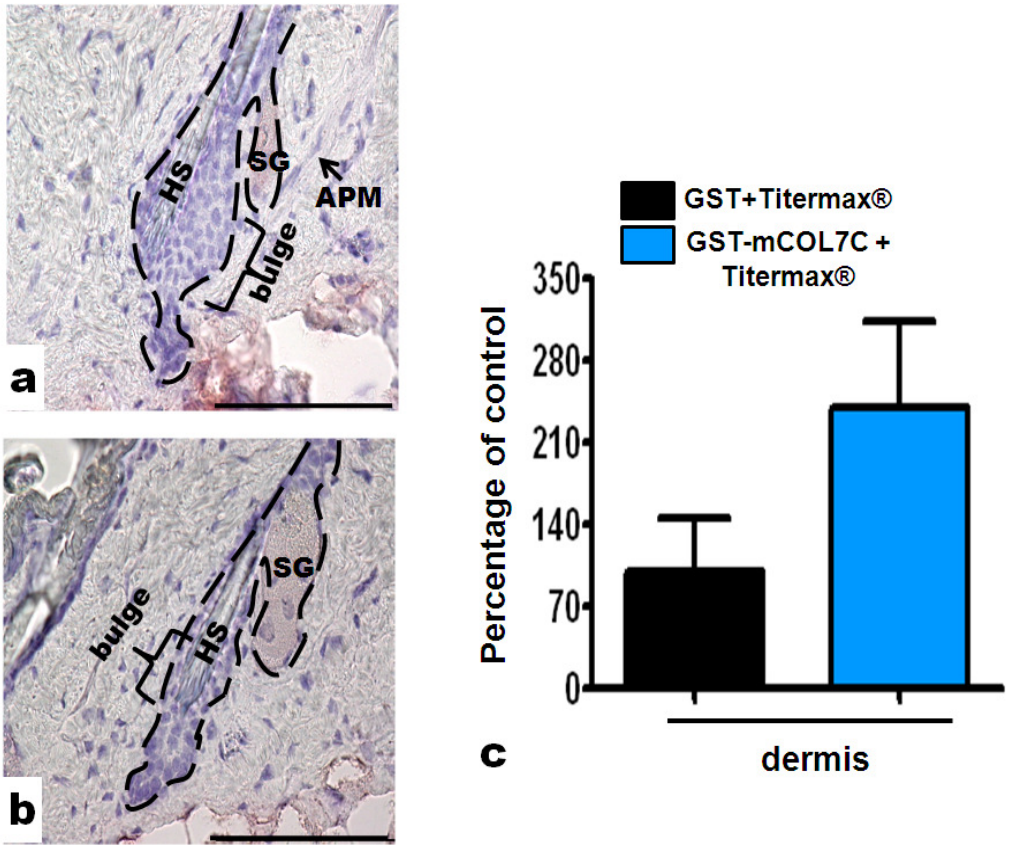

Fig.34: Increase in CD54+ cells around the hair follicle bulge of mice injected with GST-m COL7 C + Titermax®. *CD54 positive cells in the CTS surrounding the hair follicle bulge region of mice injected with GST + Titermax® (a). CD54 positive cells surrounding the hair follicle bulge of mice injected with GST-m COL7 C + Titermax® (b). Quantitative analysis reveals increase in the number of CD4+ cells in the CTS surrounding the bulge in anti COL7 IgG injected mice in comparison to control group (c).ABC - peroxidase method, DAB, brown, counterstaining using haematoxylin. N = 9-15 HF's, derived from 2-3 mice/group*

from one individual experiment, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.

3.9.4 Mast cells are slightly increased in the bulge of GST-mCOL7C+Titermax® injected mice

Considering the role of mast cells in stress-induced hair loss, autoimmunity and skin immunolgy (Moretti et.al 1966, Peters et al.2004, Peters et al. 2007, Metz et al. 2008, Stelekati et al.2009, Brown and Hatfield 2012), the numbers of degranulating and non degranulating mast cells were also measured in the different reference compartments. This showed a slight increase in the levels of both non- degranulated and degranulated mast cells in the peri-bulge dermis of the mCOL7C+Titermax® group (**Fig.35**a-c).

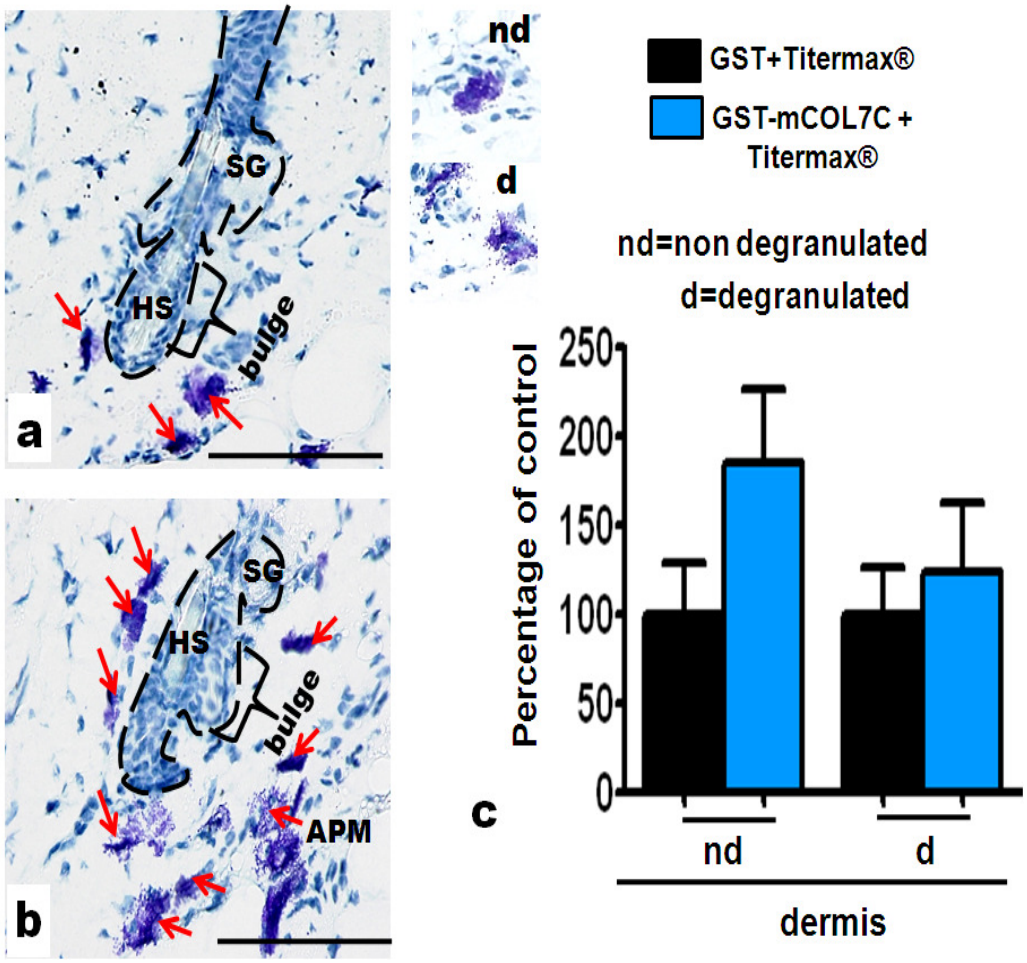

Fig.35: Increase in perifollicular mast cells in mice injected with GST-m COL7 C + Titermax®. *Toluidine blue staining shows perifollicular mast cells (red arrows) of mice injected with GST + Titermax® (red*

arrows) (a). versus mice injected with GST-m COL7 C + Titermax® (red arrows) (b). Quantitative analysis reveals an increase in the number of non-degranulated and degranulated perifollicular mast cells in the dermis in GST-m COL7 C + Titermax® injected mice in comparison to the control (c). ABC- peroxidase method, DAB, brown, counterstaining using haematoxylin.N = 14-21 HF's, derived from 2-3 mice/group from one individual experiment mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.

Taken together these results indicate the creation of a pro-inflammatory milieu by mCOL7C+Titiermax® injection that assembles CD8+ cells, CD4+ cells, and mast cells around telogen HFs and up-regulates ICAM-1 expression. Together this may mitigate the destruction of BM by this infiltrate, thus further exposing the HF stem cells in the bulge directly to inflammatory cells.

3.10 Injecting mCOL7C+Titermax® into mice leads to change in expression of cytokines in the follicular bulge

When, next, selected cytokines were examined, this showed no increase in IL15 immunoreactivity in the bulge region of the ORS of mice injected with mCOL7C+ Titermax® in comparison to the control group (**Fig.36**a-c).

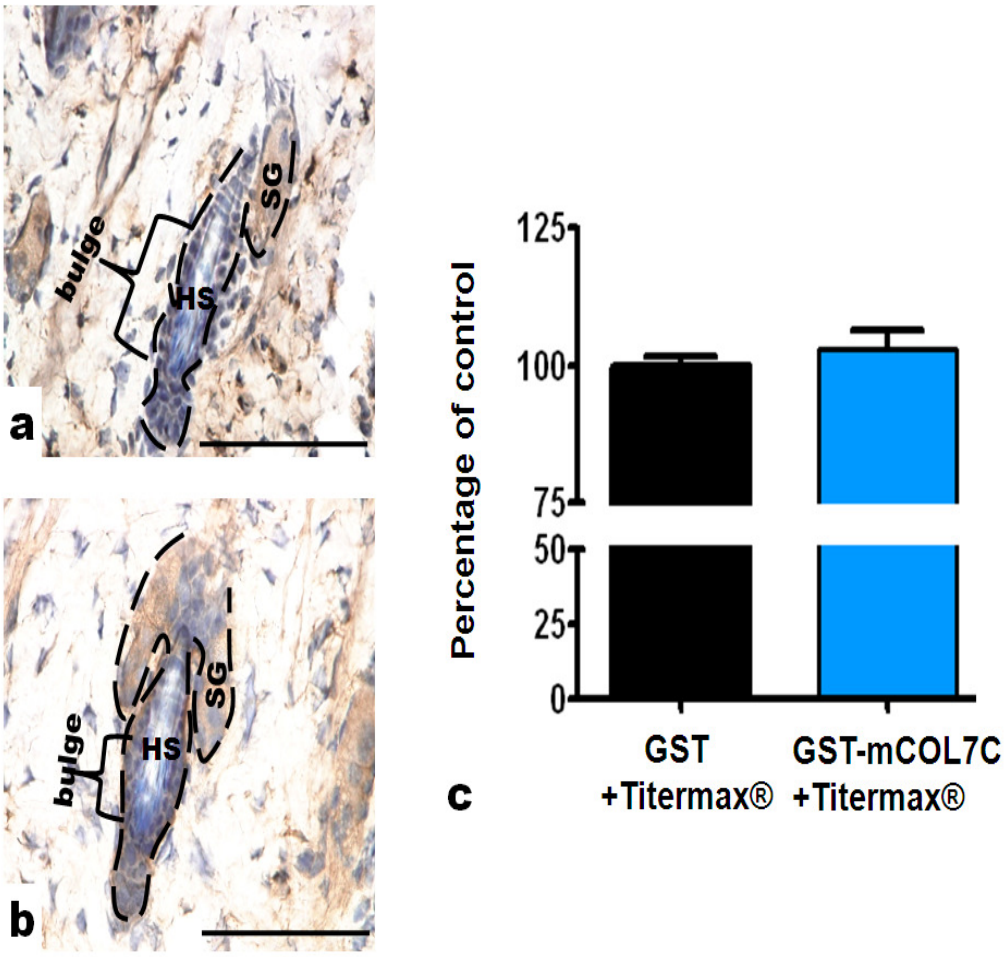

Fig.36: Marginal increase in IL15 IR in the bulge epithelium of mice injected with GST-mCOL7 C + Titermax®. *IL 15 immunoreactivity (brown) in the bulge region of mice injected with GST + Titermax (a) or GST-m COL7 C + Titermax® (b). Quantitative analysis reveals only a marginal increase in IL15 in the bulge in test in comparison to control mice (c).ABC-peroxidase method, DAB, brown, counterstaining using haematoxylin. N = 14-16 HF's derived from 2-3/group mice from one individual experiment, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.*

However, it was found that TGFβ-1 immunoreactivity was slightly decreased in the bulge ORS of mice in mice injected with mCOL7C+Titermax® in comparison to the control group (**Fig.37**a-c).

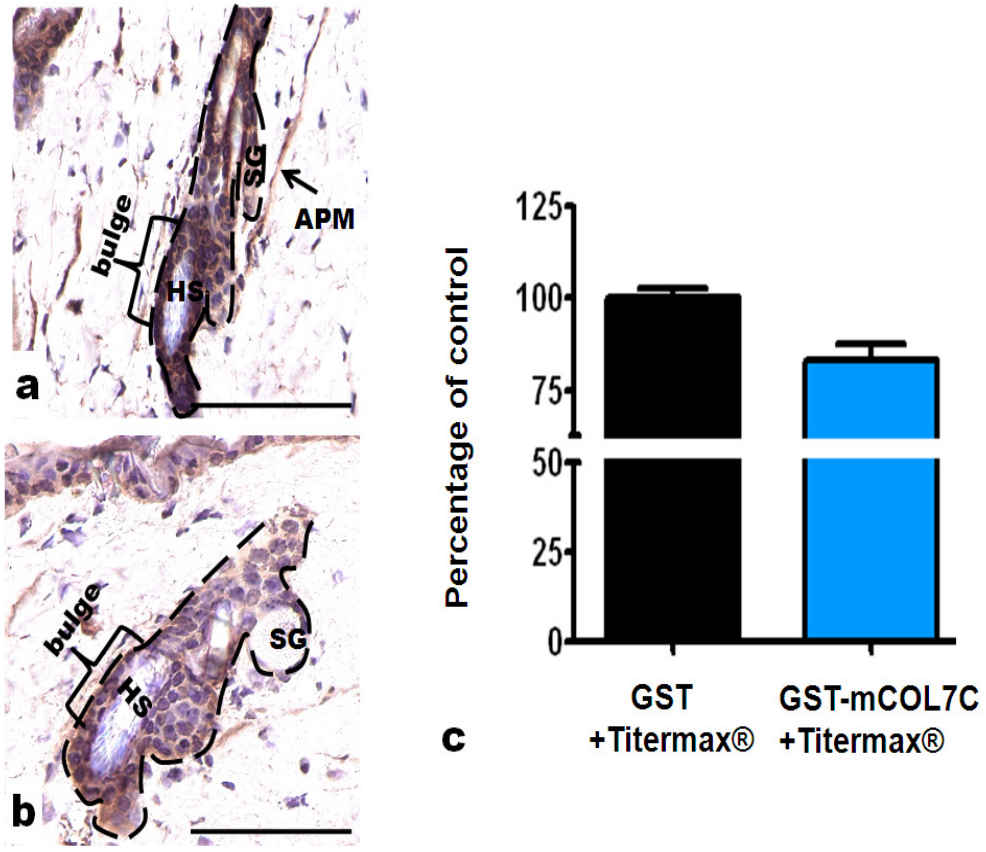

Fig.37: TGFß1 immunoreactivity decreases in the hair follicle bulge of mice injected with GST-m COL7 C + Titermax *TGFß1 immunoreactivity (brown) in the bulge region of mice injected with GST + Titermax® (a) or with GST-m COL7 C + Titermax® (b). Quantitative analysis reveals decrease in the level of bulge TGFß1 IR in test compared to control mice (c).ABC-peroxidase method, DAB, brown, counterstaining using haematoxylin. N = 5-6 HFs derived from 2-3 mice/group from one experiment mean +/-SEM,. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.*

Taken together these results suggest that, at least in these early-stage lesions, IL-15 and TGFß1 do not play a major role, although this would have to be examined by functional studies.

3.11 CD34 increases marginally in the follicular bulge of GST-mCOL7C+Titermax® injected mice

CD34 was chosen as an excellent marker to access the amount of damage to bulge stem cells (Trempus et al 2003, Ohayama et al. 2006, Trempus et al. 2007, Cotsarelis 2006, Singh & Morris 2012a, Nagao et al. 2012b, and Zhou et al.

2012) as our efforts to stain for K15 (Morris et al. 2004, Ito et al. 2005) in mice failed after multiple attempts. Histomorphometric results showed no decrease in the number of CD34+ cells; rather, there was a marginal increase in CD34 IR in the bulge ORS of mice injected with GST-mCOL7C+Titermax® in comparison to control group (**Fig.38**a-c). This suggests that the bulge stem cells in the active model of EBA are more resistant to inflammatory damage than previously thought.

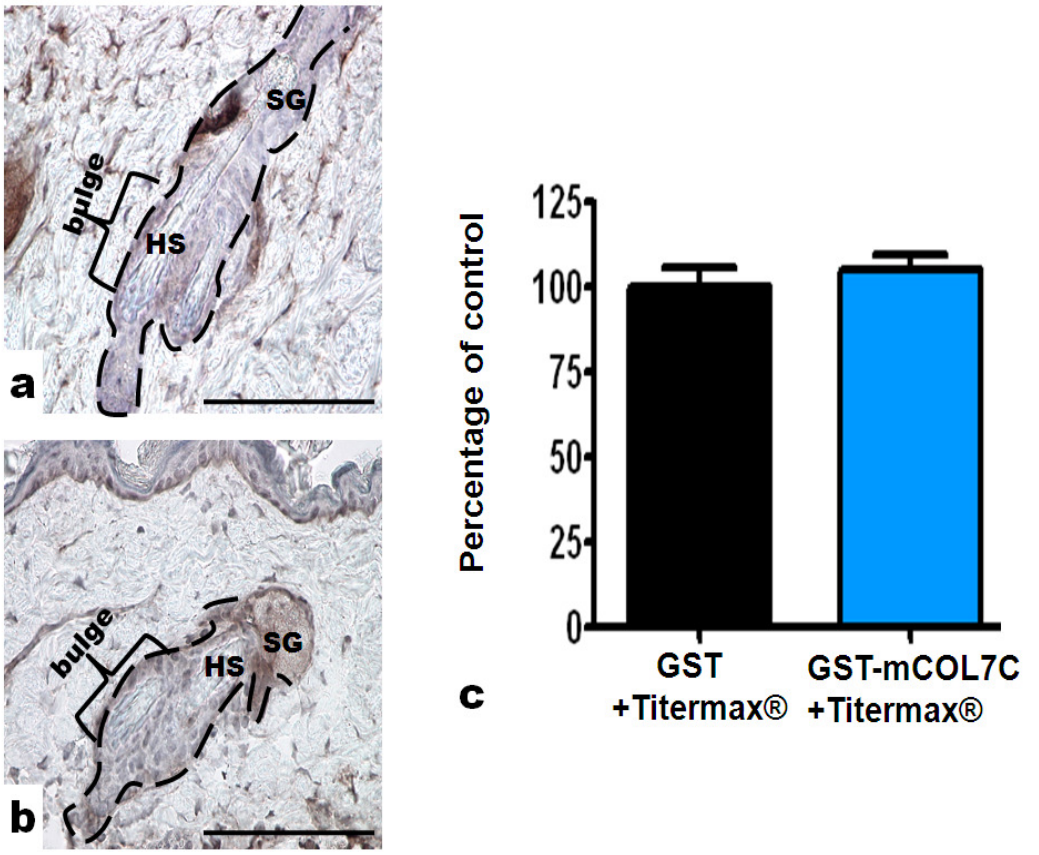

Fig.38: CD34 immunoreactivity showed marginal change in the bulge of mice injected with GST-m COL7 C + Titermax®. *CD34 immunoreactivity (brown) in the bulge region of mice injected with GST + Titermax® (a) or with GST-m COL7 C + Titermax® (b). Quantitative analysis reveals marginal changes between test and control groups (c).ABC - peroxidase method, DAB, brown, counterstaining using haematoxylin. N = 9-15 HFs derived from 2-3 mice/group derived from one experiment, mean +/- SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars = 100 µm.*

3.12 Injecting anti FcγRIV antibodies into mice prevents blister formation and scarring alopecia.

FcγRIV are inhibitory receptors that were recently discovered in mice.RIIIA forms the human ortholog of FcγRIV (Nimmerjahn et al. 2005).The role of anti FcγRIV in modulating the disease outcome was recently documented in the murine model of EBA (Passive) (Kasperkiewicz et al. 2012). In light of this evidence, it was decided to see whether simultaneously injecting anti FcγRIV receptor antibody with anti COL7IgG could prevent bulge IP collapse and affected the perifollicular inflammatory cell infiltrate. For this, non-lesional back skin from anti FcγRIV injected mice was taken and compared with anti COL IgG-injected mice and isotype 9E9 controls. With the idea of using immunoglobulins as a therapeutic tools in EBA in mind, it was asked whether simultaneous injection of anti COL7 IgG and anti FcγRIV prevents scarring alopecia and/or bulge IP collapse and/or impacts on the distribution patterns of various immune cells like MHCII+ cells, CD4+ T-cells and mast cells.

As expected, antiCOL7 IgG injected mice demonstrated deposition of anti cOL7 IgG along the basement membrane (**Fig.39**c), increased blistering, crusts and hair loss along the flanks, and HE stains showed dermal epidermal separation with HF destruction (**Fig.39**a,e). Instead, injection of anti FcγRIV in Balb/c mice along with anti COL7 IgG protected mice from blistering and hair loss (**Fig.39**b,f). Further examination by HE staining revealed no blistering and a reduced number of inflammatory infiltrates present in the dermis and CTS surrounding the hair follicle (**Fig.39**a,e).

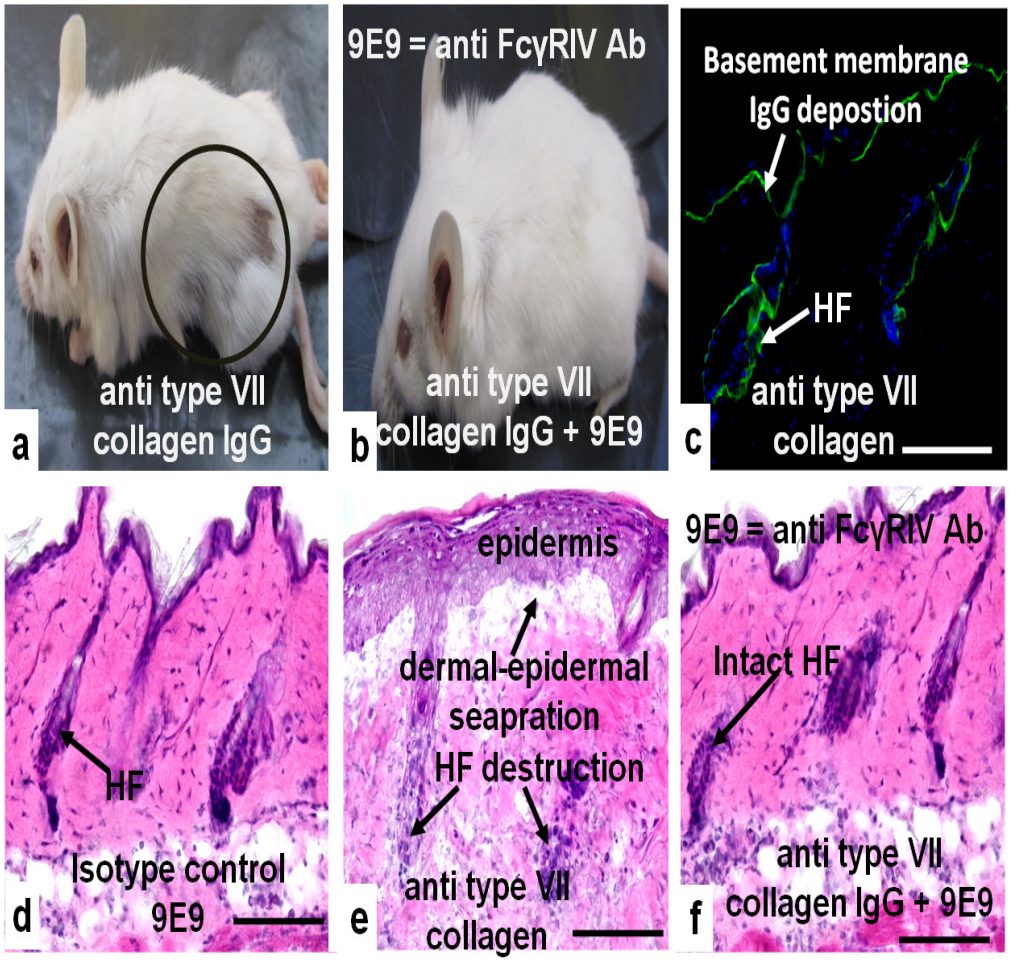

Fig.39: Disease phenotype and scarring alopecia seen in anti type VII collagen antibodies injected mice in comparison to that of mice injected with anti type VII collagen and anti FcγRIV antibodies *Lesion of scarring alopecia (black circle) seen in mice injected with anti type VII collagen (a). Absence of pathological lesion in mice injected with anti type VII collagen and anti FcγRIV antibody (b) Indirect immunofluorescent staining depicting the deposition of anti type VII throughout the basement membrane-FITC labeled antibodies in green (c). Histology depicting intact skin in mice in isotype control 9E9 mice(d).Dermal-epidermal separation seen in the skin of mice injected with anti type VII collagen(e).Intact skin seen in mice injected with both anti type VII collagen and anti FcγRIV antibodies. Scale bars=100 µM*

3.13 Injecting anti FcγRIV antibodies into mice leads to restoration of bulge immune privilege

Since it had previously been shown that normal telogen HFs enjoy relative, MHCI and CD200-dependent IP (see **Figs.40**a-d,**Fig.41**a-d), it was asked whether simultaneous injection of protective anti FcγRIV antibodies with pathogenic anti

COL7 IgG prevented HF IP collapse. The results indicated that injection of FcγRIV in fact prevented bulge IP collapse by decreasing the ectopic expression of MHCI (**Fig.40** a-d).

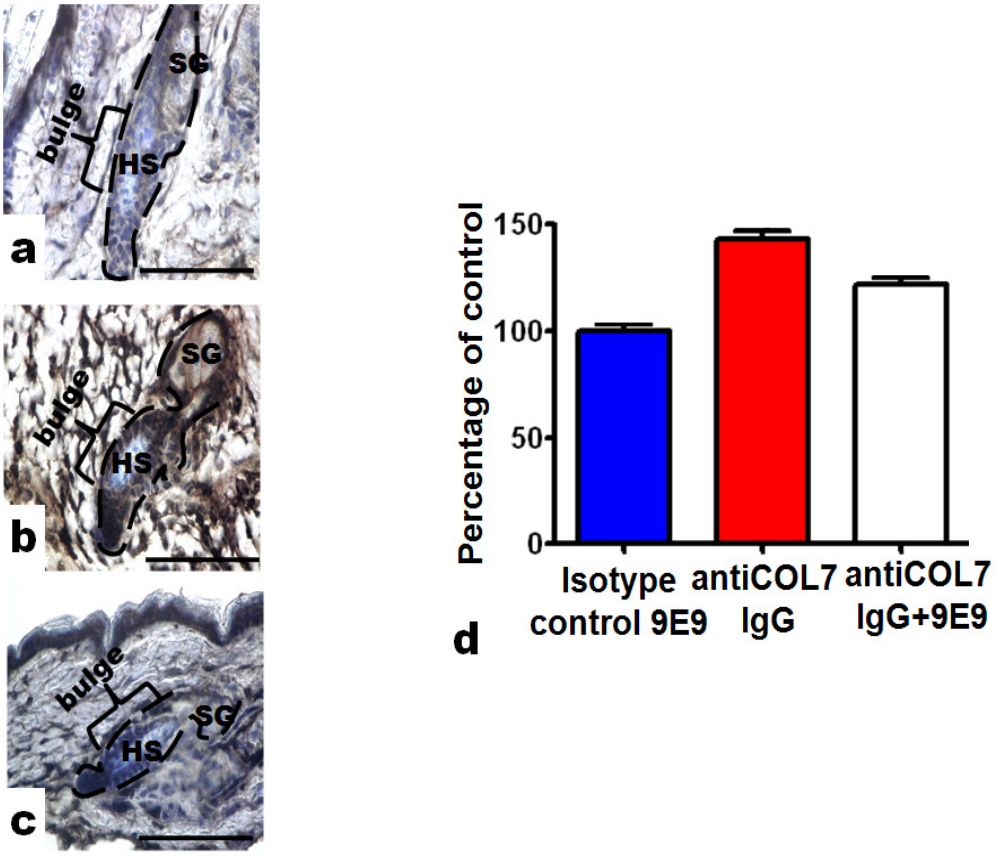

Fig.40: Simultaneous injection of anti type VII collagen and anti FcγRIV antibodies (9E9) decreases the ectopic MHC I expression in the bulge.*MHC I immunoreactivity (brown) in the bulge region of isotype control 9E9 mice (a). MHC I immunoreactivity (brown) in the hair follicle bulge of mice injected with anti type VII collagen IgG (b). MHC I immunoreactivity (brown) in the hair follicle bulge of mice injected with anti type VII collagen IgG and 9E9(c).Quantitative analysis reveals decreased levels of MHC I immunoreactivity at the level bulge in anti COL7 IgG +9E9 injected mice in comparison to isotype control 9E9 and anti type VII collagen IgG (d).ABC-peroxidase method, DAB, brown, counterstaining using haematoxylin. N=9-14 HF's, derived from 2-3 mice/group derived from one experiment, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.*

Moreover the levels of CD200 expression also increased in anti FcγRIV injected mice in comparison to mice that had only received anti COL7 IgG (**Fig.41**a-d).

These findings suggest that injection of anti FcγRIV antibodies can prevent anti-COL7 IgG-induced bulge IP collapse by restoring levels of both CD200 expression and reducing the level of MHCI expression in the bulge.

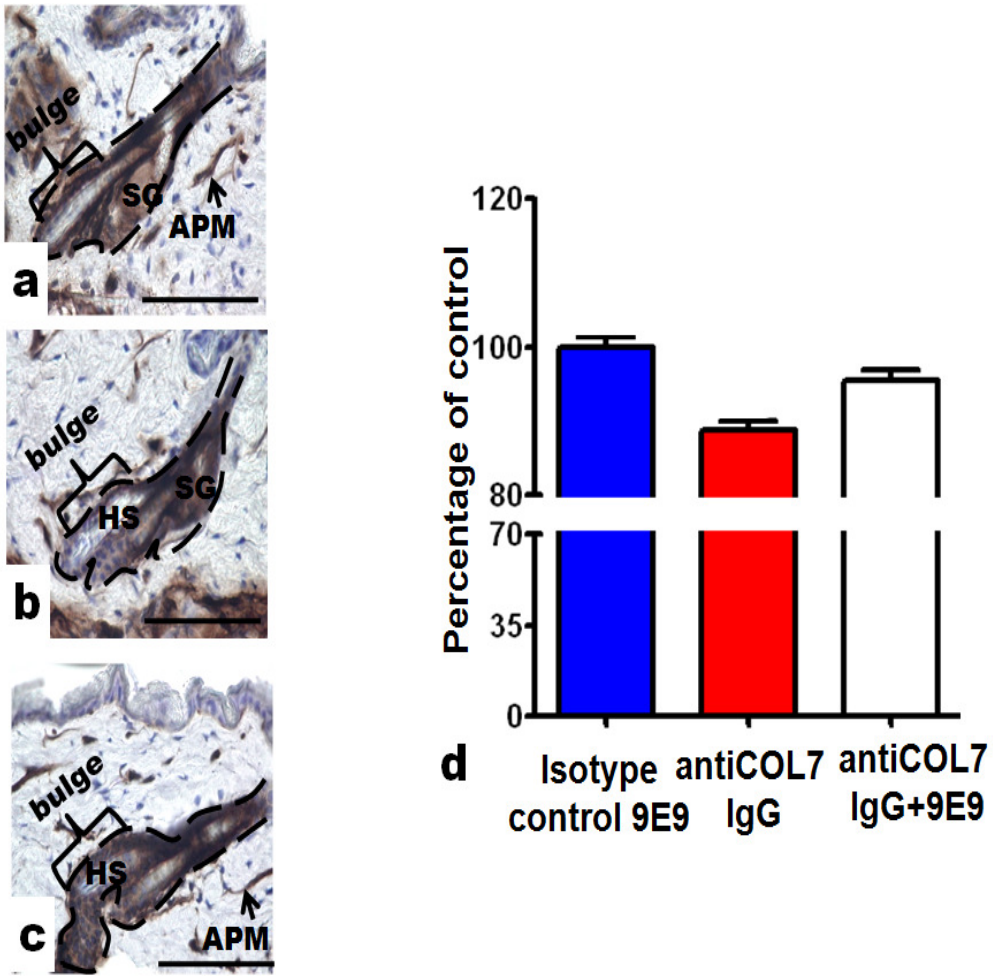

Fig.41: Simultaneous injection of anti type VII collagen and anti FcγRIV antibodies (9E9) restores bulge CD200 immunoreactivity. *CD200 immunoreactivity (brown) in the hair follicle bulge region of isotype control 9E9 mice (a).CD200 I immunoreactivity (brown) in the bulge of mice injected with anti type VII collagen IgG and anti type VII collagen plus 9E9 injected mice (b),(c). Quantitative analysis reveals considerbile increase(partial restoration) in the levels of bulge CD200 IR in anti COL7 IgG + 9E9 injected mice in comparison to Isotype control 9E9 and to anti type VII collagen IgG (d).ABC-peroxidase method, DAB, brown, counterstaining using haematoxylin. N = 34-36 HF's, derived from 3 mice/group from two individual experiments, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.*

3.14 Injecting anti FcγRIV antibodies into mice leads to decrease accumulation of inflammatory infiltrates in the bulge.

3.14.1 Injecting anti FcγRIV into mice leads to decrease in MHCII+ cells in the surrounding dermis but not in the CTS of follicular bulge region.

The injection of anti COL7 leads to accumulation of inflammatory infiltrates such as MHC II+ cells, CD4+ T cells and mast cells that bring about BM damage in the passive model of EBA. Therefore, it was investigated whether simultaneous injection of anti FcγRIV antibodies had an influence in the accumulation of inflammatory infiltrates surrounding the bulge. The distribution of MHC II+ cells, CD4+ T cells and mast cells was quantified in the ORS, CTS and 50µm into the dermis from the CTS after staining by routine immunohistochemistry and toluidine blue stainings.

Quantitative immunohistomorphometry indicated no decrease in the number of MHC II+ cells in the dermis of mice simultaneously injected with anti FcγRIV mice in comparission to anti COL7IgG treated mice in the dermis (**Fig.42** a, b, c) In contrast, in the CTS, the number of MHC II+ cells in the anti FcγRIV injected mice was higher than in anti COL7 IgG injected mice and an isotype control (**Fig.42**c,d). These results show that anti FcγRIV may slightly reduce the number of MHC II+ cells in the dermis, but fails to do so in the CTS.

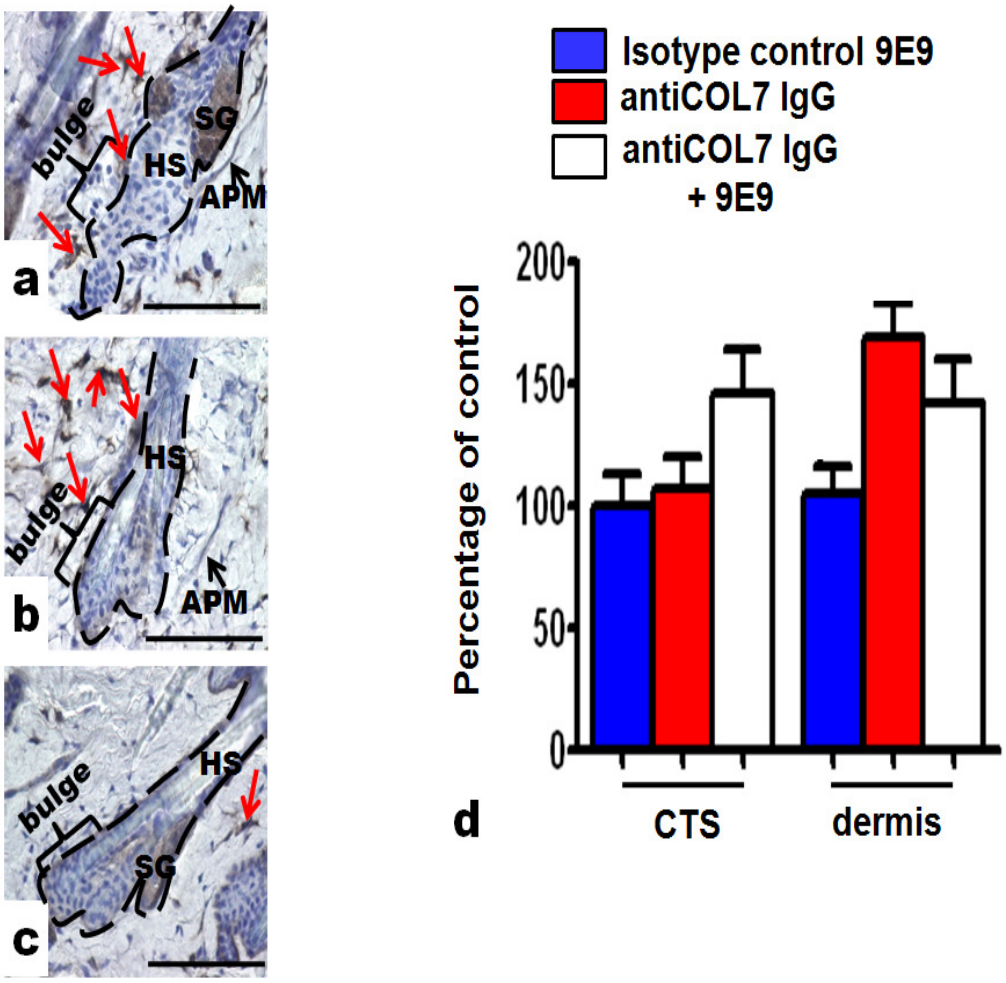

Fig.42: Simultaneous injection of anti type VII collagen and anti FcγRIV (9E9) antibodies show opposing effects in regulating MHC II+ cells in the CTS and dermis surrounding the hair follicle.*MHC II positive cells in the CTS surrounding the hair follicle bulge region of isotype control 9E9 mice (red arrows) (a). MHC II+ cells surrounding (brown) the bulge of mice injected with anti type VII collagen IgG (red arrows) (b). Perifollicular MHCII positive cells (brown) of mice injected with anti type VII collagen IgG (red arrows) (b) or with anti type VII collagen IgG plus 9E9 (c). Quantitative analysis reveals a simultaneous decrease and an increase in the number of MHC II positive cells in the dermis and CTS of anti COL7 IgG and 9E9 injected mice in comparison to antitype VII collagen IgG (d).ABC- peroxidase method, DAB in brown, counterstaining using haematoxylin. N = 33-40 HFs, derived from 2-3 mice/group from two individual experiments, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.*

3.14.2 CD4+ cells are decreased in the follicular bulge of Anti FcγRIV injected mice

However, quantitative measurement of CD4+ T-cells in FcγRIV injected mice showed a pronounced decrease in cell numbers in the CTS (**Fig.43** a,b,d). Thus, anti FcγRIV is able to down-regulate the number of CD4+ T cells in the CTS

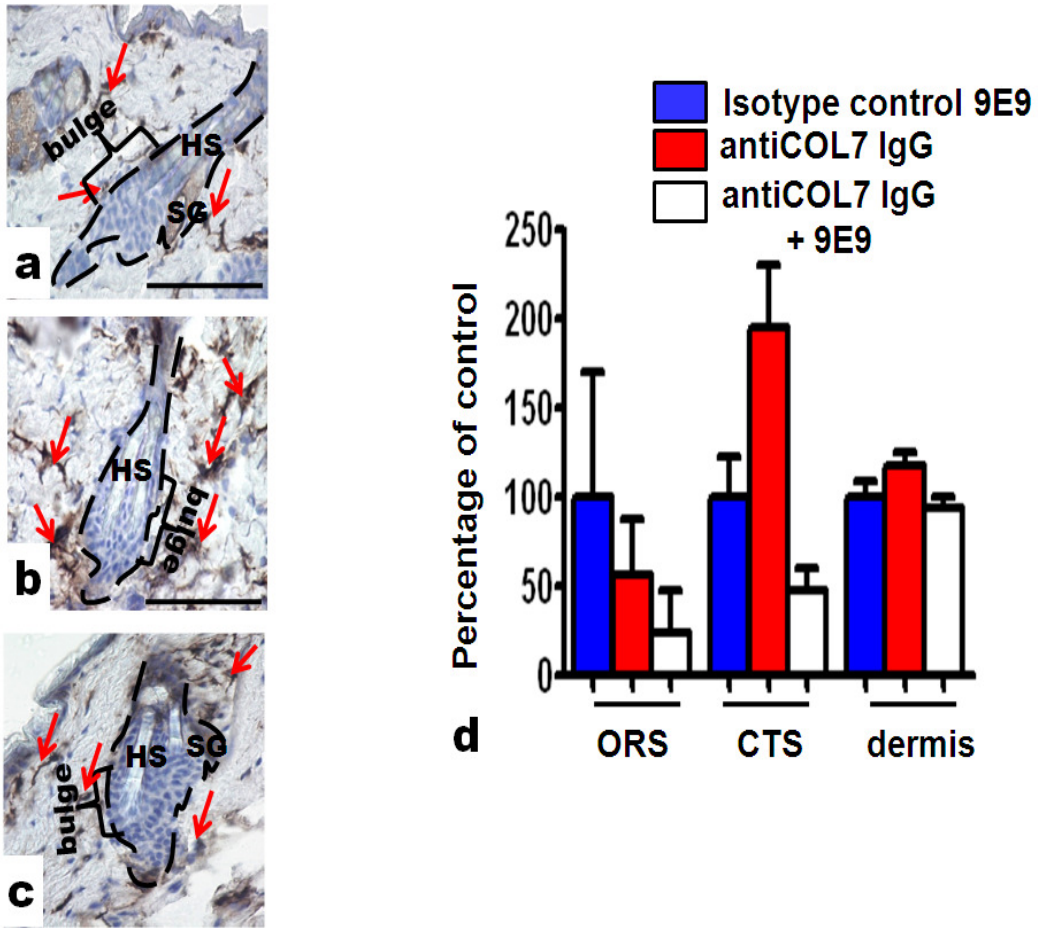

Fig.43: Simultaneous injection of anti type VII collagen and anti FcγRIV antibodies (9E9) decreases the no of CD4+ T-cells in the CTS.and dermis. *CD4 positive T-cells in the dermis and CTS of isotype control 9E9 mice (red arrows) (a). Perifollicular CD4 positive cells (brown) in mice injected with anti type VII collagen IgG (red arrows) (b)or with anti type VII collagen IgG plus 9E9 (c).Quantitative analysis reveals a decrease in the number of CD4+ cells in the CTS and dermis of anti COL7 IgG injected mice in comparison to isotype control 9E9 and anti type VII collagen IgG injected mice,The analysis also reavels a decrease in CD4+ cells in the ORS in comparison to anti COL7 IgG injected mice but not with the Isotype control(d).ABC-peroxidase method, DAB, brown, counterstaining using haematoxylin. N = 19-38 HF's, derived from 2-3 mice/group*

from two individual experiments, mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.

3.14.3 Mast cells are decreased in the Anti FcγRIV injected mice

Similarly, quantification of non degranulated and degranulated mast cells in FcγRIV injected mice in both the dermis and the CTS was undertaken. This showed a marked decrease in the percentage of degranulated mast cells in the dermis of anti FcγRIV injected mice (**Fig.44** c,d).The numbers non degranulated mast cells in the dermis were also marginally lower than both anti COL7 IgG and isotype control 9E9 mice groups(**Fig.44** c,d). Therefore, anti FcγRIV can also reduce intradermal mast cell degranulation.

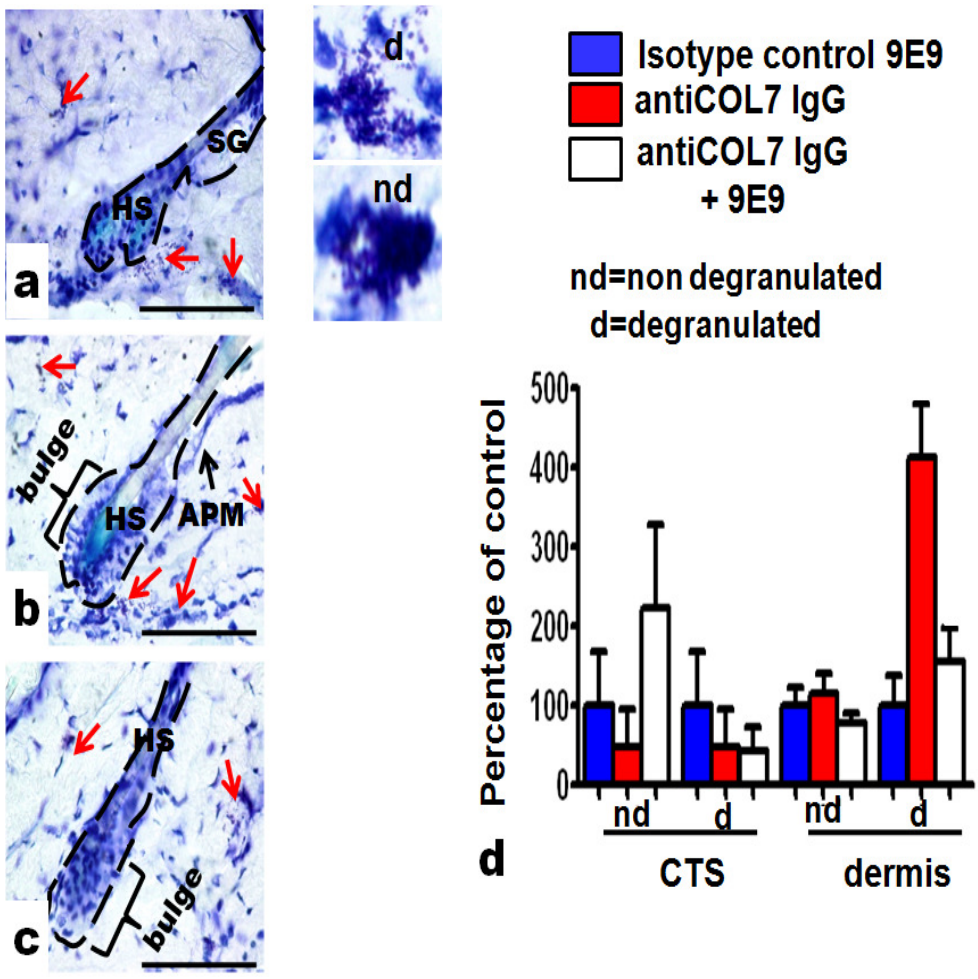

Fig.44: Simultaneous injection of anti type VII collagen and anti FcγRIV antibodies (9E9) shows opposing effects in regulating mast cell recruitment in CTS and dermis. *Toluidine blue staining shows perifollicular*

mast cells (red arrows) in isotype control 9E9 mice (red arrows) (a) and in mice injected with anti type VII collagen IgG (red arrows) (b). Perifollicular mast cells injected with anti type VII collagen IgG plus 9E9 (c) .Quantitative analysis revealed a decrease and also a sustained increase in the number of degranulated perifollicular mast cells in anti COL7 IgG and 9E9 injected mice in comparison to the anti COL7 IgG injected mice and isotype control 9E9 mice. Interestingly It also reveled higher number of recruited mast cells(non degranulated) in the anti COL7IgG plus 9E9 injected group in comparison to both anti COL7IgG and isotype control 9E9 mice(d).ABC - peroxidase method, DAB, brown, counterstaining using haematoxylin. N = 21-34 HF's, derived from 2-3 mice/group from two individual experiments mean +/-SEM. APM, arrector pili muscle, HS, hair shaft, SG, sebaceous gland, scale bars=100 µm.

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4.0 DISCUSSION

The specific questions that the present thesis project has posed have been answered as follows.

1. Murine telogen follicles **display a state of relative IP in the bulge** as shown by increased expression of CD200 and decreased MHCI expression in the bulge (**Fig.13** a-d.)

2. Injection of anti COL7 IgG and GST-mCOL7C causes **scarring alopecia with basement membrane destruction** (**Fig.18** a-b, **Fig.29**a,c,f,g,i). Binding of autoantibodies to type VII collagen **leads to bulge immune privilege collapse** (**Fig.19**a-c,**Fig.31**a-c) via **the accumulated inflammatory infiltrates** such as **CD4+**(**Fig.22**a-c,**Fig.33**a-c),**MHCII+/CD54+**(**Fig.23**a-c,**Fig.34**a-c),**mastcells**(**Fig.24**a-c,**Fig.35**a-c) and **CD8+ cells**(**Fig.21**a-c,**Fig.32**a-c) at the level of the follicular bulge. This also leads increase in key cytokines such as **IL 15** (**Fig.25**a-c, **Fig.36**a-c) and **TGF-β1** (**Fig.26**a-c, **Fig.37**a-c). But the levels of **CD34 IR (stem cell marker) showed a mild change** in both active and passive mice models (**Fig.27**a-c, **Fig.38**a-c) though the **total number of CD34+ cells decreased** in the passive model(**Fig.28**a-c).

3. Injecting anti FcγRIV prevented CA in murine EBA (**Fig.39**b, f) **restored HF IP**. This was demonstrated by a considerable **decrease in the expression of MHC I** (**Fig.40**a-d) **and increased expression of CD200**(**Fig.41**a-d) in the bulge.Moreover a decrease in the level of **CD4+ cells** (**Fig.43**a-d) and **mast cells**(**Fig.44**a-d) at the level of the bulge was also seen in mice injected with anti FcγRIV.

These experiments demonstrate for the first time that the bulge region of healthy murine telogen HFs exhibits characteristic immunohistological markers of relative IP. In both passive and active murine EBA, this bulge IP of telogen HFs is being compromised by the increased, ectopic expression of MHC I and a simultaneous decrease in expression of the immunoinhibitory “IP guardian”, CD200. This

appears to occur because of perifollicular inflammatory cell infiltrates induced by anti-collagen type VII-autoantibodies accumulate around the bulge region of non-lesional HFs in the skin of EBA mice.

Using CD34 as a bulge stem cell marker, only marginal differences were found between control and test mice in either EBA model. This suggests that the bulge damage associated with IP collapse is less pronounced and more reversible in EBA than in other forms of CA and that early therapeutic intervention permits full HF recovery. anti-FcγRIV antibodies helped to preserve bulge IP and increasingly reduced perifollicular inflammatory cell infiltrates.

In summary, these novel findings in two EBA mouse models suggest for the first time that autoantibodies directed against basement membrane antigens can induce an inflammatory infiltrate that endangers the IP of a protected intrafollicular stem cell niche (bulge) in mammalian skin, and that EBA-therapeutic (anti-FcγRIV) antibodies seem to exert HF and bulge-protective functions. Taken together, this sheds new light on the impact of autoantibody-induced inflammation on intracutaneous epithelial stem cells and the special immunoprivileged niche these reside in, and suggests potential therapeutic interventions.

Mice injected with anti COL7 IgG and GST-mCOL7C showed scarring alopecia mainly on the snout, head, back and flanks. The degree of alopecia was more pronounced in the anti COL7 IgG injected mice than in the GST-mCOL7C mice which is indicative that pathogenic antibodies are specific and fast acting while the active model takes longer time to synthesize and bind to target protein. This is also evident from the differences in fluorescent staining intensity at the level of basement membrane (**Fig.12**c, **Fig.29**b) and might reflect a quantitatively low amount of binding of the antibody. This might perhaps be one of the key reasons why some key parameters did not show higher degree of change in staining intensity as seen in the passive model (**Fig.30**a-c, **Fig.36**a-c, and **Fig.37**a-c). Nevertheless the presence of visible hair loss with blisters should serve to study the mechanism of hair loss in the active model of EBA.

Neutrophils have been previously shown to play an active role in the disease process in these mice models of EBA.(Sitaru et al.2005,Chiriac et al.2007,) Interestingly and to our surprise performing routine HE and preliminary staining (Ly-6G and Ly-6C) on the non lesional back skin of both the passive and active models did not show neutrophils. This shows that the disease has not yet progressed to where neutrophils can be detected, or that their impact is so transitory that it was missed in the current study. This also indicates that other inflammatory infiltrates may also play an important role in initiating the disease process, at least with respect to the induction of HF damage.

Moreover it was also found that not all the follicles in the back skin are uniformly targeted and may reflect the differential secretary activities in various follicles. The BM stabilizes and protects the follicle from surrounding infiltrates in the dermis by expressing various structural proteins (Chuang et al.2003, Joubeh et al.2003, Fujiwara et al. 2011).The PAS staining used to detect BM destruction showed decreased intensity in the affected follicles in both passive and active mice models (**Fig18**a-b, **Fig.29** i).This is considered to be an important step for immune cells to access the follicular bulge in the affected follicles.

The current findings underscore that T cells play important role in the pathogenesis of EBA (Muller et al.2010, Sitaru et al. 2010) Since it was also noticed that many of the HFs do not undergo uniform destruction, it appears advisable to further characterize the specific type of T cells (Konig et al.2002) and MHCII+ cells that attack the HF to better understand how HF damage is brought about in these EBA mice models.

After injection of pathogenic antibody, perifollicular mast cells also showed a vigorous activation response (**Fig.24**a-c, **Fig.35**a-c). Though large numbers of mice and tissue sections need to be evaluated, ideally also in repeat experiments, this suggests a more important role for mast cells in (non-IgE) antibody-induced HF inflammation. This calls to mind the hypothesis that, under physiological circumstances, perifollicular mast cells may largely exert an immunoinhibitory, HF IP-preserving influence (Waldmann H Nature 2006, Bertolini et al.2012). Instead, once given appropriate stimuli, mast cells may turn into autoimmunity-promoting cells (Christy et al. 2012, Brown & Hatfield 2012,

and Walker et al. 2012). It is conceivable that this occurs also in alopecia areata (Bertolini et al. 2012, Gilhar et al.2012) and some forms of lichen planopilaris (Harries et al. 2013) - and possibly also in EBA-associated alopecia.

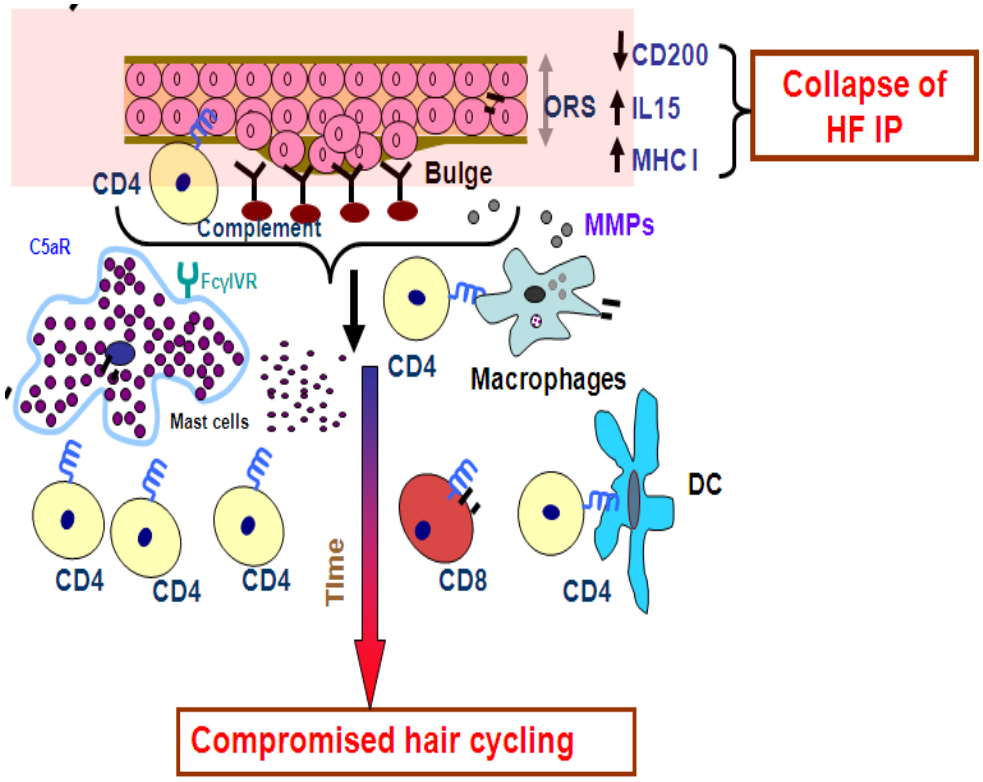
The increase in TGF-β1(**Fig.26**a-c) in the bulge suggests that the deposition of anti-collagen VII antibody at the follicular BM triggers induces the potent immunoinhibitory and IP-protective growth factor,TGFß1 (Wahl et al. 2006, Niederkorn 2002) in the passive EBA model,possibly as a damage-response strategy geared at reestablishing HF IP. However, TGFß1 is also fibrosis-promoting (Penn et al.2012). Together, this may both regulate the perifollicular inflammatory cell infiltrate while attempting to maintain or restore the endangered HF IP and suppressing epithelial HF stem cell apoptosis, yet risks the activation of scarring-promoting pathways. This hypothetical scenario now remains to be probed in additional *in vivo*-studies that test the HF effects of anti COL7IgG versus NRIgG in the presence or absence of IL-15- or TGF- ß1-neutralizing antibodies.

In line with collapsed IP, increase in infiltrates and change cytokine profiles, the bulge stem cell marker CD34+ cells (Morris et al. 2004, Ito et al. 2005,Ohyama et al.2006,Tiede et al.2007a,b)were analysed. Preliminary analysis of staining intensity did not rule out gross stem cells damage (**Fig.27**a-c,**Fig.38**a-c) but a closer look at the total number of CD34+ cells in the follicular bulge in the passive model revealed decreased levels(**Fig.28**a-c) of CD34+ cells in the follicular bulge of anti COL7IgG in comparison to NRIgG. If these trends were to be followed by measuring greater number of follicles it would perhaps lead to adecrease in the follicular stem cells though this could also represent a temporary phenomenon. Having said that, the regrowth of hair follicles in mice after two months post injection of anti COL7IgG (**Fig.17**b) also suggests that HF epithelial stem cells may be more resistant to inflammatory damage than previously thought. This phenomenon also implies that the present EBA mouse model might not represent a true CA model system, as CA typically results from irreversible stem cell destruction.

Though these murine models of EBA serve to provide important insights into the underlying mechanism for HF destruction in this disease, important differences

were also found between the passive and active EBM models. Visibly the active model had smaller and localized patches alopecia in comparison to the passive models and this in fact correlated with the relative amount of pathogenic IgG binding to basement membrane as visualized by IF. Additionally, some of the important parameters in the non lesional back skin of these models also showed marked differences in their staining intensity. In the passive model MHCI, IL15 and TGF-β1 IR increased predominantly in the bulge, but in the active model IL15 and MHCI increased marginally, while TGF-β1 IR markedly declined because the mechanism of induction of disease varies considerably in these two models systems. Furthermore the current HF immunopathology findings suggest that the passive EBA model represents more of an active, but short-lived disease, while active model shows a slow disease process, with only a very mild hair phenotype in non-lesional skin.

Though experimentally the treatment did reverse the blistering process without hair loss, the increase in inflammatory cells especially in the CTS of both the isotype control group and that of the anti COL7IgG plus 9E9 treated mice suggests that some of the immune cells may nonspecifically interact with the anti FcγRIV antibody. This may be abrogated by using an appropriate antibody that complements with the mouse system in repeated experiments, thus permitting better insight into the role of inflammatory cells as mediators of HF stem cell damage. **Fig. 45** depicts a hypothetical scenario that attempts to explain, on the basis of the observations made in the current thesis project, how anti-COL7 (auto-)antibodies may sequentially damage the HF BM and endanger the bulge IP, thus risking HF bulge stem cell damage that leads to EBA-associated alopecia:



45: Hypothetical scenario: How inflammatory events may lead to irreversible destruction of the HF bulge in the examined murine models of EBA.*The accumulation of inflammatory infiltrates (MHC II+,CD4+,Mast cells, and CD8+ cells) leads to bulge immune privilege collapse(increase in MHCI and decrease in CD200 IR) and change in cytokine profile of the follicular bulge. This further potentiate the differentiation follicular stem cells into scar tissue under the influence of a week but a sustained inflammatory process in the long term*

In summary, the findings in two EBA mouse models reported here suggest that a) autoantibodies directed against basement membrane antigens can induce an inflammatory infiltrate that endangers the IP of a protected intrafollicular stem cell niche (bulge) in mammalian skin, b) the bulge epithelium may counteract IP collapse by the up-regulation of IP- and stem cell-protective cytokines, and that c) EBA-therapeutic anti- FcγRIV antibodies may exert HF- and bulge-protective functions. Taken together, these novel insights from two EBA mouse models have shed new light on the impact of autoantibody-induced inflammation on

intracutaneous epithelial stem cells and the special immunoprivileged niche these reside in, and suggest potential therapeutic interventions.

Follow-up studies could carry the current work into the human system, e.g. by testing teh effects of anti-COL7 antisera from EBA patients on microdissected, organ-cultured human scalp hair follicle and their stem cell zone. Moreover isolated ORS bulge stem cells can be transfected to express green fluorescent protein (GFP) under the control of the keratin 15 promoter directly monitor HF stem cells with live fluorescence imaging (Tiede et al. 2009a) after exposure to EBA patient antisera, in the presence or absence of defined immunocyte populations. This can be combined with laser capture microdissection and subsequent DNA microarray analysis of the stem cell region, both in non lesional human and mouse skin and in organ-cultured human scalp HFs (Panicker et al. 2012).

Given the observed increase in MHC II+ cells and CD4+ cells in murine EBA skin, it is also important to determine the exact phenotype of these cells, e.g. by double immunostaining (CD68 / CD4+,CD25, Foxp3+ cells).

5.0 SUMMARY

Cicatricial alopecia (CA) is a rare group of permanent hair loss disorders that occurs due to the irreversible destruction of epithelial hair follicle stem cells. It is also seen as a secondary event in infectious diseases and autoimmune bullous dermatoses. Along with the death of the epithelial hair follicle (HF) stem cells, a sustained inflammatory process associated with fibrosis is mainly seen in the hair follicle stem cell region. In the current thesis project, the CA that occurs in a particularly well-defined autoimmune bullous dermatosis, epidermolysis bulluosa acquisita (EBA) caused by autoantibodies to basement membrane-associated collagen type VII, was examined in a passive and an active EBA mouse model.

Since it had previously only been established in human HFs that the bulge region represents an area of relative immune privilege (IP), whose collapse may contribute to the pathogenesis of CA, the first question addressed was whether there is immunophenomenological evidence that the bulge region of murine HFs also is immunoprivileged. Second, the type of inflammatory cell infiltrates in HFs of EBA mice was characterized by quantitative (immuno-) histomorphometry, comparing results from the passive and the active EBA mouse model. Third, this infiltrate was correlated with evidence of a) HF epithelial stem cell damage and b) bulge IP collapse. Fourth and finally, based on prior research in these EBA mouse models, which had suggested a key role for a specific IgG receptor (FcγRIV) in murine EBA pathogenesis,it was tested whether the blocking of FcγRIV by appropriate antibodies impacted on CA and HF damage.

These experiments demonstrate for the first time that the bulge region of healthy murine telogen HFs exhibit characteristic immunohistological markers of relative IP. Second, in both passive and active murine EBA, this bulge IP of telogen HFs is being compromised by the increased, ectopic expression of MHC I and a simultaneous decrease in expression of the immunoinhibitory “IP guardian”, CD200. This appears to occur as a result of anti-collagen type VII-autoantibody-induced perifollicular inflammatory cell infiltrates (namely MHC class II+, CD4+ and/or CD8+ lymphocytes, and mast cells) which accumulate around the bulge

region of non-lesional HFs in the skin of EBA mice. Interestingly the active model mice had decreased lesional areas and also showed a decrease in CD200 and TGF-β1 in comparison the passive model mice. Interestingly, using CD34 as a bulge stem cell marker, only marginal differences were found between control and test mice in either EBA model. This suggests that the bulge damage associated with IP collapse is less pronounced and more reversible in EBA than in other forms of CA and that early therapeutic intervention permits full HF recovery. Moreover, the bulge region in passive EBA mice showed increased expression of transforming growth factor-ß1. Since the after a major endogenous immunosuppressant with a recognized key role in HF IP, this invites the hypothesis that overexpression of TGFβ-1 may represent a protective mechanism by with the bulge region may protect itself from inflammation-associated stem cell damage. Finally, we showed that mice injected with anti-FcγRIV antibodies along with pathogenic anti-collagen type VII antibodies showed no signs of CA, along with largely preserved bulge IP and with a reduction in perifollicular inflammatory cell infiltrates.

In summary, these novel findings in two EBA mouse models suggest for the first time that a) autoantibodies directed against basement membrane antigens can induce an inflammatory infiltrate that endangers the IP of a protected intrafollicular stem cell niche (bulge) in mammalian skin, b) the bulge epithelium may counteract IP collapse by the up-regulation of IP- and stem cell-protective cytokines, and that c) EBA-therapeutic (anti-FcγRIV) antibodies also seem to exert HF and bulge-protective functions. Taken together, this sheds new light on the impact of autoantibody-induced inflammation on intracutaneous epithelial stem cells and the special immunoprivileged niche these reside in, and suggests potential therapeutic interventions.

6.0 ZUSAMMENFASSUNG

Vernarbende (cicatrizielle) Alopezien (CA) umfassen eine seltene Gruppe von Erkrankungen mit permanentem Haarausfall, die durch eine irreversible Zerstörung epithelialer Haarfollikelstammzellen ausgelöst wird. CA treten auch als sekundäres Ereignis nach Infektionskrankheiten und autoimmunbullösen Dermatosen auf. Zusammen mit dem Tod der epithelialen Haarfollikel (HF)-Stammzellen tritt eine anhaltende Entzündung auf, assoziiert mit Fibrose, hauptsächlich im Stammzellbereich des HF, dem sog. Wulst (bulge).In der vorliegenden Arbeit wird die CA, die in einer gut beschriebenen autoimmunbullösen Dermatose auftritt, der Epidermolysis bullosa acquisita (EBA). Diese wird durch Autoantikörper gegen das Basalmembran-assoziierte Kollagen Typ VII verursacht. Hierzu werden ein passives und ein aktives EBA-Mausmodell untersucht.

Da es zuvor nur in menschlichen HFs festgestellt wurde, dass die Wulst-Region einen relatives Immunprivileg (IP) aufweist, deren Zusammenbruch zur Pathogenese der CA wesentlich beitragen könnte, war die erste zu klärende Frage, ob es immunophenomenologische Hinweise darauf gibt, daß die Wulst-Region muriner Telogen-HF auch immunologisch privilegiert ist. Zweitens wurde das entzündliche Zellinfiltrat in HFn von EBA-Mäusen mittels quantitativer (Immun-)Histomorphometrie anaylsiert, wobei die Ergebnisse aus dem passiven und dem aktiven EBA Mausmodell miteinander verglichen wurden. Drittens wurde dieses Infiltrat genauer untersucht hinsichtlich Schäden im Bereich der Wulstzone und nach Hinweisen auf einen IP-Zusammenbruch. Basierend auf früheren Forschungen in diesen EBA-Mausmodellen, in denen eine entscheidende Rolle für einen spezifischen IgG-Rezeptor (FcγRIV) in der Pathogenese der murinen EBA ermittelt worden war, wurde viertens getestet, ob eine Blockade des FcγRIV durch entsprechende Antikörper Einfluß auf die HF-Schädigung und die CA hat .

Diese Experimente zeigen zum ersten Mal, dass die Wulstzone von gesunden murinen Telogen-HF charakteristische immunhistologische Marker eines relativen IP aufweisen. Ferner konnte gezeigt werden, dass sowohl im passiven wie wie auch im aktiven EBA-Mausmodell, dieses Wulst-IP - des Telogen HFs

kompromittiert wird. Dies zeigt sich an einer erhöhten und ektopischen Expression von MHC KlasseI-Molekülen und einer gleichzeitigen Abnahme der Expression des immuninhibitorischen "IP-Wächtermoleküls", CD200. Dies scheint aus der durch Anti-Kollagen Typ VII-Autoantikörper induzierten perifollikulären Entzündung zu resultieren.

Diese Entzündungsinfiltrate bestehen überwiegend aus MHC-Klasse II+ Zellen (aktivierte Makrophagen), CD4+ und / oder CD8+ Lymphozyten und aus Mastzellen, die um die Wulstregion von zunächst unbefallenen HFn in der Haut von EBA- Mäusen akkumulieren. Interessanterweise zeigten die Mäuse im aktiven EBA-Modell auch eine signifikante Abnahme der CD200- und der TGF-β1-Expression in läsionalen HFn im Vergleich zu den Mäusen im passiven Modell.

Interessant war auch, dass unter Verwendung des Stammzellmarkers CD34 nur marginale Unterschiede zwischen Kontroll-und Test-Mäusen in beiden EBA-Modellen gefunden werden konnten. Dies deutet darauf hin, dass der HF-Stammzellschaden nach dem (Auto-)Antikörper-induzierten Zusammenbruch des Wulst-IP bei der experimentellen murinen EBA weniger ausgeprägt und eher reversibel ist als bei anderen Formen der CA. Dies paßt gut zu der klinischen Beobachtung bei EBA-Mäusen, daß der „vernarbende“ Haarausfall hier oft viel reversibler ist, als dies für eine CA typisch ist.

Hieraus ergibt sich die wichtige, klinisch relevante Erkenntnis, dass eine frühzeitige therapeutische Intervention – im Gegensatz zu anderen CAs (wie z.B. Lichen planopilaris oder CDLE-assoziierter CA), wo dies selbst bei rechtzeitiger und aggressiver Therapie kaum gelingt - durchaus eine volle HF-Regeneration mit komplettem Haarnachwuchs ermöglichen sollte.

Darüber hinaus zeigt der Wulstbereich von HFn im passiven EBA-Mausmodell eine erhöhte Expression von TGF-ß1. Da letzteres ein wichtiger endogener Immuninhibitor mit einer anerkannten Schlüsselrolle im IP des HFs ist, laden diese Befunde zu der Hypothese ein, dass die Überexpression von TGF-ß1 einen Schutzmechanismus darstellen könnte,mit dem sich der HF-Wulst möglicherweise vor Entzündungs-assoziierten Stammzellen-Schäden zu schützen sucht. Falls dieses Prinizip sich auch im Humansystem bestätigt, könnte hieraus eine neue

therapeutische Strategie zur Protektion von epithelialen Stammzellen – möglicherweise auch außerhalb der Haut – abgeleitet werden.

Schließlich konnte noch gezeigt werden, dass Mäuse, die gemeinsam mit pathogenen Anti-Kollagen Typ VII Antikörpern auch mit FcγRIV -blockierenden Antikörpern behandelt wurden, keine Anzeichen von CA zeigten. Dies ging einher mit einem (zumindest immunphänomenologisch) weitgehend erhaltenen Wulst-IP und mit einer starken Reduzierung der perifollikulären entzündlichen Zellinfiltrate in dieser Region. Sollten diese Ergebnisse aus dem passiven EBA-Mausmodell auf die klinische Situation bei EBA-Patienten übertragbar sein, liesse sich hieraus ableiten, dass FcγRIV -blockierende Antikörper – neben ihrem therapeutischenNutzen für die EBA insgesamt – auch potente protektive Effekte auf Basalmembran-nahe, sensible epitheliale Stammzellpopulationen entfalten und vor dem Auftreten einer irreversiblen CA effektiv schützen könnten.

Zusammenfassend zeigen diese neuen, translational relevanten Erkenntnisse aus zwei experimentellen EBA Mausmodellen erstmals, dass a) gegen Basalmembran-assoziierte Antigene gerichtete Antikörper ein entzündliches Infiltrat induzieren können, welches die intrafollikuläre Stammzellnische in der Haut von Säugetieren (HF-Wulst) angreift und dessen IP gefährdet, b) diese Stammzellregion gegenregulatorische Maßnahmen ergreift (z.B. Hochregulation von TGF-ß1), c) möglicherweise deswegen nur geringe Verluste ihrer Stammzellpopulation erleidet, so daß die Harsausfall bei EBA viel weniger irreversibel ist als dies für CA üblich ist, und d) daß anti- FcγRIV Antikörper bei EBA auch eine HF-und Wulst-Schutzfunktionen ausüben.

Insgesamt ergeben sich hieraus wichtige pathobiologische und therapeutische Perspektiven, die über die EBA hinausweisen und für andere Autoimmunerkranklungen relevant sein könnten, bei denen es zur Schädigung von epithelialen Stammzellen kommt.

7.0 REFERENCES

Alonso L, Fuchs E: The hair cycle.J Cell Sci 119,391-393(2006).

Al-Refu K: Stem cells and alopecia: a review of pathogenesis.Br J Dermatol 167,479-484(2012).

Al-Refu K, Goodfield M: Hair follicle stem cells in the pathogenesis of the scarring process in cutaneous lupus erythematosus.Autoimmun Rev 8,474-477(2009).

Al-Zaid T, Vanderweil S, Zembowicz A, Lyle S:Sebaceous gland loss and inflammation in scarring alopecia: A potential role in pathogenesis.J Am Acad Dermatol 65,597-603(2011)

Amoh Y, Mii S, Aki R, Hamada Y, Kawahara K, Hoffman RM, Katsuoka K:Multipotent nestin-expressing stem cells capable of forming neurons are located in the upper, middle and lower part of the vibrissa hair follicle.Cell cycle 11,3513-3517(2012).

Ansell DM, Kloepper JE, Thomason HA, Paus R, Hardman MJ: Exploring the "hair growth-wound healing connection": anagen phase promotes wound re-epithelialization.J Invest Dermatol 131,518-528(2011).

Apte RS, Sinha D, Mayhew E, Wistow GJ, Niederkorn JY:Cutting edge: role of macrophage migration inhibitory factor in inhibiting NK cell activity and preserving immune privilege.J Immunol 160,5693-5696(1998).

Arck PC, Handjiski B, Hagen E, Joachim R, Klapp BF, Paus R:Indications for a 'brain-hair follicle axis (BHA)': inhibition of keratinocyte proliferation and up-regulation of keratinocyte apoptosis in telogen hair follicles by stress and substance P.FASEB J 15,2536-2538(2001).

Bardazzi F, Fanti PA, Orlandi C, Chieregato C, Misciali C: Psoriatic scarring alopecia: observations in four patients.Int J Dermatol 38,765-768(1999).

Beck B, Blanpain C: Mechanisms regulating epidermal stem cells.EMBO J 31,2067-2075(2012).

Berard M, Brandt K, Bulfone-Paus S, Tough DF: IL-15 promotes the survival of naive and memory phenotype CD8+ T cells.J Immunol 170,5018-5026(2003).

Bernhagen J, Bacher M, Calandra T, Metz CN, Doty SB, Donnelly T, Bucala R:

An essential role for macrophage migration inhibitory factor in the tuberculin delayed-type hypersensitivity reaction.J Exp Med 183,277-282(1996).

Berzofsky JA: A push-pull vaccine strategy using Toll-like receptor ligands, IL-15, and blockade of negative regulation to improve the quality and quantity of T cell immune responses.Vaccine 30,4323-4327(2012).

Bieber K, Sun S, Ishii N, Kasperkiewicz M, Schmidt E, Hirose M, Westermann J, Yu X, Zillikens D, Ludwig RJ: Animal models for autoimmune bullous dermatoses.Exp Dermatol 19,2-11(2010).

Billingham RE: Transplantation immunity evoked by skin homografts and expressed in intact skin.Adv Biol Skin 11,183-198(1971).

Blanpain C, Lowry WE, Geoghegan A, Polak L, Fuchs E:Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche.Cell 118,635-648(2004).

Böhm M, Apel M, Sugawara K, Brehler R, Jurk K, Luger TA, Haas H, Paus R, Eiz-Vesper B, Walls AF, Ponimaskin E, Gehring M, Kapp A, Raap U:Modulation of basophil activity: a novel function of the neuropeptide α-melanocyte-stimulating hormone.J Allergy Clin Immunol129,1085-1093(2012).

Bohm M, Luger TA: The role of melanocortins in skin homeostasis.Horm Res 54,287-293(2000).

Bos JD, Zonneveld I, Das PK, Krieg SR, van der Loos CM, Kapsenberg ML:The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin.J Invest Dermatol 88,569-573(1987).

Botchkarev VA, Botchkareva NV, Roth W, Nakamura M, Chen LH, Herzog W, Lindner G, McMahon JA, Peters C, Lauster R, McMahon AP, Paus R:Noggin is a mesenchymally derived stimulator of hair-follicle induction.Nat Cell Biol 1,158-164(1999).

Botchkarev VA, Eichmuller S, Johansson O, Paus R: Hair cycle-dependent plasticity of skin and hair follicle innervation in normal murine skin.J Comp Neurol 386,379-395(1997).

Botchkarev VA, Paus R: Molecular biology of hair morphogenesis:development and cycling.J Exp Zool B Mol Dev Evol 298,164-180(2003).

Botchkarev VA, Paus R, Czarnetzki BM, Kupriyanov VS, Gordon DS, Johansson O: Hair cycle-dependent changes in mast cell histochemistry in murine

skin.Arch Dermatol Res 287,683-686(1995).

Botchkareva NV, Botchkarev VA, Chen LH, Lindner G, Paus R: A role for p75 neurotrophin receptor in the control of hair follicle morphogenesis.Dev Biol 216,135-153(1999).

Botchkareva NV, Botchkarev VA, Gilchrest BA: Fate of melanocytes during development of the hair follicle pigmentary unit.J Investig Dermatol Symp Proc 8,76-79(2003).

Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, Lazetic S, Young NT, Bell JI, Phillips JH, Lanier LL, McMichael AJ:HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature 391,795-799(1998).

Breitkopf T, Leung G, Yu M, Wang E, McElwee KJ: The basic science of hair biology: what are the causal mechanisms for the disordered hair follicle? Dermatol.Clin.31,1-19(2013).

Brown MA, Hatfield JK: Mast Cells are Important Modifiers of Autoimmune Disease: With so Much Evidence, Why is There Still Controversy? Front Immunol 3,147(2012).

Bruckner-Tuderman L, Hopfner B, Hammami-Hauasli N: Biology of anchoring fibrils: lessons from dystrophic epidermolysis bullosa.Matrix Biol 18, 43-54(1999).

Bruno R, Sabater L, Sospedra M, Ferrer-Francesch X, Escudero D, Martinez-Caceres E, Pujol-Borrell R: Multiple sclerosis candidate autoantigens except myelin oligodendrocyte glycoprotein are transcribed in human thymus.Eur J Immunol 32,2737-2747(2002).

Brzoska T Fau - Luger TA, Luger Ta Fau - Maaser C, Maaser C Fau - Abels C, Abels C Fau - Bohm M, Bohm M:Alpha-melanocyte-stimulating hormone and related tripeptides: biochemistry, antiinflammatory and protective effects in vitro and in vivo, and future perspectives for the treatment of immune-mediated inflammatory diseases.Endocr Rev. 29,581-602(2008)

Bubier JA, Sproule TJ, Alley LM, Webb CM, Fine JD, Roopenian DC, Sundberg JP: A mouse model of generalized non-Herlitz junctional epidermolysis bullosa.J Invest Dermatol 130,1819-1828(2010).

Bukovsky A, Presl J, Zidovsky J, Mancal P: The localization of Thy-1.1, MRC OX 2 and Ia antigens in the rat ovary and fallopian tube.Immunology 48,587-596(1983).

Bulfone-Paus S, Ungureanu D, Pohl T, Lindner G, Paus R, Rückert R, Krause H, Kunzendorf U: Interleukin-15 protects from lethal apoptosis in vivo.Nat Med 3,

1124-1128(1997).

Cameli N, Picardo M, Tosti A, Perrin C, Pisani A, Ortonne JP:Expression of integrins in human nail matrix.Br J Dermatol 130,583-588(1994).

Chan LS:Human skin basement membrane in health and in autoimmune diseases.Front Biosci 2, d343-352(1997)a.

Chan LS, Majmudar AA, Tran HH, Meier F, Schaumburg-Lever G, Chen M, Anhalt G, Woodley DT, Marinkovich MP:Laminin-6 and laminin-5 are recognized by autoantibodies in a subset of cicatricial pemphigoid.J Invest Dermatol 108,848-853(1997)b.

Chen L, Peterson JD, Zheng WY, Lin SX, Chan LS: Autoimmunity to type VII collagen in SKH1 mice is independent of regulatory T cells.Clin Exp Immunol 145,322-331(2006).

Chen M, Kim GH, Prakash L, Woodley DT:Epidermolysis bullosa acquisita: autoimmunity to anchoring fibril collagen.Autoimmunity 45, 91-101(2012).

Chen PW, Mellon JK, Mayhew E, Wang S, He YG, Hogan N, Niederkorn JY:Uveal melanoma expression of indoleamine 2,3-deoxygenase: establishment of an immune privileged environment by tryptophan depletion.Exp Eye Res 85,617-625(2007).

Chiriac MT, Roesler J, Sindrilaru A, Scharffetter-Kochanek K, Zillikens D, Sitaru C: NADPH oxidase is required for neutrophil-dependent autoantibody-induced tissue damage.J Pathol 212,56-65(2007).

Christoph T, Muller-Rover S, Audring H, Tobin DJ, Hermes B, Cotsarelis G, Ruckert R, Paus R:The human hair follicle immune system: cellular composition andimmune privilege.Br J Dermatol 142,862-873(2000).

Christy AL, Brown MA: The multitasking mast cell: positive and negative roles in the progression of autoimmunity. J Immunol 179,2673-2679(2007).

Christy AL, Walker ME, Hessner MJ, Brown MA: Mast cell activation and neutrophil recruitment promotes early and robust inflammation in the meninges in EAE.J Autoimmun (2012).

Clark DA, Keil A, Chen Z, Markert U, Manuel J, Gorczynski RM: Placental trophoblast from successful human pregnancies expresses the tolerance signaling molecule, CD200 (OX-2). Am J Reprod Immunol 50,187-195(2003).

Cobbold SP, Adams E, Graca L, Daley S, Yates S, Paterson A, Robertson NJ, Nolan KF, Fairchild PJ, Waldmann H:Immune privilege induced by regulatory T cells in transplantation tolerance.Immunol Rev 213,239-255(2006).

Cone RD, Lu D, Koppula S, Vage DI, Klungland H, Boston B, Chen W, Orth DN, Pouton C, Kesterson RA:The melanocortin receptors: agonists, antagonists, and the hormonal control of pigmentation.Recent Prog Horm Res 51,287-317;discussion 318(1996).

Conrad F, Ohnemus U, Bodo E, Biro T, Tychsen B, Gerstmayer B, Bosio A, Schmidt-Rose T, Altgilbers S, Bettermann A, Saathoff M, Meyer W, Paus R:Substantial sex-dependent differences in the response of human scalp hair follicles to estrogen stimulation in vitro advocate gender-tailored management of femaleversus male pattern balding.J Invest Dermatol Symp Proc 10,243-246(2005).

Cotsarelis G: Epithelial stem cells: a folliculocentric view. J Invest Dermatol 126,1459-1468(2006).

Cotsarelis G, Sun TT, Lavker RM: Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis.Cell 61,1329-1337(1990).

Csorba K, Sitaru S, Sitaru C: Granulocyte-dependent autoantibody-induced skin blistering.).J Vis Exp 12,4250(2012)

Derynck R, Lindquist PB, Lee A, Wen D, Tamm J, Graycar JL, Rhee L, Mason AJ, Miller DA, Coffey RJ, et al.:A new type of transforming growth factor-beta, TGF-beta 3.EMBO J 7,3737-3743(1988).

Di Meglio P, Perera GK, Nestle FO:The multitasking organ: recent insights into skin immune function.Immunity 35,857-869(2011).

Draborg AH, Duus K, Houen G: Epstein-Barr virus and systemic lupus erythematosus. Clin Dev Immunol 2012,370516(2012).

Dry FW: The coat of the mouse (Mus musculus).Journal of Genetics 16,287-340(1926).

Duheron V, Hess E, Duval M, Decossas M, Castaneda B, Klopper JE, Amoasii L, Barbaroux JB, Williams IR, Yagita H, Penninger J, Choi Y, Lezot F, Groves R, Paus R, Mueller CG:Receptor activator of NF-kappaB (RANK) stimulates the proliferation of epithelial cells of the epidermo-pilosebaceous unit.Proc Natl Acad Sci U S A 108,5342-5347(2011).

Eichmuller S, van der Veen C, Moll I, Hermes B, Hofmann U, Muller-Rover S, Paus R: Clusters of perifollicular macrophages in normal murine skin: physiological degeneration of selected hair follicles by programmed organ deletion.J Histochem Cytochem 46,361-370(1998).

Fan L, Busser BW, Lifsted TQ, Lo D, Laufer TM: Antigen presentation by keratinocytes directs autoimmune skin disease.PNAS 100, 3386-3391(2003).

Fernando MM, Stevens CR, Walsh EC, De Jager PL, Goyette P, Plenge RM, Vyse TJ, Rioux JD: Defining the role of the MHC in autoimmunity: a review and pooled analysis.PLoS Genet 4, e1000024 (2008).

Filippini A, Riccioli A, Padula F, Lauretti P, D'Alessio A, De Cesaris P, Gandini L, Lenzi A, Ziparo E:Control and impairment of immune privilege in the testis and in semen. Hum Reprod Update 7,444-449(2001).

Fine JD, Bauer EA, McGuire J, Moshell A. Epidermolysis Bullosa: Clinical, Epidemiologic, and Laboratory Advances and the Findings of the National Epidermolysis Bullosa Registry. Baltimore: Md: Johns Hopkins University Press(1999).

Fine JD, Eady RA, Bauer EA, Bauer JW, Bruckner-Tuderman L, Heagerty A, Hintner H, Hovnanian A, Jonkman MF, Leigh I, McGrath JA, Mellerio JE, Murrell DF, Shimizu H, Uitto J, Vahlquist A, Woodley D, Zambruno G: The classification of inherited epidermolysis bullosa (EB): Report of the Third International Consensus Meeting on Diagnosis and Classification of EB.J Am Acad Dermatol 58,931-950(2008).

Fine JD, Mellerio JE: Extracutaneous manifestations and complications of inherited epidermolysis bullosa: part I. Epithelial associated tissues.J Am Acad Dermatol 61,367-384; quiz 385-366(2009).

Fine JD, Mellerio JE: Extracutaneous manifestations and complications of inherited epidermolysis bullosa: part II.Other organs.J Am Acad Dermatol 61,387- 402;quiz 403-384(2009).

Foitzik K, Lindner G, Mueller-Roever S, Maurer M, Botchkareva N, Botchkarev V, Handjiski B, Metz M, Hibino T, Soma T, Dotto GP, Paus R:Control of murine hair follicle regression (catagen) by TGF-beta1 in vivo.FASEB J 14,752-760(2000).

Forouzandeh F, Jalili RB, Germain M, Duronio V, Ghahary A: Differential immunosuppressive effect of indoleamine 2,3-dioxygenase (IDO) on primary human CD4+ and CD8+ T cells.Mol Cell Biochem 309, 1-7(2008).

Fritsch A, Loeckermann S, Kern JS, Braun A, Bosl MR, Bley TA, Schumann H, von Elverfeldt D, Paul D, Erlacher M, Berens von Rautenfeld D, Hausser I, Fassler R, Bruckner-Tuderman L:A hypomorphic mouse model of dystrophic epidermolysis bullosa reveals mechanisms of disease and response to fibroblast therapy. J Clin Invest 118, 1669-1679(2008).

Fruci D, Lo Monaco E, Cifaldi L,Locatelli F, Tremante E, Benevolo M, Giacomini P: T and NK cells: two sides of tumor immunoevasion. J transla med, doi: 10.1186/1479-5876-11-30. (2013).

Fuchs E: Scratching the surface of skin development.Nature 445,834-842(2007).

Fuchs E: The tortoise and the hair: slow-cycling cells in the stem cell race.Cell 137,811-819(2009).

Fujiwara H, Ferreira M, Donati G, Marciano DK, Linton JM, Sato Y, Hartner A, Sekiguchi K, Reichardt LF, Watt FM:The basement membrane of hair follicle stem cells is a muscle cell niche.Cell 144,577-589(2011).

Galat A, Riviere S, Bouet F: Purification of macrophage migration inhibitory factor (MIF) from bovine brain cytosol.FEBS Lett 319,233-236(1993).

Gao GF, Tormo J, Gerth UC, Wyer JR, McMichael AJ, Stuart DI, Bell JI, Jones EY,Jakobsen BK:Crystal structure of the complex between human CD8alpha(alpha) and HLA-A2.Nature 387,630-634(1997).

Gáspár E, Nguyen-Thi KT, Hardenbicker C, Tiede S, Plate C, Bodó E, Knuever J, Funk W, Bíró T, Paus R:Thyrotropin-releasing hormone selectively stimulates human hair follicle pigmentation.J Invest Dermatol 131,2368-2377(2011).

Gelse K, Poschl E, Aigner T: Collagens--structure, function, and biosynthesis.Adv Drug Deliv Rev 55, 1531-1546(2003).

Geyfman M, Gordon W, Paus R, Andersen B: Identification of telogen markers underscores that telogen is far from a quiescent hair cycle phase.J Invest Dermatol 132,721-724(2012).

Gilfillan AM, Tkaczyk C: Integrated signalling pathways for mast-cell activation. Nat Rev Immunol 6,218-230(2006).

Gilhar A, Etzioni A, Paus R: Alopecia areata. N Engl J Med 366,1515-1525(2012)

.**Gilliver SC, Emmerson E, Bernhagen J, Hardman MJ**: MIF: a key player in cutaneous biology and wound healing.Exp Dermatol 20, 1-6(2011).

Goldstein J, Horsley V:Home sweet home: skin stem cell niches.Cell Mol Life Sci 69,2573-2582(2012).

Gonzalez ME:Evaluation and treatment of the newborn with epidermolysis bullosa. Semin Perinatol 37,32-39(2013).

Griffin LL, Michaelides C, Griffiths CEM, Paus R, Harries MJ: Primary cicatricial

alopecias: a U.K. survey. Br J Dermatol167,694-697(2012).

Gupta R, Woodley DT, Chen M:Epidermolysis bullosa acquisita. Clin Dermatol 30,60-69(2012).

Hainz U, Jürgens B, Heitger A: The role of indoleamine 2,3-dioxygenase in transplantation.Transpl Int 20,118-127(2007).

Hammers CM, Bieber K, Kalies K, Banczyk D, Ellebrecht CT, Ibrahim SM, Zillikens D, Ludwig RJ, Westermann J:Complement-fixing anti-type VII collagen antibodies are induced in Th1-polarized lymph nodes of epidermolysis bullosaacquisita-susceptible mice.J Immunol 187,5043-5050(2011).

Harries MJ, Meyer K, Chaudhry I, Kloepper J, Poblet E, Griffiths CEM, Paus R:Lichen planopilaris is characterized by immune privilege collapse of the hair follicle's epithelial stem cell niche. J Pathol doi: 10.1002/path.4233 (2013).

Harries MJ, Meyer KC, Chaudhry IH, Griffiths CE, Paus R: Does collapse of immune privilege in the hair-follicle bulge play a role in the pathogenesis of primary cicatricial alopecia? Clin Exp Dermatol 35,637-644(2009).

Harries MJ, Paus R: The pathogenesis of primary cicatricial alopecias.Am J Pathol 177,2152-2162(2010).

Harrist TJ, Ruiter DJ, Mihm MC, Jr., Bhan AK: Distribution of major histocompatibility antigens in normal skin.Br J Dermatol 109,623-633(1983).

Harvima IT, Nilsson G: Mast Cells as Regulators of Skin Inflammation and Immunity.Acta Derm Venereol 91, 644-650(2011)

Hashimoto T, Ishii N, Ohata C, Furumura M: Pathogenesis of epidermolysis bullosa acquisita, an autoimmune subepidermal bullous disease.J Pathol 228, 1- 7(2012).

Head JR, Billingham RE:Immunologically privileged sites in transplantation immunology and oncology.Perspect Biol Med 29,115-131(1985).

Headington JT: Transverse microscopic anatomy of the human scalp- A basis for a morphometric approach to disorders of the hair follicle.Arch Dermatol 120,449-456(1984).

Hendrix S, Handjiski B, Peters EMJ, Paus R: A guide to assessing damage response pathways of the hair follicle: lessons from cyclophosphamide-induced alopecia in mice.J Invest Dermatol 125, 42-51(2005).

Hennino A, Vocanson M, Toussaint Y, Rodet K, Benetiere J, Schmitt AM, Aries

MF, Berard F, Rozieres A, Nicolas JF:Skin-infiltrating CD8+ T cells initiate atopic dermatitis lesions.J Immunol 178, 5571-5577(2007).

Hibino T, Nishiyama T: Role of TGF-beta2 in the human hair cycle.J Dermatol Sci 35, 9-18(2004).

Hirose M, Vafia K, Kalies K, Groth S, Westermann J, Zillikens D, Ludwig RJ, Collin M, Schmidt E: Enzymatic autoantibody glycan hydrolysis alleviates autoimmunity against type VII collagen.J Autoimmun 39,304-314(2012).

Hoi CSL, Lee SE, Lu S-Y, McDermitt DJ, Osorio KM, Piskun CM, Peters RM, Paus R, Tumbar T:Runx1 directly promotes proliferation of hair follicle stem cells and epithelial tumor formation in mouse skin. Mol Cell Biol 30, 2518-2536(2010).

Holm TL, Markholst H: Confirmation of a disease model of pemphigus vulgaris: characterization and correlation between disease parameters in 90 mice. Exp Dermatol 19,e158-165(2010).

Holub BS, Kloepper JE, Tóth BI, Bíro T, Kofler B, Paus R: The neuropeptide galanin is a novel inhibitor of human hair growth. Br J Dermatol167,10-16(2012).

Hordinsky M: Cicatricial alopecia: discoid lupus erythematosus.Dermatol Ther 21,245-248(2008).

Hsu Y-C, Fuchs E: A family business: stem cell progeny join the niche to regulate homeostasis.Nat Rev Mol Cell Biol 13,103-114(2012).

Intong LR, Murrell DF: Inherited epidermolysis bullosa: new diagnostic criteria and classification.Clin Dermatol 30, 70-77(2012).

Ishii N, Hamada T, Dainichi T, Karashima T, Nakama T, Yasumoto S, Zillikens D, Hashimoto T: Epidermolysis bullosa acquisita: what's new? J Dermatol 37,220-230(2011).

Ito M, Liu Y, Yang Z, Nguyen J, Liang F, Morris RJ, Cotsarelis G:Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis Nat Med 11, 1351-1354(2005).

Ito N, Ito T, Kromminga A, Bettermann A, Takigawa M, Kees F, Straub RH, Paus R:Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol.FASEB J 19,1332-1334(2005).

Ito N, Sugawara K, Bodo E, Takigawa M, van Beek N, Ito T, Paus R: Corticotropin-releasing hormone stimulates the in situ generation of mast cells from precursors in the human hair follicle mesenchyme.J Invest Dermatol 130,995-1004(2010).

Ito T, Ito N, Bettermann A, Tokura Y, Takigawa M, Paus R:Collapse and restoration of MHC class-I-dependent immune privilege: exploiting the human hair follicle as a model. Am J Pathol 164,623-634(2004).

Ito T, Ito N, Saathoff M, Stampachiacchiere B, Bettermann A, Bulfone-Paus S, Takigawa M, Nickoloff BJ, Paus R:Immunology of the Human Nail Apparatus: The Nail Matrix Is a Site of Relative Immune Privilege.J Investig Dermatol 125, 1139-1148(2005).

Ito T, Ito N, Saatoff M, Hashizume H, Fukamizu H, Nickoloff BJ, Takigawa M, Paus R:Maintenance of hair follicle immune privilege is linked to prevention of NK cell attack.J Invest Dermatol 128,1196-1206(2008).

Jackow CM, Papadopoulos E, Nelson B, Tschen JA, Heatherington G, Duvic M:Follicular mucinosis associated with scarring alopecia, oligoclonal T-cell receptor V beta expansion, and Staphylococcus aureus: when does follicular mucinosis become mycosis fungoides? J Am Acad Dermatol 37,828-831(1997).

Jackson JM, Callen JP: Scarring alopecia and sclerodermatous changes of the scalp in a patient with hepatitis C infection.J Am Acad Dermatol 39,824-826(1998).

Jahoda CA, Reynolds AJ:Dermal-epidermal interactions. Adult follicle-derived cell populations and hair growth.Dermatol Clin 14,573-583(1996).

Jalili RB, Rayat GR, Rajotte RV, Ghahary A:Suppression of islet allogeneic immune response by indoleamine 2,3 dioxygenase-expressing fibroblasts.J Cell Physiol 213,137-143(2007).

Jaworsky C, Kligman AM, Murphy GF:Characterization of inflammatory infiltrates in male pattern alopecia: implications for pathogenesis.Br J Dermatol127,239-246(1992).

Jiang S, Zhao L, Teklemariam T, Hantash BM:Small cutaneous wounds induce telogen to anagen transition of murine hair follicle stem cells.J Dermatol Sci 60,143-150(2010).

Joachim RA, Handjiski B, Blois SM, Hagen E, Paus R, Arck PC:Stress-induced neurogenic inflammation in murine skin skews dendritic cells towards maturation and migration: key role of intercellular adhesion molecule-1/leukocyte function-associated antigen interactions.Am J Pathol 173,1379-1388(2008).

Joubeh S, Mori O, Owaribe K, Hashimoto T:Immunofluorescence analysis of the basement membrane zone components in human anagen hair follicles. Experimental dermatology 12,365-370(2003).

Karnik P, Stenn K:Cicatricial alopecia symposium 2011: lipids, inflammation and stem cells.J Invest Dermatol 132,1529-1531(2012).

Karnik P, Tekeste Z, McCormick TS, Gilliam AC, Price VH, Cooper KD, Mirmirani P:Hair follicle stem cell-specific PPARgamma deletion causes scarring alopecia.J Invest Dermatol 129,1243-1257(2009).

Karsten CM, Pandey MK, Figge J, Kilchenstein R, Taylor PR, Rosas M, McDonald JU, Orr SJ, Berger M, Petzold D, Blanchard V, Winkler A, Hess C, Reid DM, Majoul IV, Strait RT, Harris NL, Köhl G, Wex E, Ludwig R, Zillikens D, Nimmerjahn F, Finkelman FD, Brown GD, Ehlers M, Köhl J:Anti- inflammatory activity of IgG1 mediated by Fc galactosylation and association of FcγRIIB and dectin-1.Nat Med 18,1401-1406(2012).

Kasperkiewicz M, Muller R, Manz R, Magens M, Hammers CM, Somlai C, Westermann J, Schmidt E, Zillikens D, Ludwig RJ, Orosz A:Heat-shock protein 90 inhibition in autoimmunity to type VII collagen: evidence that nonmalignant plasma cells are not therapeutic targets.Blood 117,6135-6142(2011).

Kasperkiewicz M, Nimmerjahn F, Wende S, Hirose M, Iwata H, Jonkman MF, Samavedam U, Gupta Y, Moller S, Rentz E, Hellberg L, Kalies K, Yu X, Schmidt E, Hasler R, Laskay T, Westermann J, Kohl J, Zillikens D, Ludwig RJ:Genetic identification and functional validation of FcgammaRIV as key molecule in autoantibody-induced tissue injury.J Pathol 228,8-19(2012).

Kaufman G, d'Ovidio R, Kaldawy A, Assy B, Ullmann Y, Etzioni A, Paus R, Gilhar A:An unexpected twist in alopecia areata pathogenesis: are NK cells protective and CD49b+ T cells pathogenic? Exp Dermatol 19,e347-349(2010).

Khalil N:TGF-beta: from latent to active.Microbes Infect 1,1255-1263(1999).

Kinori M, Bertolini M, Funk W, Samuelov L, Meyer KC, Emelianov VU, Hasse S, Paus R:Calcitonin gene-related peptide (CGRP) may award relative protection from interferon-γ-induced collapse of human hair follicle immune privilege.Exp Dermatol 21,223-226(2012).

Kinori M, Kinori M. Kloepper JE. Paus R: "Can the hair follicle become a model for studying selected aspects of human ocular immune privilege?" Invest Ophthalmol Vis Sci 52,4447-4458(2011).

Kligman AM:The human hair cycle. J Invest Dermatol 33,307-316(1959).

Kloepper JE, Kawai K, Bertolini M, Kanekura T, Paus R:Loss of gammadelta T Cells Results in Hair Cycling Defects.J Invest Dermatol 133,1666-9 (2013).

Kloepper JE, Tiede S, Brinckmann J, Reinhardt DP, Meyer W, Faessler R, Paus R:Immunophenotyping of the human bulge region: the quest to define useful in-*situ* markers for human epithelial hair follicle stem cells and their niche.Exp Dermatol 17,592-609(2008).

Knuever J, Poeggeler B, Gáspár E, Klinger M, Hellwig-Burgel T, Hardenbicker C, Tóth BI, Bíró T, Paus R: Thyrotropin-releasing hormone controls mitochondrial biology in human epidermis.J Clin Endocrinol Metab 97,978-986(2012).

König R, Shen X, Maroto R, Denning TL:The role of CD4 in regulating homeostasis of T helper cells.Immunol Res 25,115-130(2002).

Kumamoto T, Shalhevet D, Matsue H, Mummert ME, Ward BR, Jester JV, Takashima A:Hair follicles serve as local reservoirs of skin mast cell precursors. Blood 102,1654-1660(2003).

Lander AD, Kimble J, Clevers H, Fuchs E, Montarras D, Buckingham M, Calof AL, Trumpp A, Oskarsson T: What does the concept of the stem cell niche really mean today? BMC Biol 10,19(2012).

Lau K, Paus R, Tiede S, Day P, Bayat A: Exploring the role of stem cells in cutaneous wound healing.Exp Dermatol 18,921-933(2009).

Langan EA, Vidali S, Pigat N, Funk W, Lisztes E, Bíró T, Goffin V, Griffiths CE, Paus R.: Tumour Necrosis Factor Alpha, Interferon Gamma and Substance P Are Novel Modulators of Extrapituitary Prolactin Expression in Human Skin. PLoS ONE 8 e60819. doi:10.1371/journal.pone.0060819(2013)

Lee J, Tumbar T: Hairy tale of signaling in hair follicle development and cycling. Semin Cell Dev Biol 23,906-916(2012).

Leech M, Metz C, Hall P, Hutchinson P, Gianis K, Smith M, Weedon H, Holdsworth SR, Bucala R, Morand EF:Macrophage migration inhibitory factor in rheumatoid arthritis: evidence of proinflammatory function and regulation by glucocorticoids.Arthritis Rheum 42,1601-1608(1999).

Licarete E, Ganz S, Recknagel MJ, Di Zenzo G, Hashimoto T, Hertl M, Zambruno G, Hundorfean G, Mudter J, Neurath MF, Bruckner-Tuderman L, Sitaru C:Prevalence of collagen VII-specific autoantibodies in patients with autoimmune and inflammatory diseases.BMC Immunol 13,16(2012).

Limat A, Wyss-Coray T, Hunziker T, Braathen LR:Comparative analysis of surface antigens in cultured human outer root sheath cells and epidermal keratinocytes: persistence of low expression of class I MHC antigens in outer root sheath cells in

vitro.Br J Dermatol 131,184-190(1994).

Lindner G, Botchkarev VA, Botchkareva NV, Ling G, van der Veen C, Paus R:Analysis of apoptosis during hair follicle regression (catagen).Am J Pathol 151,1601-1617(1997).

Lu B, Finn OJ:T-cell death and cancer immune tolerance.Cell Death Differ 15,70-79(2008).

Ludwig RJ:Model systems duplicating epidermolysis bullosa acquisita: a methodological review.Autoimmunity 45,102-110(2012).

Ludwig RJ, Recke A, Bieber K, Muller S, Marques Ade C, Banczyk D, Hirose M, Kasperkiewicz M, Ishii N, Schmidt E, Westermann J, Zillikens D, Ibrahim SM:Generation of antibodies of distinct subclasses and specificity is linked to H2s in an active mouse model of epidermolysis bullosa acquisita.J Invest Dermatol 131,167-176(2011).

Ludwig RJ, Schmidt E: Cytokines in autoimmune bullous skin diseases. Epiphenomenaor contribution to pathogenesis? G Ital Dermatol Venereol 144,339-349(2009).

Ludwig RJ, Zillikens D: Pathogenesis of epidermolysis bullosa acquisita. Dermatol Clin 29,493-501(2011).

Luger TA, Brzoska T: alpha-MSH related peptides: a new class of anti- inflammatory and immunomodulating drugs.Ann Rheum Dis 66, Suppl 3,iii,52-55(2007).

Luger TA,Scholzen TE, Brzoska T, Bohm M, Bohm M:New insights into the functions of alpha-MSH and related peptides in the immune system. Ann N Y Acad Sci.994,133-40(2003).

Marzano AV, Cozzani E, Fanoni D, De Pità O, Vassallo C, Berti E, Parodi A, Crosti C, Cugno M:Diagnosis and disease severity assessment of epidermolysis bullosa acquisita by ELISA for anti-type VII collagen autoantibodies: an Italian multicentre study.Br J Dermatol168,80-84(2013).

Maurer M, Opitz M, Henz BM, Paus R: The mast cell products histamine and serotonin stimulate and TNF-alpha inhibits the proliferation of murine epidermal keratinocytes in situ.J Dermatol Sci 16,79-84(1997).

McElwee KJ: Etiology of cicatricial alopecias: a basic science point of view.Dermatol Ther, 21,212-220(2008).

Mecklenburg L, Tobin DJ, Muller-Rover S, Handjiski B, Wendt G, Peters EM,

Pohl S, Moll I, Paus R:Active hair growth (anagen) is associated with angiogenesis. J Invest Dermatol 114,909-916(2000).

Meier N, Langan D, Hilbig H, Bodó E, Farjo NP, Farjo B, Armbruster FP, Paus R:Thymic peptides differentially modulate human hair follicle growth.J Invest Dermatol 132,1516-1519(2012).

Metz M, Maurer M:Mast cells-key effector cells in immune responses. Trends Immunol 28,234-241(2007).

Metz M, Siebenhaar F, Maurer M: Mast cell functions in the innate skin immune system. Immunobiology 213, 251-260(2008).

Meyer KC, Klatte JE, Dinh HV, Harries MJ, Reithmayer K, Meyer W, Sinclair R, Paus R:Evidence that the bulge region is a site of relative immune privilege in human hair follicles.Br J Dermatol 159,1077-1085(2008).

Meyer W, Gorgen S: Development of hair coat and skin glands in fetal porcine integument. J Anat 144, 201-220(1986).

Mihai S, Chiriac MT, Takahashi K, Thurman JM, Holers VM, Zillikens D, Botto M, Sitaru C:The alternative pathway of complement activation is critical for blister induction in experimental epidermolysis bullosa acquisita.J Immunol 178,6514-6521(2007).

Miteva M, Murrell DF, Tosti A:Hair loss in autoimmune cutaneous bullous disorders. Dermatol Clin 29,503-509,(2011)

Mobini N, Tam S, Kamino H: Possible role of the bulge region in the pathogenesis of inflammatory scarring alopecia: lichen planopilaris as the prototype.J Cutan Pathol 32, 675-679(2005).

Mohamadzadeh M, Berard F, Essert G, Chalouni C, Pulendran B, Davoust J, Bridges G, Palucka AK, Banchereau J:Interleukin 15 skews monocyte differentiation into dendritic cells with features of Langerhans cells.J Exp Med 194,1013-1020(2001).

Moretti G, Rebora A, Giacometti C, Boido V, Rampini E, Cipriani C: The quantitative behavior of cutaneous histamine and mast cells in the hair cycles of rats. J Invest Dermatol 46,231-239(1966).

Morris RJ, Liu Y, Marles L, Yang Z, Trempus C, Li S, Lin JS, Sawicki JA, Cotsarelis G: Capturing and profiling adult hair follicle stem cells.Nat Biotechnol 22,411-417(2004).

Müller R, Dahler C, Möbs C, Wenzel E, Eming R, Messer G, Niedermeier A, Hertl M:

T and B cells target identical regions of the non-collagenous domain 1 of type VII collagen in epidermolysis bullosa acquisita. Clin Immunol 135,99- 107(2010).

Muller-Rover S, Bulfone-Paus S, Handjiski B, Welker P, Sundberg JP, McKay IA, Botchkarev VA, Paus R:Intercellular adhesion molecule-1 and hair follicle regression.J Histochem Cytochem 48,557-568(2000)

Muller-Rover S, Handjiski B, van der Veen C, Eichmuller S, Foitzik K, McKay IA, Stenn KS, Paus R:A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages.J Invest Dermatol 117,3-15(2001).

Munro JM:Endothelial-leukocyte adhesive interactions in inflammatory diseases. Eur Heart J 14 Suppl K,72-77(1993).

Murauer EM, Gache Y, Gratz IK, Klausegger A, Muss W, Gruber C, Meneguzzi G, Hintner H, Bauer JW:Functional correction of type VII collagen expression in dystrophic epidermolysis bullosa. J Invest Dermatol 131,74-83(2011).

Murphy K.Janeway's Immunobiology: The Immune System. 8th ed,Garland Science 2011.

Myung P, Ito M: Dissecting the bulge in hair regeneration.Journal of Clinical Investigation 122,448-454(2012).

Nagao K, Kobayashi T, Moro K, Ohyama M, Adachi T, Kitashima DY, Ueha S, Horiuchi K, Tanizaki H, Kabashima K, Kubo A, Cho Y-h, Clausen BE, Matsushima K, Suematsu M, Furtado GC, Lira SA, Farber JM, Udey MC, Amagai M:Stress-induced production of chemokines by hair follicles regulates the trafficking of dendritic cells in skin.Nat Immunol 13,744-752(2012)a.

Nagao K, Kobayashi T, Ohyama M, Akiyama H, Horiuchi K, Amagai M: Brief report: requirement of TACE/ADAM17 for hair follicle bulge niche establishment. Stem Cells 30,1781-1785(2012)b.

Nepom GT, Erlich H: MHC class-II molecules and autoimmunity.Annu Rev Immunol 9, 493-525(1991).

Niederkorn JY: Anterior chamber-associated immune deviation.Chem Immunol 73, 59-71(1999).

Niederkorn JY: Immune privilege in the anterior chamber of the eye.Crit Rev Immunol 22, 13-46(2002).

Niederkorn JY: Mechanisms of immune privilege in the eye and hair follicle.J Investig Dermatol Symp Proc 8,168-172(2003).

Niederkorn JY: See no evil, hear no evil, do no evil: the lessons of immune privilege.NatImmunol 7,354-359(2006).

Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV: FcgammaRIV: a novel FcR with distinct IgG subclass specificity.Immunity 23, 41-51(2005).

Nimmerjahn F, Ravetch JV: FcgammaRs in health and disease.Curr Top Microbiol Immunol 350,105-125(2011).

Nishimura EK: Melanocyte stem cells: a melanocyte reservoir in hair follicles for hair and skin pigmentation.Pigment Cell Melanoma Res 24,401-410(2011).

Norde WJ, Hobo W, Voort Rvd, Dolstra H: Coinhibitory molecules in hematologic malignancies: targets for therapeutic intervention.Blood 120,728- 736(2012).

Ohyama M, Terunuma A, Tock CL, Radonovich MF, Pise-Masison CA, Hopping SB, Brady JN, Udey MC, Vogel JC:Characterization and isolation of stem cell-enriched human hair follicle bulge cells. J Clin Invest 116,249-260(2006).

Olsen E, Stenn K, Bergfeld W, Cotsarelis G, Price V, Shapiro J, Sinclair R, Solomon A, Sperling L, Whiting D:Update on cicatricial alopecia. J Investig Dermatol Symp Proc 8,18-19(2003).

Orinska Z, Maurer M, Mirghomizadeh F, Bulanova E, Metz M, Nashkevich N, Schiemann F, Schulmistrat J, Budagian V, Giron-Michel J, Brandt E, Paus R, Bulfone-Paus S:IL-15 constrains mast cell-dependent antibacterial defenses by suppressing chymase activities.Nat Med 13,927-934(2007).

Panicker SP, Ganguly T, Consolo M, Price V, Mirmirani P, Honda K, Karnik P:Sterol intermediates of cholesterol biosynthesis inhibit hair growth and trigger an innate immune response in cicatricial alopecia.PLoS One 7,e38449(2012).

Panteleyev AA, Jahoda CA, Christiano AM: Hair follicle predetermination. J Cell Sci 114,3419-3431(2001).

Paus R: Frontiers in the (neuro-) endocrine controls of hair growth.J Investig Dermatol Symp Proc 12, 20-22(2007).

Paus R:A neuroendocrinological perspective on human hair follicle pigmentation. Pigment Cell Melanoma Res 24,89-106(2011).

Paus R, and, Pecker S. 2003. Biology of Hair and Nail,' In Bolognia, J. L., Jorizzo, J. L., Rapini, R. P,eds Dermatology Mosby-Wolfe London.

Paus R, Botchkarev VA, Botchkareva NV, Mecklenburg L, Luger T, Slominski A: The

skin POMC system (SPS). Leads and lessons from the hair follicle.Ann N Y Acad Sci 885,350-363(1999)a.

Paus R, Cotsarelis G:The biology of hair follicles.N Engl J Med 341,491-497(1999)b.

Paus R, Czarnetzki BM: Bulge-activation hypothesis (continued). Am J Dermatopathol 16,458-460(1994)b.

Paus R, Czarnetzki BM: The "bulge-activation hypothesis" does not explain hair follicle cycling but may still be valid. Am J Dermatopathol 16,112-114(1994)a.

Paus R, Eichmüller S, Hofmann U, Czarnetzki BM, Robinson P:Expression of classical and non-classical MHC class I antigens in murine hair follicles.Br J Dermatol131,177-183(1994)c.

Paus R, Foitzik K:In search of the "hair cycle clock": a guided tour.Differentiation 72,489-511(2004).

Paus R, Foitzik K, Welker P, Bulfone-Paus S, Eichmüller S: Transforming growth factor-beta receptor type I and type II expression during murine hair follicle development and cycling. J Invest Dermatol 109,518-526(1997)a.

Paus R, Hofmann U, Eichmüller S, Czarnetzki BM: Distribution and changing density of gamma-delta T cells in murine skin during the induced hair cycle.Br J Dermatol130,281-289(1994)d

Paus R, Ito N, Takigawa M, Ito T: The hair follicle and immune privilege. J Investig Dermatol Symp Proc 8,188-194(2003).

Paus R, Maurer M, Slominski A, Czarnetzki BM: Mast cell involvement in murine hair growth.Dev Biol 163,230-240(1994)e.

Paus R, Nickoloff BJ, Ito T: A 'hairy' privilege. Trends Immunol 26,32-40(2005).

Paus R, Peters EM, Eichmüller S, Botchkarev VA: Neural mechanisms of hair growth control.J Investig Dermatol Symp Proc 2,61-68(1997)b

.**Paus R, van der Veen C, Eichmuller S, Kopp T, Hagen E, Muller-Rover S, Hofmann U**:Generation and cyclic remodeling of the hair follicle immune system in mice.J Invest Dermatol 111,7-18(1998).

Penn JW, Grobbelaar AO, Rolfe KJ: The role of the TGF-β family in wound healing, burns and scarring: a review.Int J Burns Trauma 2, 18-28(2012).

Peters EM, Botchkarev VA, Botchkareva NV, Tobin DJ, Paus R:Hair-cycle-associated remodeling of the peptidergic innervation of murine skin, and hair growth modulation by

neuropeptides.J Invest Dermatol 116,236-245(2001).

Peters EM, Handjiski B, Kuhlmei A, Hagen E, Bielas H, Braun A, Klapp BF, Paus R, Arck PC:Neurogenic inflammation in stress-induced termination of murine hair growth is promoted by nerve growth factor.Am J Pathol 165,259-271(2004).

Peters EM, Liotiri S, Bodo E, Hagen E, Biro T, Arck PC, Paus R: Probing the effects of stress mediators on the human hair follicle: substance P holds central position. Am J Pathol 171,1872-1886(2007).

Petukhova L, Duvic M, Hordinsky M, Norris D, Price V, Shimomura Y, Kim H, Singh P, Lee A, Chen WV, Meyer KC, Paus R, Jahoda CAB, Amos CI, Gregersen PK, Christiano AM:Genome-wide association study in alopecia areata implicates both innate and adaptive immunity.Nature 466,113-117(2010).

Pfendner, E. G. and A. W. Lucky: Dystrophic Epidermolysis Bullosa. GeneReviews.Seattle (WA), University of Washington, Seattle, 1993-2013, 2006

Philpott MP, Sanders D, Westgate GE, Kealey T: Human hair growth in vitro: a model for the study of hair follicle biology.J Dermatol Sci 7 Suppl, S55-72(1994).

Pi Lq Fau - Jin X-H, Jin Xh Fau - Hwang ST, Hwang St Fau - Lee W-S, Lee WS:Effects of calcitonin gene-related peptide on the immune privilege of human hair follicles.Neuropeptides 47,51-57(2013)

Plikus MV, Chuong C-M: Complex hair cycle domain patterns and regenerative hair waves in living rodents. J Invest Dermatol 128, 1071-1080(2008)a.

Plikus MV, Mayer JA, de la Cruz D, Baker RE, Maini PK, Maxson R, Chuong C-M:Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration.Nature 451,340-344(2008)b.

Pollack Bp Fau - Sapkota B, Sapkota B Fau - Cartee TV, Cartee TV: Epidermal growth factor receptor inhibition augments the expression of MHC class I and II genes. Clin Cancer Res.17, 4400-4413(2011)

Poole BD, Templeton AK, Guthridge JM, Brown EJ, Harley JB, James JA: Aberrant Epstein-Barr viral infection in systemic lupus erythematosus.Autoimmun Rev 8, 337-342(2009).

Powell BC, Rogers GE: The role of keratin proteins and their genes in the growth, structure and properties of hair. EXS 78, 59-148(1995).

Powell JJ, Dawber RP, Gatter K: Folliculitis decalvans including tufted folliculitis: clinical, histological and therapeutic findings.Br J Dermatol 140,328-33(1999)

Pozdnyakova O, Mahalingam M: Involvement of the bulge region in primary scarring alopecia.J Cutan Pathol 35,922-925(2008).

Ramot Y, Tiede S, Bíró T, Abu Bakar MH, Sugawara K, Philpott MP, Harrison W, Pietilä M, Paus R: Spermidine promotes human hair growth and is a novel modulator of human epithelial stem cell functions.PLoS One 6,e22564-e22564(2011).

Rausch A, Hessmann M, Hölscher A, Schreiber T, Bulfone-Paus S, Ehlers S, Hölscher C: Interleukin-15 mediates protection against experimental tuberculosis: a role for NKG2D-dependent effector mechanisms of CD8+ T cells.Eur J Immunol 36,1156-1167(2006).

Recke A, Sitaru C, Vidarsson G, Evensen M, Chiriac MT, Ludwig RJ, Zillikens D:Pathogenicity of IgG subclass autoantibodies to type VII collagen: induction of dermal-epidermal separation.J Autoimmun 34,435-444(2010).

Remington J, Chen M, Burnett J, Woodley DT: Autoimmunity to type VII collagen: epidermolysis bullosa acquisita.Curr Dir Autoimmun 10,195-205(2008).

Reynolds AJ, Jahoda CA: Hair matrix germinative epidermal cells confer follicle-inducing capabilities on dermal sheath and high passage papilla cells. Development 122,3085-3094(1996).

Reynolds AJ, Lawrence C, Cserhalmi-Friedman PB, Christiano AM, Jahoda CA:Trans-gender induction of hair follicles.Nature 402,33-34(1999).

Rompolas P, Deschene ER, Zito G, Gonzalez DG, Saotome I, Haberman AM, Greco V: Live imaging of stem cell and progeny behaviour in physiological hair-follicle regeneration.Nature 487,496-499(2012).

Rongioletti F, Christana K: Cicatricial (scarring) alopecias: an overview of pathogenesis, classification, diagnosis, and treatment.Am J Clin Dermatol 13,247-260(2012).

Rosenblum MD, Olasz EB, Yancey KB, Woodliff JE, Lazarova Z, Gerber KA, Truitt RL:Expression of CD200 on epithelial cells of the murine hair follicle: a role in tissue-specific immune tolerance?J Invest Dermatol 123,880-887(2004).

Rosenblum MD, Yancey KB, Olasz EB, Truitt RL: CD200, a "no danger" signal for hair follicles.J Dermatol Sci 41,165-174(2006).

Rousselle P, Keene DR, Ruggiero F, Champliaud MF, Rest M, Burgeson RE: Laminin 5 binds the NC-1 domain of type VII collagen.J Cell Biol 138,719-728(1997).

Ruckert R, Asadullah K, Seifert M, Budagian VM, Arnold R, Trombotto C, Paus R, Bulfone-Paus S: Inhibition of keratinocyte apoptosis by IL-15: a new parameter in the

pathogenesis of psoriasis? J Immunol 165, 2240-2250(2000)

Rygiel TP, Meyaard L: CD200R signaling in tumor tolerance and inflammation: A tricky balance.Curr Opin Immunol 24,233-238(2012).

Saleh MA, Ishii K, Kim Y-J, Murakami A, Ishii N, Hashimoto T, Schmidt E, Zillikens D, Shirakata Y, Hashimoto K, Kitajima Y, Amagai M:Development of NC1 and NC2 domains of type VII collagen ELISA for the diagnosis and analysis of the time course of epidermolysis bullosa acquisita patients.J Dermatol Sci 62,169-175(2011).

Samavedam UKSRL, Kalies K, Scheller J, Sadeghi H, Gupta Y, Jonkman MF, Schmidt E, Westermann J, Zillikens D, Rose-John S, Ludwig RJ:Recombinant IL-6 treatment protects mice from organ specific autoimmune disease by IL-6 classical signalling-dependent IL-1ra induction. Journal of Autoimmunity, 40,74–85(2013)

Sarita M, Kumari R, Rajesh NG, Thappa DM:Multiple skin nodules over the scalp and trunk. Cylindroma. Indian J Dermatol Venereol Leprol 78,517-518(2012).

Sayed BA, Christy A, Quirion MR, Brown MA:The master switch: the role of mast cells in autoimmunity and tolerance.Annu Rev Immunol 26,705-739(2008).

Schmidt E, Höpfner B, Chen M, Kuhn C, Weber L, Bröcker EB, Bruckner-Tuderman L, Zillikens D:Childhood epidermolysis bullosa acquisita: a novel variant with reactivity to all three structural domains of type VII collagen.Br J Dermatol147, 592-597(2002).

Schneider MR, Schmidt-Ullrich R, Paus R: The hair follicle as a dynamic miniorgan. Curr Biol 19, R132-142(2009).

Sesarman A, Sitaru AG, Olaru F, Zillikens D, Sitaru C: Neonatal Fc receptor deficiency protects from tissue injury in experimental epidermolysis bullosa acquisita.J Mol Med (Berl) 86,951-959(2008).

Sesarman A, Sitaru C: Induction of experimental epidermolysis bullosa acquisita by immunization with murine collagen VII.Methods Mol Biol 961,371-387(2013).

Severs GA, Griffin T, Werner-Wasik M: Cicatricial alopecia secondary to radiation therapy: case report and review of the literature.Cutis 81,147-153(2008).

Shimizu T: Role of macrophage migration inhibitory factor (MIF) in the skin.J Dermatol Sci 37,65-73(2005)a.

Shimizu T, Ogata A, Honda A, Nishihira J, Watanabe H, Abe R, Zhao Y, Shimizu H:Expression of macrophage migration inhibitory factor in rat skin during embryonic development.Exp Dermatol14,819-823(2005)b.

Singh A, Morris RJ: Innate immunity and the regulation and mobilization of keratinocyte stem cells: are the old players playing a new game? Exp Dermatol 21,660-664(2012)a.

Singh AM, Reynolds D, Cliff T, Ohtsuka S, Mattheyses AL, Sun Y, Menendez L, Kulik M, Dalton S: Signaling network crosstalk in human pluripotent cells: a Smad2/3-regulated switch that controls the balance between self-renewal and differentiation.Cell Stem Cell 10,312-326(2012)b.

Sitaru AG, Sesarman A, Mihai S, Chiriac MT, Zillikens D, Hultman P, Solbach W, Sitaru C:T cells are required for the production of blister-inducing autoantibodies in experimental epidermolysis bullosa acquisita.J Immunol 184,1596-1603(2010).

Sitaru C, Chiriac MT, Mihai S, Buning J, Gebert A, Ishiko A, Zillikens D: Induction of complement-fixing autoantibodies against type VII collagen results in subepidermal blistering in mice.J Immunol 177,3461-3468(2006).

Sitaru C, Mihai S, Otto C, Chiriac MT, Hausser I, Dotterweich B, Saito H, Rose C, Ishiko A, Zillikens D:Induction of dermal-epidermal separation in mice by passive transfer of antibodies specific to type VII collagen.J Clin Invest 115,870-878(2005).

Sitaru C, Mihai S, Zillikens D: The relevance of the IgG subclass of autoantibodies for blister induction in autoimmune bullous skin diseases. Arch Dermatol Res 299,1-8(2007).

Slominski A, Botchkareva NV, Botchkarev VA, Chakraborty A, Luger T, Uenalan M, Paus R:Hair cycle-dependent production of ACTH in mouse skin. Biochim Biophys Acta 1448,147-152(1998).

Slominski A, Wortsman J, Luger T, Paus R, Solomon S:Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. Physiol Rev 80,979-1020(2000).

Slominski A, Wortsman J, Plonka PM, Schallreuter KU, Paus R, Tobin DJ:Hair follicle pigmentation. J Invest Dermatol 124,13-21(2005).

Somani N, Bergfeld WF:Cicatricial alopecia: classification and histopathology. Dermatol Ther 21,221-237(2008).

Sompayrac LM. How the Immune System Works, Edition. 4 ed, 2012, Wiley-Blackwell.(2012)

Sperling LC, Solomon AR, Whiting DA: A new look at scarring alopecia.Arch Dermatol 136, 235-242(2000).

Stefanato CM: Histopathology of alopecia: a clinicopathological approach to diagnosis.Histopathology 56, 24-38(2010)

Stelekati E, Bahri R, D'Orlando O, Orinska Z, Mittrücker H-W, Langenhaun R, Glatzel M, Bollinger A, Paus R, Bulfone-Paus S:Mast cell-mediated antigen presentation regulates CD8+ T cell effector functions.Immunity 31,665-676(2009).

Stenn KS: Insights from the asebia mouse: a molecular sebaceous gland defect leading to cicatricial alopecia.J Cutan Pathol 28,445-447(2001).

Stenn KS, Cotsarelis G, Price VH: Report from the cicatricial alopecia colloquium. J Invest Dermatol 126,539-541(2006).

Stenn KS, Sundberg JP, Sperling LC: Hair follicle biology, the sebaceous gland, and scarring alopecias.Arch Dermatol 135,973-974(1999).

Sugawara K, Bíró T, Tsuruta D, Tóth BI, Kromminga A, Zákány N, Zimmer A, Funk W, Gibbs BF, Zimmer A, Paus R:Endocannabinoids limit excessive mast cell maturation and activation in human skin.J Allergy Clin Immunol129,726-738.e728-726-738.e728(2012).

Sundberg JP, Boggess D, Sundberg BA, Eilertsen K, Parimoo S, Filippi M, Stenn K:Asebia-2J (Scd1(ab2J)): a new allele and a model for scarring alopecia. Am J Pathol 156,2067-2075(2000).

Sundberg JP, Peters EMJ, Paus R: Analysis of hair follicles in mutant laboratory mice.J Investig Dermatol Symp Proc 10,264-270(2005).

Suto H, Nakae S, Kakurai M, Sedgwick JD, Tsai M, Galli SJ: Mast cell-associated TNF promotes dendritic cell migration.J Immunol 176, 4102-4112(2006).

Taylor AW, Kitaichi N: The diminishment of experimental autoimmune encephalomyelitis (EAE) by neuropeptide alpha-melanocyte stimulating hormone (alpha-MSH) therapy.Brain Behav Immun 22,639-646(2008).

Taylor AW, Lee DJ:The alpha-melanocyte stimulating hormone induces conversion of effector T cells into treg cells.J Transplant 2011,(2011).

Tiede S, Kloepper JE, Bodo E, Tiwari S, Kruse C, Paus R: Hair follicle stem cells: walking the maze.Eur J Cell Biol 86, 355-376(2007)a.

Tiede S, Kloepper JE, Whiting DA, Paus R: The 'follicular trochanter': an epithelial compartment of the human hair follicle bulge region in need of further characterization.Br J Dermatol 157, 1013-1016(2007)b.

Tiede S, Koop N, Kloepper JE, Fässler R, Paus R:Nonviral in situ green fluorescent protein labeling and culture of primary, adult human hair follicle epithelial progenitor cells.Stem Cells 27,2793-2803(2009)a.

Tiede S, Kloepper JE, Ernst N, Poeggeler B, Kruse C,Paus R: Nestin in human skin: exclusive expression in intramesenchymal skin compartments and regulation by leptin.J Invest Dermatol. 129, 2711-2720(2009)b

Tobin DJ, Gunin A, Magerl M, Handijski B, Paus R: Plasticity and Cytokinetic Dynamics of the Hair Follicle Mesenchyme: Implications for Hair Growth Control.J Investig Dermatol 120,895-904(2003).

Topfer K, Kempe S, Muller N, Schmitz M, Bachmann M, Cartellieri M, Schackert G, Temme A:Tumor evasion from T cell surveillance.J Biomed Biotechnol 2011, 918471(2011).

Torres-Alvarez B, Mesa-Garza IG, Castanedo-Cazares JP, Fuentes-Ahumada C, Oros-Ovalle C, Navarrete-Solis J, Moncada B:Histochemical and immunohistochemical study in melasma: evidence of damage in the basal membrane. Am J Dermatopathol 33,291-295(2011).

Tosti A, Duque-Estrada B, Murrell DF: Alopecia in epidermolysis bullosa.Dermatol Clin 28,165-169(2010).

Trempus CS, Morris RJ, Bortner CD, Cotsarelis G, Faircloth RS, Reece JM, Tennant RW: Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34.J Invest Dermatol 120,501-511(2003).

Trempus CS, Morris RJ, Ehinger M, Elmore A, Bortner CD, Ito M, Cotsarelis G, Nijhof JG, Peckham J, Flagler N, Kissling G, Humble MM, King LC, Adams LD, Desai D, Amin S, Tennant RW:CD34 expression by hair follicle stem cells is required for skin tumor development in mice.Cancer Res 67,4173-4181(2007).

Tripathi P, Naik S, Agrawal S: HLA-E and immunobiology of pregnancy.Tissue Antigens 67,207-213(2006).

Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, Fuchs E: Defining the epithelial stem cell niche in skin.Science 303,359-363(2004).

Ujiie H, Shimizu H: Evidence for pathogenicity of autoreactive T cells in autoimmune bullous diseases shown by animal disease models.Exp Dermatol 21,901-905(2012).

Varki R, Sadowski S, Pfendner E, Uitto J: Epidermolysis bullosa. I. Molecular genetics of the junctional and hemidesmosomal variants.J Med Genet 43,641-652(2006).

Varki R, Sadowski S, Uitto J, Pfendner E: Epidermolysis bullosa. II. Type VII collagen mutations and phenotype-genotype correlations in the dystrophic subtypes.J Med Genet 44,181-192(2007).

Veraitch O, Ohyama M, Yamagami J, Amagai M: Alopecia as a rare but distinct manifestation of pemphigus vulgaris.J Eur Acad Dermatol Venereol 27, 86-91(2013).

Wahl SM, Wen J, Moutsopoulos N: TGF-beta: a mobile purveyor of immune privilege.Immunol Rev 213,213-227(2006).

Walker ME, Hatfield JK, Brown MA: New insights into the role of mast cells in autoimmunity: evidence for a common mechanism of action? Biochim Biophys Acta 1822,57-65(2012).

Watt FM, Fujiwara H: Cell-extracellular matrix interactions in normal and diseased skin. Cold Spring Harb Perspect Biol 3,(2011).

Weber A, Knop J, Maurer M: Pattern analysis of human cutaneous mast cell populations by total body surface mapping. Br J Dermatol148,224-228(2003).

Weedon D: Weedon's Skin Pathology, 3rd.Edition, Churchill Livingstone 2009.

Weller K, Foitzik K, Paus R, Syska W, Maurer M: Mast cells are required for normal healing of skin wounds in mice.FASEB J 20, 2366-2368(2006).

Whiting DA: Cicatricial alopecia: clinico-pathological findings and treatment.Clin Dermatol 19,211-225(2001).

Whiting DA. The Structure of Human Hair Follicle: Light Microscopy of Vertical and Horizontal Sections of Scalp Biopsies. 4th edition, New Jersey, Canfield publishing, 2004

Wong KY, Rajora N, Boccoli G, Catania A, Lipton JM:A potential mechanism of local anti-inflammatory action of alpha-melanocyte-stimulating hormone within the brain: modulation of tumor necrosis factor-alpha production by human astrocytic cells.Neuroimmunomodulation 4,37-41(1997).

Woodley DT, Ram R, Doostan A, Bandyopadhyay P, Huang Y, Remington J, Hou Y, Keene DR, Liu Z, Chen M:Induction of epidermolysis bullosa acquisita in mice by passive transfer of autoantibodies from patients.J Invest Dermatol 126, 1323-1330(2006).

Wozniak K, Hashimoto T, Ishii N, Koga H, Huczek M, Kowalewski C:Fluorescence overlay antigen mapping using laser scanning confocal microscopy differentiates linear IgA bullous dermatosis from epidermolysis bullosa acquisita mediated by IgA.Br J Dermatol168,634-638(2013).

Wright GJ, Cherwinski H, Foster-Cuevas M, Brooke G, Puklavec MJ, Bigler M, Song Y, Jenmalm M, Gorman D, McClanahan T, Liu MR, Brown MH, Sedgwick JD, Phillips JH, Barclay AN:Characterization of the CD200 receptor family in mice and humans and their interactions with CD200.J Immunol 171, 3034-3046(2003).

Wucherpfennig KW, Strominger JL: Selective binding of self peptides to disease-associated major histocompatibility complex (MHC) molecules: a mechanism for MHC-linked susceptibility to human autoimmune diseases.J Exp Med 181, 1597-1601(1995).

Xu X, Solky B, Elenitsas R, Cotsarelis G: Scarring alopecia associated with mastocytosis.J Cutan Pathol 30,561-565(2003).

Yano K, Brown LF, Detmar M: Control of hair growth and follicle size by VEGF-mediated angiogenesis. J Clin Invest 107,409-417(2001).

Yeoman H, Ramrakha PS, Sharp RJ, Stanley MA: The role of antigen-presenting cells in the survival of murine-cultured keratinocyte allografts. Transplant Proc 21, (1989).

Zhou Y, Jiang X, Gu P, Chen W, Zeng X, Gao X: Gsdma3 mutation causes bulge stem cell depletion and alopecia mediated by skin inflammation. Am J Pathol 180,763-774(2012).

8.0 Appendix

List of reagents and solutions

1. Eosin (0.1%)

100 mg of eosin in 100 ml of ethanol

2. Meyer's Hematoxylin:

Dissolve 50 g alum in 1000 mls of distilled water and add 1g of hematoxylin, 0.2 g of sodium iodate and 20 ml of glacial acetic acid boil and filter on cooling. Dispense the solution into an air tight brown glass bottle and store it at room temperature.

3. Naphthol AS-D chloroacetate solution:

1ml N,N dimethyl formamide mixed with 35 ml Sörensens working solution and 200 ul of nitrosylated pararosaniline.

4. Pararosaniline hydrochloride (pH-6.3,):

0.5 g of pararosaniline hydrochloride powder (Merck,Germany) in 20 ml distilled water and 2.5 ml HCl heated, filtered and stored in the fridge.

5. Periodic acid Schiff (PAS):

0.5 mg in 100 mls of distilled water

6. Phosphate buffered saline (pH-7.2)

0.8 g of sodium chloride (NaCl) and 1.8 g of sodium dihydrogen phosphate monohydrate (NaH_2PO_4) mixed in 1 L of distilled water. pH was adjusted using 1N NaOH.

7. Sodium veronal working solution:

Made from suspending 5 ml vernol stock in 100 ml distilled water

8. Sörsen's working solution:

32.8ml of Solution A (2.73 g Na_2HPo_4 in 250 ml distilled water) mixed with 7.2 ml solution B (2.27 g KH_2Po_4 in 250 ml distilled water).

9. Toluidine blue (TB):

1 g of toluidine blue mixed with 0.1g of disodium tetraborate 10 hydrate and 0.2 g paraformaldehyde in 100 ml distilled water.

10. Tris-borate buffered saline (pH-7.6)

8.8 g of sodium chloride (NaCl) and 6.19 g of Tris Borate mixed in 1L of distilled water. Corresponding pH was adjusted using 1N NaOH.

11. Tris-NaCl-Tween buffer (pH-7.5)

15.76 gms of Tris HCl and 8.76 g of NaCl added to 1L of distilled water. Additionally 0.05% Tween 20 is added and the pH was adjusted using 1N NaOH

12. Veronol acetate buffer:

Sodium veronal stock solution was made by mixing 2.89 g sodium vernol and 1.15 g sodium acetate in 100 ml distilled water and stored at 4^0 C

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Project titile: Immune privilege collapse and epithelial stem cell damage during hair follicle inflammation in murine epidermolysis bullosa acquisita (EBA)

Abstracts published as part of the PhD thesis project

- **Uppalapati SK**, Meyer KC, Hirose M, Mihai S, Zillikens D, Ludwig R.

  Antibody-induced cicatricial alopecia in epidermolysis bullosa acquisita (EBA): Lessons on the immunopathology of epithelial stem cell deletion from a passive EBA mouse model. *J Invest Dermatol* 129: (2009), S-68

- **S. K.Uppalapati**, K. C. Meyer, M. Hirose, S. Mihai, F. Nimmerjahn, D.Zillikens, R. J. Ludwig and R. Paus Lessons on the immunopathology of epithelial stem cell destruction and antibody-induced cicatricial alopecia from a passive transfer epidermolysis bullosa acquisita (EBA) mouse model *Exp Dermatol* 19: (2010) issue 2, page 191.

Other associated abstracts and publications during my thesis work

- **S. K. Uppalapati**, M. Bertolini, K. C. Meyer, S. Tiede, P. Peterson and R. Paus.Gene and protein expression of AIRE (Autoimmune regulator) in AA c ompared to normal human scalp skin. *Exp Dermatol* 19: (2010) issue 2, page 191

Other publications made during the course of the thesis project:

- Reithmayer K, Meyer KC, Kleditzsch P, Tiede S, **Uppalapati SK**, Gläser R, Harder J, Schröder JM, Paus R. Human hair follicle epithelium has an antimicrobial defense system that includes the inducible antimicrobial peptide psoriasin (S100A7) and RNase 7. *Br J Dermatol* 161: (2009) 78-89.

- Rakers S, Gebert M, **Uppalapati SK**, Meyer W, Maderson P, Sell AF, and Kruse C, Paus R. 'Fish matters': the relevance of fish skin biology to investigative dermatology. *Exp Dermotol* 19: (2010) 313-24.