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“The contribution of IL-17A and IL-17F in the pathogenesis of antibody-induced Epidermolysis Bullosa Acquisita”

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Zusammenfassung

Epidermolysis bullosa acquisita (EBA) ist eine chronische Autoimmunerkrankung der Haut, die durch Autoantikörper gegen Kollagen Typ VII verursacht wird und durch Neutrophile in der dermal-epidermalen Junktion gekennzeichnet ist. Das Interleukin (IL)-17A vermittelt infektiöse und autoimmune Erkrankungen, indem es die Rekrutierung und Aktivierung von Neutrophilen fördert. Zudem wurde angedeutet, dass IL-17A zur Pathogenese der EBA beiträgt. Übereinstimmend zeigen unsere Daten bisher, dass IL-17A-deletierte ($^{-/-}$) Mäuse resistent gegenüber Antikörper-induzierter EBA sind. In der vorliegenden Arbeit wurden Beteiligung und Mechanismen von IL-17A und IL-17F zur Pathogenese von Antikörper-induzierter EBA untersucht. Jedoch konnte in dieser Studie der Verlauf der Krankheit nur zum Teil in IL-17 Rezeptor (R) a$^{-/-}$ und IL-17A/F$^{-/-}$ Mäusen reduziert werden. Da IL-17A, IL-17E und IL-17F ihre Signale durch den IL-17Ra vermitteln, deuten diese Ergebnisse an, dass IL-17E keine Rolle bei experimenteller EBA spielt. Im Gegensatz weiste IL-17F eine pathogene Rolle auf, da IL-17F$^{-/-}$ Mäuse eine verminderte EBA aufzeigten. Die anscheinend inkonsequenten Ergebnisse aus Versuchen mit IL-17A$^{-/-}$, IL-17F$^{-/-}$, IL-17A/F$^{-/-}$ und IL-17Ra$^{-/-}$ Mäusen deuten an, dass möglicherweise weitere Faktoren zu dem resistenten Phänotyp von IL-17A$^{-/-}$ Mäusen beitragen. Während der genetische Hintergrund von IL-17A$^{-/-}$ Mäusen aus Embryostammzellen von 129 Mäusen basiert, zeigen genetische Analysen dieser Studie, dass Gen-Cluster in IL-17A$^{-/-}$ von 129 Mäusen stammen, welche resistent gegenüber EBA sind und somit implizieren, dass die von 129 Mäusen stammenden Gene zur EBA Resistenz beitragen. In dieser Arbeit wurde die pathogene Rolle von IL-17A durch die Neutralisierung dieses Zytokins mittels monoklonalen Antikörpern bestätigt. Mechanistisch wurden γδT Zellen als Hauptproduzenten von IL-17A identifiziert und IL-1β als stärkster Vermittler für die IL-17A Produktion. Als Reaktion auf IL-17A induzierten Keratinozyten und Fibroblasten das Chemokin CXCL-1, welches die Migration von Neutrophilen beschleunigen und schließlich die Entwicklung von EBA fördern kann. Diese Studie zeigt erstmals die pathogene Rolle von IL-17A und IL-17F in der durch Antikörper induzierten Pathogenese der EBA auf. Das von γδT Zellen produzierte IL-17A spielt eine zentrale Rolle in der experimentellen EBA indem es die Freilassung der Chemokine in Keratinozyten und Fibroblasten induziert, um die Infiltration von Neutrophilen zu fördern und anschließend die Blasenbildung in diesen Krankheiten zu verschärfen.

Abstract

Epidermolysis bullosa acquisita (EBA) is a chronic autoimmune skin disease caused by autoantibodies against type VII collagen and characterized by neutrophils in the dermal-epidermal junction (DEJ). Interleukin (IL)-17A is reported to mediate infectious and autoimmune diseases by promoting the recruitment and activation of neutrophils: it is implied that IL-17A may contribute to the pathogenesis of EBA. Consistently, our preliminary data showed that antibody-induced EBA was completely abrogated in IL-17A-deficient ($^{-/-}$) mice. This thesis investigates the relative contribution and mechanism of IL-17A and IL-17F to the pathogenesis of antibody-induced EBA. IL-17A is postulated to be essential in mediating subepidermal bullous skin disorders. However, in the present study, EBA severity was only partially reduced in IL-17 receptor (R) a$^{-/-}$ and IL-17A/F$^{-/-}$ mice. Because IL-17A, IL-17E and IL-17F signals are mediated through IL-17Ra, these results implied that IL-17E had no impact on experimental EBA, whereas IL-17F may play a protective role in EBA. To the contrary, it revealed a pathogenic role of IL-17F in EBA, since IL-17F$^{-/-}$ mice had ameliorated antibody-induced EBA. The apparently inconsistent results obtained in IL-17A$^{-/-}$, IL-17F$^{-/-}$, IL-17A/F$^{-/-}$, and IL-17Ra$^{-/-}$ mice suggested other factors may contribute to the resistant phenotype of IL-17A$^{-/-}$ mice. Whereas IL-17A$^{-/-}$ mice were generated in embryonic stem cells from a 129 mice genetic background, genetic analysis revealed that clusters of genes in IL-17A$^{-/-}$ mice originated from 129 mice which are resistant to EBA, implying that genes derived from 129 mice may contribute to EBA resistance in IL-17A$^{-/-}$ mice. Nevertheless, the pathogenic role of IL-17A in the development of EBA was confirmed by neutralization of IL-17A with monoclonal antibodies. Mechanistically, γδT cells were identified as the major IL-17A producing cells, and IL-1β the most potent mediator for the production of IL-17A. In response to IL-17A, keratinocytes and fibroblasts resulted to induce chemokine CXCl-1, which could accelerate the migration of neutrophil and promote the development of EBA. This study for the first time reveals the pathogenic role of IL-17A and IL-17F in the pathogenesis of antibody-induced EBA. IL-17A derived from γδT cells, plays a pivotal role in experimental EBA by the induction of chemokine release in keratinocytes and fibroblasts to promote neutrophil infiltration and eventually aggravate blistering disease.

1. Appendix

1.1. List of abbreviation

AhR：aryl hydrocarbon receptor

APC：antigen present cell

Batf: basic leucine zipper transcription factor

BD-2：Beta-defensin-2

BMZ: basement membrane zone

BP: bullous pemphigoid

BP180: 180-kD bullous pemphigoid antigen, type XVII collagen

BP230: 230-kD bullous pemphigoid antigen

C/EBPs：CCAAT/enhancer binding proteins

C3a: complement 3a

C5a: complement 5a

CCL: chemokine ligand

CD: cluster of differentiation

cDNA：complementary deoxyribonucleic acid

CIA: collagen induced arthritis

CMP: acartilage matrix protein

Col7: type VII collagen

CTLA-8: cytotoxic T lymphocyte

CXCL: chemokines containing chemokine ligand

CXCR: chemokines containing chemokine receptor

DC：dentritic cell

DEJ: dermal-epidermal junction

DETC：dendritic morphology

DNA：deoxyribonucleic acid

DSS：dextran sulfate sodium

EAE: Experimental Autoimmune Encephalomyelitis

EBA: Epidermolysis bullosa acquisita

eGFP：enhanced green fluorescent protein

ES：embryonic stem

FNIII: fibronectin III

G-CSF: granulocyte colony-stimulating factor

GM-CSF: granulocyte macrophage colony-stimulating factor

GST: Glutathione S-transferase

IBD: inflammatory bowel disease

IC: immune complexes

IFN-γ : interferon-γ

IgA: immunoglobulin A

IgG: immunoglobulin G

IkBf: inhibitor zeta

IL: interleukin

IL-17R：IL-17 receptor

JAK：Janus kinase

LAD: linear IgA bullous dermatosis

LTi：lymphoid tissue inducer

MAPKs：mitogen-activated protein kinases

mCol7: murine type VII collagen

MMP: matrix metalloproteinase

mRNA： messager ribonucleic acid

MS: Multiple Sclerosis

NC domain: non-collagenous domain

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B

NKT cell: natural killer T cell

NR-IgG: normal rabbit IgG

PAMPs: pathogen-associated molecular patterns

PGE2：prostaglandin E2

PI3K：phosphoinositide 3-kinases

PPR: pattern recognition receptors

RA: rheumatoid arthritis

RORa: Retinoic acid receptor related orphan receptor a

ROR γ t: Retinoic acid receptor related orphan receptor γ t

ROS: reactive oxygen species

S100A7：S100 calcium-binding protein A7

SEFIR：SEFs and IL17Rs

SLE: systemic lupus erythematosus

Stat3: signal transducer and activator of transcription 3

T1DM: type 1 diabetes mellitus

Tc17 cell：CD8+ T cell

TCR: T cell receptor

TGF-β1: transforming growth factor-β1

Th cell: helper T cells

Timp-1：tissue inhibitor of metalloproteinases 1

TLR2: Toll-like receptor 2

TNF-α: tumor necrosis factor-α

TRAF6：tumor necrosis factor receptor associated factor 6

TUNEL：Terminal deoxynucleotidyl transferase dUTP nick end labeling

vWF: von Willebrand factor

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2. Introduction

2.1. Immunity and autoimmunity

2.1.1. The immune system

In response to pathogenic micro-organisms around us, the body's immune system has evolved to protect it from all kinds of potential pathogens, such as bacteria, parasites and viruses. Based on specificity and memorability, the immune system can be divided into two major branches: the innate immune system and the adaptive immune system [1].

The innate immune system, as the first line of defense, primarily consists of physical barriers and innate immune cells [2],[3]. The physical barriers formed by skin and mucosa protect the body from most pathogenic organisms. In some conditions, where the barriers are damaged and pathogenic organisms invade the body, a panel of innate immune cells, such as neutrophils, macrophages and eosinophils, migrate into the injured sites and erase these "invaders" by phagocytosis and secreting anti-pathogen substances [2]. To recognize the pathogens, innate immune cells develop a family of receptors, pattern recognition receptors (PPR), which can specifically bind to certain molecules expressed on pathogens (pathogen-associated molecular patterns (PAMPs)) and initiate immune responses immediately [3],[4]. However, since the diversity of the receptors is comparatively low, their specificity is limited [5]. Furthermore, the innate immune system functions as the initiator of the adaptive immune response [6]. Using professional antigen-presenting cells, the innate immune system can present molecules of pathogenic organisms to T-cells, a major component of the adaptive immune system, which subsequently trigger a series of adaptive immune responses [7]. The adaptive immune system is another indispensable part of immune surveillance. Its major components include T-cells and B-cells, which drive the cellular and humoral immune responses respectively. Upon binding with pathogen molecules presented by the innate immune system, T-cells are activated and differentiate into effected T-cells, which can contribute to the immune response in several ways, such as enhancing the pathogen-killing activities of innate immune cells, generating cytotoxicity on

pathogen-infected cells, promoting antibody productions by B-cells and modulating immune responses by secreting cytokines [8]. Since the activation of the adaptive immune responses requires antigen presentation by the innate immune system and more inter-cellular communication is involved in this process, the response time to pathogens of the adaptive immune system is much longer than that of the innate immune system. However, the pathogen recognition receptors of T-cells and B-cells are highly heterogeneous, making the adaptive immune system able to recognize and respond to a large spectrum of pathogens. In addition, during the adaptive immune response, memory T-cells and B-cells are also produced, which can react rapidly without the help of the innate immune system a second time [9].

2.1.2. Self-tolerance and autoimmunity

Besides protecting against pathogenic organisms, another critical function of the immune system is self-tolerance [10]. The immune system can recognize molecules of its own body and not generate an immune response against them. This is a self-protecting mechanism, by which the immune system can kill and erase non-self substances but not damage the body. The major mechanism involved in this process is negative selection [11]. During the proliferation and development of T-cells, a vast spectrum of T-cell receptors (TCR) are expressed on the T-cells, which enables the T-cells to recognize hundreds and thousands of molecules. However, some T-cells can also recognize the molecules of their own body. To delete these autoreactive T-cells, during the maturation process of T-cells in the thymus, epithelial cells express a variety of molecules of the body, autoreactive T-cells recognize these self-molecules and activate a self-death program [11],[12]. Although there is no such negative selection during the generation of B-cells, the primary function of B-cells largely relies on T-cells [13]. Therefore, by this mechanism the autoreactive B-cells are not activated and thus do not damage the body.

Although self-tolerance is generally well-maintained, it can be dysregulated by some factors, such as chemicals, viruses and genetic mutations [14],[15]. In this case, autoimmunity can be induced, in which the body's own organs or proteins are damaged by autoreactive immune

cells. For instance, during T-cell maturation in the thymus, the expression of human molecules by the thymus epithelial cells is critical. If the function of these cell is dysregulated and these molecules are not expressed properly, autoreactive T-cells can escape negative selection [16]. After leaving the thymus, these autoreactive T-cells are activated by self-molecules (self-antigen), and trigger a series of self-damaging processes, including over-expression of cytokines and promotion of autoantibody production. Since multiple factors are involved in maintaining the homeostasis of self-tolerance, the autoimmune diseases are complicated and thus the mechanisms underlying autoimmunity are still largely unknown.

2.1.3. Autoimmune diseases

As introduced above, when self-tolerance is dysregulated, autoimmunity can be induced and autoimmune diseases develop. Since the mechanisms involved in this process are complicated, there are large numbers of autoimmune diseases in which distinctive disease features are manifested and different pathogeneses are involved [17]. Up to now, more than 80 autoimmune diseases have been reported [18],[19], the total prevalence is 12.5±7.9% of the whole population [20], and women are more commonly affected [21]. Since our understanding of the pathogenesis of autoimmune disease is poor, there are no effective treatments for most autoimmune diseases, which leads to considerable morbidity and mortality.

Autoimmune diseases can affect virtually every part of the body, such as the endocrine system, the connective tissue, the gastrointestinal tract, the heart, the skin, and the kidneys. According to the extent of the tissues affected, autoimmune diseases can be categorized into organ-specific and systemic diseases [22]. In organ-specific autoimmune diseases, autoreactive immune cells recognize certain self-antigens and attack a single organ; therefore, the disease mechanisms are relatively better investigated. Many autoimmune diseases can be classified in this category, such as type I diabetes mellitus, in which the pancreas is targeted and damaged by immune cells [23], multiple sclerosis, which is a central nervous

system-specific autoimmune disorder [24], and pemphigoid disease, in which the skin is the predominant targeted organ [25]. In contrast to organ-specific autoimmune disease, systemic autoimmune diseases are characterized by multiple organ involvement and a more complicated disease pathogenesis. For example, systemic sclerosis, an autoimmune disease with a poorly understood pathogenesis, is characterized by inflammation, fibrosis, and vasculopathy in multiple organs, such as the lung, skin, heart and esophagus [26]. It is of note that the above categorization of autoimmune diseases does not reflect the pathogenesis and etiology of the disease, being based primarily on the clinical involvement of organs instead of the expression pattern of self-antigens that are targeted by autoimmunity. Furthermore, autoimmune diseases can be further classified as autoimmune cell-mediated disorders or autoantibody-induced diseases according to the specific immune reaction [22]. Rheumatoid arthritis (RA) is a typical systemic autoimmune disease caused by T-cells [27]. Pemphigoid diseases are autoantibody-induced skin-specific autoimmune disorders [28]. However, autoimmune diseases frequently involve innate immune cells as well as the adaptive immune system, and both autoimmune cells and autoantibodies contribute to the pathogenesis of the autoimmune response.

2.2. Epidermolysis Bullosa Acquisita (EBA)

Epidermolysis bullosa acquisita (EBA) was firstly reported by Elliott [29], who characterized patients with the disease by severe skin blistering throughout the body. EBA is a chronic autoimmune blistering disease (AIBD) clinically characterized by features resembling those of hereditary dystrophic epidermolysis bullosa on the skin and mucosal surfaces [29]. In further studies, autoantibodies against type VII collagen (Col7) were identified as the major mediator of EBA [30]. Like many other autoimmune diseases, the prevalence of EBA is low, ranging from 0.2 to 0.5 per million people per year, and EBA is prone to developing in the elderly and female population [31],[32],[33]. Although EBA has been studied for more than a century, many questions on its pathogenesis still remain, which greatly hinder the treatment of the disease.

2.2.1. The structure of skin

The skin is the largest organ of the body, and the first line of the innate immune system. It forms a physical barrier that protects from pathogens. Moreover, the skin also secretes several anti-bacterial substances, which further augment its protective function [34]. Besides its important role in immune defense, the skin is indispensable for maintaining the homeostasis of the body. With the large surface area and thermal regulating elements, the skin can help to adjust the body temperature to the appropriate level essential for the performance of most organs [35],[36].

The skin is composed of two primary layers: the epidermis and the dermis (Figure 1) [37],[38],[39]. The epidermis is the outermost layer of skin, which primarily consists of keratinocytes, melanocytes, Merkel cells and Langerhans cells. Keratinocytes are major components of the epidermis, accounting for more than 90% of the total cell number [39],[40]. On the basis of the morphological features of maturating keratinocytes, the epidermis can be further subdivided into the following layers: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum [39]. Due to the high proportion of keratinocytes, the epidermis is tough and resilient, which enables the skin to protect the body from a number of harmful substances, such as chemicals, bacteria, viruses, and fungi. Beneath the epidermis is the dermis [39]. It is mainly composed of fibroblast and collagen, which provides mechanical strength to the skin. According to the arrangement of collagen, the dermis is divided into two areas: the superficial papillary dermis containing a thin arrangement of collagen fibers, and the deeper reticular dermis made of thick collagen fibers containing epidermal appendages, embedded nerves, blood vessels, fibroblasts, and various immune cells [39],[41].

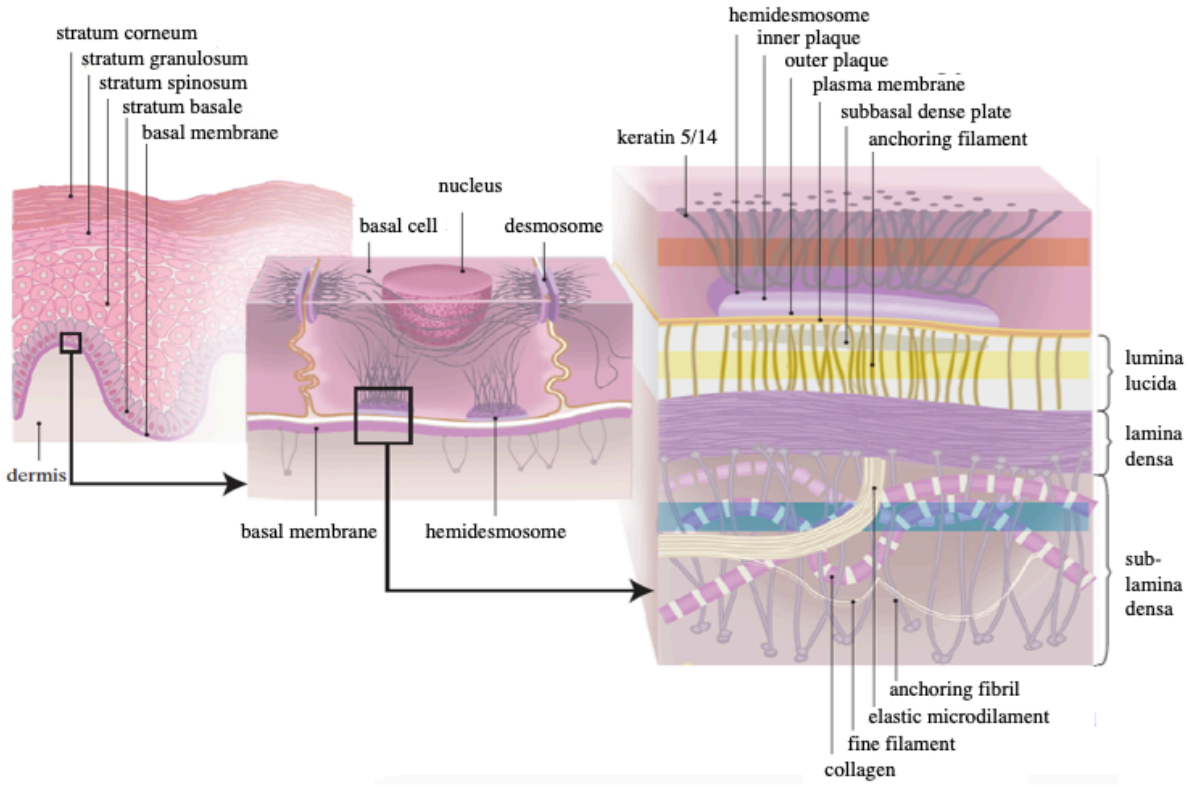

Figure 1. Schematic view of the skin structure. The dermis and epidermis are separated by the basement membrane zone called the dermal-epidermal junction (DEJ). Illustration of the epidermis (left), lower aspect of the basal keratinocyte with the epidermal basement membrane zone (middle), and the hemidesmosome adhesion complex (right). (figure adapted from Nederlands Tijdschrift voor Geneeskunde. 2003;147:1108-13) [42]

Between the lower part of the epidermis and the top layer of the dermis is a basement membrane zone called the dermal-epidermal junction (DEJ), which tightly connects the epidermis and dermis [43],[37]. Ultrastructurally, the DEJ can be further divided into four layers: 1) the cell membranes of basal keratinocytes, which contain hemidesmosomes; 2) the lamina lucida, an electron-lucent region which anchoring filaments traverse; 3) the lamina densa, an electron-dense area, which interacts with dermal collagens (Type I, III, V); 4) the sublamina densa, which contains anchoring fibrils, dermal microfibril bundles, and collagen fibers [44]. Besides providing the structural integrity of the skin, the DEJ can regulate epithelial-mesenchymal interactions and the permeability of the barrier, participate in signal transduction, and provide protection against shearing forces. More importantly, several structural proteins in the skin DEJ are auto-antigens in autoimmune blistering diseases [45],[46]. BP180 (type XVII collagen) linked to Laminin 332 has been identified as an

autoantigen of bullous pemphigoid [47],[48],[49]. BP230 located in the hemidesmosomal electron-dense inner plaque is one target antigen of linear IgA pemphigoid disease [50],[51]. Type VII collagen as main anchoring fibrils in the sublamina have been demonstrated to be the specific target antigens initiating a process that leads to the separation of the epidermis and dermis in EBA [52],[30].

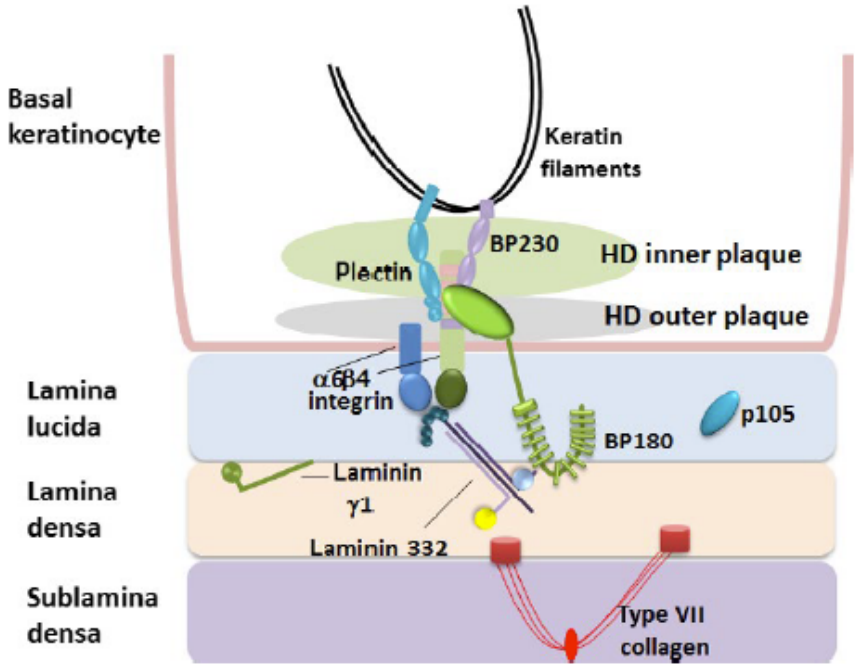

Figure 2. Schematic diagram of the components in the dermal-epidermal junction. Localization of major target antigens of pemphigoid diseases including plectin, bullous pemphigoid (BP) antigens BP180 andBP230, α6β4 integrin, laminin 332, the laminin γ1 chain and epidermolysis bullosa acquisita (EBA) antigen type VII collagen is depicted (Figure from *Exp. Dermatol.* August, 1154–1162, 2017) [46].

2.2.2. Type VII collagen as autoantigen

In 1984, type VII collagen (Col7) was identified by Woodley as a major component of the anchoring fibrils that provide stability to dermal-epidermal adhesion [30]. Col7 is primarily synthesized by keratinocytes and fibroblasts, and is distributed in a number of organs, including the skin, cornea, oral mucosa, cervix, esophagus, colon, anus and chorioamnion [52],[53]. Like other collagens, Col7 is a trimer with a triple-helical structure. It consists of

three identical αchains (Figure 3) [54]. Each chain contains a repeating Gly-X-Y sequence that is separated by a 39-amino acid non-collagenous hinge region. Moreover, the collagenous triple-helical domain of Col7 is flanked by two non-collagenous (NC) domains: the amino-terminal globular domain (NC1) and carboxy-terminal globular domain (NC2) [55]. The NC1 domain has several subdomains that are structurally similar to adhesive proteins: a cartilage matrix protein (CMP) domain, nine fibronectin III-like (FNIII) domains, a von-Willebrand-factor A-like (vWF) domain, and a cysteine and proline-rich domain [56],[57]. The seventh to ninth FNIII domains of NC1 bind to laminin-332 and the vWF domain binds to type I collagen [58]. In contrast to NC1, the NC2 domain does not provide binding sites to other proteins, but it has a similar structure to the Kunitz protease inhibitor molecule, thus may be involved in stabilization of the collagen [59].

Notably, Col7 has been reported to be an autoantigen in EBA, and the NC1 domain has been identified as a major pathogenic epitope [60],[61]. However, the antibody's reactivity to the collagenous domain or the NC2 domain has rarely been found [62],[63].

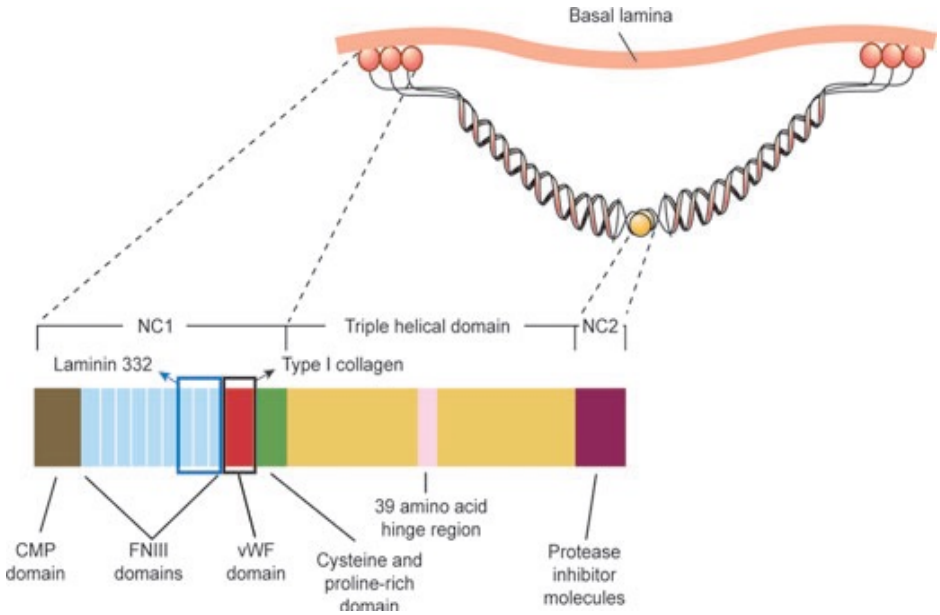

Figure 3. Schematic diagram of human type VII collagen. The collagenous triple-helical domain of type VII collagen (Col7) is flanked by two non-collagenous (NC) domains, NC1 and NC2. The NC1 domain is composed of a cartilage matrix protein (CMP) domain, nine fibronectin III (FNIII)-like domains and a von-Willebrand-factor-like domain (vWF). The Kunitz-BPTI (bovine pancreatic trypsin inhibitor) domain of the NC2 domain is structurally similar to protease inhibitors (Figure from Journal of the European Academy of Dermatology and Venereology 2013, 27, 1204–1213) [54].

2.2.3. Manifestation of EBA

EBA patients are characterized by a panel of manifestations which distinguish EBA from other bullous diseases [31],[64]. Clinically, fragility, tense blisters, and scarring are commonly observed in the skin of EBA patients. When direct immunofluorescence analysis is performed, subepidermal cleavage and a linear tissue-bound IgG or IgA and complement along dermal-epidermal junction can be detected in the affected skin of the patient, which serve as strong diagnostic evidence for EBA [64],[65]. In sera, high levels of autoantibodies against Col7, another typical sign of EBA, are produced in EBA patients [66],[67],[68].

Despite the general manifestation described above, EBA is a clinically heterogeneous disease and can be divided into two clinical subtypes: inflammatory EBA, which is the major subtype and affects more than 60% of total EBA patients, and non-inflammatory EBA [54],[69]. Based on the location of the affected skin, inflammatory EBA can be further categorized into four

types [70],[54]: (1) bullous pemphigoid (BP)-like EBA, which is the most common type of inflammatory EBA and manifests as pruritic erythematous vesicobullous eruptions that can occur in any area including the trunk, central body, extremities and oral mucosa [71]; (2) mucous membrane pemphigoid, which predominantly involves the mucous membranes and can lead to symblepharon and blindness [72]; (3) presentation reminiscent of Brunsting-Perry pemphigoid with scarring lesions predominantly localized in the head and neck [73]; (4) presentation similar to linear IgA bullous dermatosis (LAD) characterized by linear IgA deposition on the dermal side of the basement membrane zone (BMZ) [74].

In contrast to the inflammatory subtype, the non-inflammatory form is less frequently observed in EBA patients. Patients with this type of EBA often develop skin manifestations such as fragility, tense blisters, scarring, and milia, in trauma-prone sites and the extensor skin surface [29]. In addition, nail dystrophy and pigmentation are also frequently observed in non-inflammatory EBA. The clinical presentation of mild disease is similar to porphyria cutanea tarda, while severe cases are comparable to hereditary recessive dystrophic epidermolysis bullosa [75].

2.2.4. Treatment of EBA

Due to the low prevalence of EBA and limited knowledge of the disease mechanisms, therapy for EBA usually proves to be very difficult and lengthy [64]. Current recommendations for EBA treatment are, therefore, solely based on the clinical expertise of clinicians specialized in autoimmune bullous dermatoses [76]. Systemic treatment with corticosteroids (0.5-1.0 mg/kg/day) is considered as the first choice for moderate and severe conditions [77]. Because of the strong side effects of high-dose therapy, lower-dose corticosteroid treatment is usually combined with other modulatory immunosuppressive strategies such as Azathioprine, Mycophenolate, Cyclosphorin, Dapsone, Cyclophosphamide, and Methotrexate to lower the corticosteroid dose [78]. The use of colchicine is described to be successful for mild disease, although the adverse effects of gastrointestinal complaints can make it difficult for patients to achieve the prescribed dosage [79]. Rituximab (anti-CD20 monoclonal antibody) and

high-dose intravenous immunoglobulin have been described to reduce symptoms in refractory EBA patients [80].

2.2.5. Pathogenesis of EBA

As mentioned above, autoantibodies against Col7 were identified as the major driver of EBA [30]. After the generation of autoantibodies in EBA patients, a series of pathogenic processes follow, which lead to pathological changes in the patient [31],[81],[82]. Although the etiology is elusive, it is widely believed that $CD4^+$ T-cells are required for the production of anti-Col7 antibodies [83]. After being released into the blood, the anti-Col7 autoantibodies deposit in the dermal-epidermal junction and bind to Col7. Upon binding to the target, the autoantibodies trigger the activation of the complement cascade, which generates the cleavage products, C3a and C5a [84],[85]. These products have strong chemotactic properties, and can recruit immune cells, mainly neutrophils, into the DEJ [86]. After the neutrophils bind to Fc part of the anti-Col7 complex, they are activated and several protein degradation substances are released, such as reactive oxygen species (ROS), matrix metalloproteinase (MMP), elastase, and gelatinase, which hydrolyze the protein in DEJ and cause subepidermal blister formation [87],[88]. Moreover, during this pathological process, other immune cells and resident cells, such as mast cells, eosinophils and keratinocytes, are activated and produce cytokines and chemokines, which in turn recruit more immune cells and augment the tissue-damaging effect of the neutrophils [82],[89],[90],[91].

2.2.6. Experimental EBA models

Animal models are powerful tools for studying the pathogenesis of diseases. Several mouse models have been established to investigate EBA. These EBA models can be classified into active models and passive models, each of which represents diseases features resembling those in EBA patients and is used to explore the patho-mechanisms of the disease [92].

Since autoantibodies against Col7 have been reported to strongly correlate with the development of EBA, the active EBA mouse model was established to validate the pathogenicity of the antibodies and identify the pathogenic epitopes. To trigger the production of anti-Col7 antibodies, GST tagged recombinant murine NC1 domain of Col7 (GST-mCol7C) is used for immunization [93]. After immunization, these mice generate circulating anti-mCol7 antibodies, which can bind to the DEJ. Consequently, EBA-like pathological alteration in the form of subepidermal cleavage are observed in immunized mice [93]. Using this EBA mouse model, the GST-mCol7C has been validated as a pathogenic epitope and the pathogenicity of anti-Col7 autoantibodies demonstrated. Besides GST-mCol7C, von-willebrand-factor-A2-like subdomain (VWFA-2) of Col7, which is a new antigenic epitope, has also been used to establish the EBA model [94]. In response to immunization with VWFA-2, mice generated autoantibodies against Col7, and showed a deposition of anti-Col7 IgG autoantibodies and C3 at the DEJ. Furthermore, skin blister and erosion accompanied by neutrophil infiltration developed in mice immunized with VWFA-2, which mimicked the pathophysiological features of the human disease [94]. Since these active EBA models mimic the full disease course of EBA, they can be used to investigate not only the pathological processes of the effecter phase but also the events involved in the initial phase.

Beside active EBA models, EBA can also be induced in mice by passive transfer of anti-Col7 autoantibodies. In 2005, a passive EBA model was firstly reported by two research groups. One group immunized rabbits with recombinant protein of the NC1 domain of murine Col7 to generate anti-Col7 autoantibodies, and transferred the total IgG from immunized rabbits into C57Bl/6 or BALB/c mice [95]. Two to four days after receiving these polyclonal antibodies, the mice developed subepidermal blisters and erosions in a dose-dependent manner, and exhibited deposition of C3 and anti-Col7 antibodies at the DEJ, all of which resemble the disease in EBA patients [95]. Similarly, the second group applied the same establishment procedure, but with some modifications. They immunized rabbits with the recombinant NC1 domain of human Col7, instead of murine Col7, and transferred the isolated IgG to SKH1 mice, which are hairless, making it easy to evaluate the disease severity [96]. This model recapitulated several EBA-like features, including skin blistering and erosion, as well as IgG

and complement deposition in the DEJ [96]. Moreover, other passive EBA models have also been established, such as the transfer of autoantibodies from patients to induce EBA disease in mice [97],[98],[99]. Antibody transfer-induced EBA mouse models primarily resemble the effecter phase of the disease, and are widely used to investigate the mechanisms involved in autoantibody-induced tissue damage, such as Fc receptor binding [100], complement activation [84],[95], the effect of reactive oxygen species induction [81], as well as the contribution of cytokines and chemokines in blister formation [101],[102],[103].

The abovementioned EBA models facilitate studies on the pathogenesis of EBA. However, these models have limitations: (1) all of the models are induced by immunization of epitopes or transfer of IgG, which very likely is not the trigger for anti-Col7 production in humans, thus these models give little information on how the pathogenic autoantibodies are generated in patients; (2) In human patients, EBAs are categorized into two subtypes, inflammatory and non-inflammatory. The current EBA models generally mimic the features of inflammatory EBA, therefore are not suitable for studies on the pathogenesis of non-inflammatory EBA.

2.3. Interleukin 17

2.3.1. Cytokines in autoimmunity

Cytokines are proteins, peptides, or glycoproteins secreted by various cells that mediate the interactions and communications between cells [104]. Cytokines can be classified as interleukins (leukocyte modulation) as Interleukin-1(IL-1), IL-4, IL-10, IL-17; growth factors (growth and maturation of immune cells) such as tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), and chemokines containing chemokine (C-X-C motif) ligand 1 (CXCL1), chemokine (C-C motif) ligand 1 (CCL1) [104],[105]. All these molecules have interactive and multifunctional roles in immune cell development, immunoregulation and immune effector functions [106]. Cytokines are made by many cell populations, but the predominant producers are helper T-cells (Th) and innate immune cells [104]. They are often produced in a cascade and act as a network to target various cell types, while the activity on cells depends on the expression of cytokine specific receptors on the cell

membrane. Cytokines have double faces of functions, pro-inflammatory as well as anti-inflammatory, depending on the condition under which they are released from the cells [105].

Accordingly, the role of cytokines had been extensively studied in the pathogenesis of autoimmune disease. If immune tolerance is lost and host tissue antigens become autoimmune targets, cytokines can impact inflammation in autoimmune diseases [107]. In Experimental Autoimmune Encephalomyelitis (EAE) and Multiple Sclerosis (MS), IL-1 has been implicated as being pro-inflammatory in terms of disease pathogenesis and is also well-known for the promotion of inflammatory responses [108]. In contrast, some cytokines such as IL-10 mediate the immunosuppressive and immunoregulatory effects of inflammation [109]. The balance between the production of pro- and anti-inflammatory cytokines is the key to explaining the mechanisms underlying autoimmunity.

2.3.2. IL-17A and IL-17F in autoimmune diseases

The IL-17 cytokine family is the most-described set of molecules which are associated with many autoimmune diseases. To date, IL-17 family consists of six members including IL-17A (commonly referred to as IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F [110],[111],[112],[113],[114] (Figure 4).

In 1993, IL-17A, originally named CTLA-8, was first cloned from a murine cytotoxic T lymphocyte hybridoma library [115]. Until 1995, IL-17A was confirmed as a cytokine according to its structural evidence coupled with its ability [116],[117]. Murine IL-17A is a 21 kDa glycoprotein that consists of a 19 amino acid signal sequence followed by a 136 amino acid mature segment [115], sharing 63% identity with human IL-17A which contains 155 amino acids [115]. After identifying the structure of IL-17A, the biological function and immune regulation were well-investigated. Increased IL-17A level has been detected in the synovial fluids and synovium of RA patients [118],[119]. Consistent with the clinical study, mouse models of RA have demonstrated a key role for IL-17A in the progression of this disease. A deficiency of IL-17A markedly suppresses disease severity from collagen-induced

arthritis (CIA) [120],[121]. A blockade of IL-17 after disease onset effectively prevents bone and cartilage erosion and reduces the severity of clinical symptoms [122], while over-expression of IL-17A exacerbating the disease progression [123]. Apart from RA, up-regulated IL-17A has been detected in the brain lesion regions of MS patients [124], and increased IL-17A-producing T-cells are associated with disease activity [125]. In an EAE study, IL-17A deficient mice and anti-IL-17A antibody treatment exhibited significantly delayed onset and progression of disease compared to control mice [126],[127]. Emerging evidence is proving the central role of IL-17A in the pathogenesis of autoimmune diseases.

Among all the IL-17 family members, IL-17F exhibits the highest homology with IL-17A [128],[129]. Moreover, the genes encoding IL-17A and IL-17F are close to each other and are located on the same chromosome in both mice (chromosome 1) and humans (chromosome 6) [130]. Like IL-17A，the IL-17F protein contains 163 amino acids and is expressed as a disulphide-linked homodimer. In addition to the IL-17A and IL-17F homodimers, a heterodimeric IL-17A/F molecule has been shown to be produced [131]. Unlike for IL-17A, research on the function of IL-17F in autoimmune diseases is limited. IL-17F is highly expressed in both psoriatic skin biopsies and serum [132],[133],[134],[135]. The coincident finding was acquired in a psoriasis-like mice model, where mRNA expression of IL-17F increased in diseased mice. Despite its similarity to IL-17A, the contribution of IL-17F to the pathogenesis of autoimmune diseases has been shown to be extremely limited. IL-17F was not required for the initiation of EAE, but IL-17F$^{-/-}$ mice showed a slight delay in disease onset and progression compared to wild-type mice [136]. Using IL1RN knockout mice, the spontaneous development of arthritis was found to be only partially suppressed in mice that were also IL-17F deficient [121]. These studies have indicated that IL-17F seems to overlap functionally with IL-17A, but the exact role of IL-17F in the pathogenesis of autoimmune diseases still remains to be determined.

2.3.3. Other IL-17 family cytokines

IL-17E (also called IL-25) is the most distant and only shares a 23% sequence identity with IL-17A. Correspondingly, unlike IL-17A and IL-17F, IL-17E can enhance the Th2 immune response by the introduction of Th2 cytokines in allergic and infective inflammation [137],[138],[139],[140]. IL-17E was considered a 'double edged sword', because it was reported to have the ability to down-regulate localized destructive inflammatory responses such as inactivating the function of Th17 cells [141]. However, IL-17E has also been demonstrated to be associated with a pro-inflammatory response in autoimmune diseases [142]. Furthermore, IL-17E increases expression of nuclear factor kappa-light-chain-enhancer of activated B (NF-κB)-responsive luciferase reporter gene activity in addition to inducing the release of IL-8 [143].

IL-17C is another important member of the IL-17 family. IL-17C is up-regulated during inflammation and is detected in the lung [144]. It has been shown to play a major role in innate immunity[129]. In addition to its functions in mucosal immunity [145],[146], IL-17C is a critical factor in the immune response of the central nervous system [147]. IL-17B and IL-17D are the least characterized members of the IL-17 family. With regard to structure, IL-17B and IL-17D share structural identity with each other. The functions of IL-17B and IL-17D still remain largely elusive, although sporadic research has postulated these two cytokines to have pro-inflammatory effects under certain conditions [148],[112].

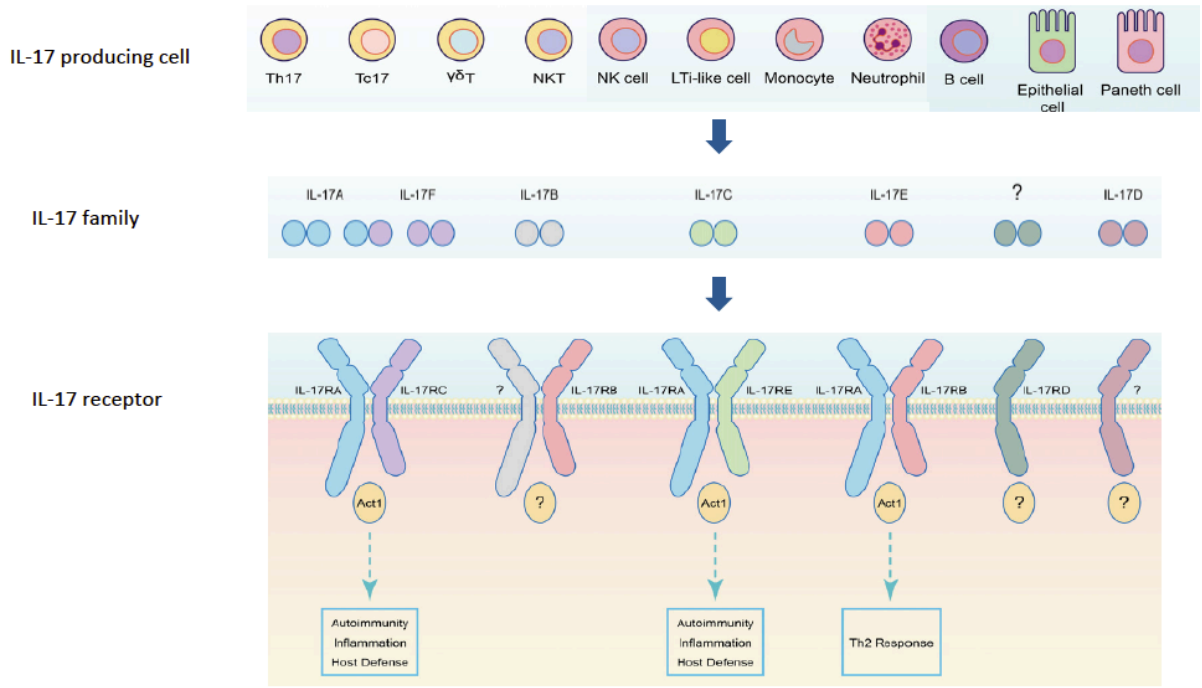

Figure 4. Overview of IL-17 family cytokines, cellular sources and receptors. IL-17 family cytokines consist of six members IL-17A to IL17F, while the receptor family contains five members, IL-17RA to IL-17RE. The main cellular sources of IL-17 are Th17 cells and other immune cells such as γδT cells, lymphoid tissue inducer cells (LTi), innate lymphoid cells type 3 (ILC3s), natural killer cells (NK), monocytes, neutrophils and B-cells. Non-immune cells such as intestinal Paneth cells and colonic epithelial cells also have ability to produce IL-17 cytokines (Figure adapted from *Cell signal*, vol 25, 2335-2347, Dec.2013 and *Oral Dis*, vol 23, 854-865, 2012). [149],[150]

2.3.4. Cellular sources of IL-17A and IL-17F

IL-17A is primarily released by a distinct $CD4^+$ T-cell subset, named Th17-cells [151]. The discovery of the Th17-cell subset with its signature cytokines has been one of the most important advances in T-cell immunology since the discovery of Th1 and Th2 cells [152],[153]. Th17 cells preferentially produce IL-17A, IL-17F, IL-21, and IL-22 [154],[155], whereas TH1 and Th2 cells mainly produce interferon-γ(IFN-γ) and IL-4 respectively. As a distinct population, Th17 cells are specialized by the unique identification of differentiation regulators and transcription factors. In general, IL-23 is considered a key factor to maintaining Th17 cells. Besides that, differentiation of Th17 cells is dependent on TGF-β, IL-6, IL-1 β, and TNF-α [156]. The retinoic acid receptor related orphan receptor γt (RORγt), was firstly

identified as the Th17 linage-specific transcription factor [157]. Other critical transcription factors associated with the commitment of Th17 cells include the signal transducer and activator of transcription 3 (Stat3), retinoic acid receptor related orphan receptor α(RORα), nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) cells, inhibitor zeta (IkBf) and basic leucine zipper transcription factor (Batf) [158].

Apart from Th17 cells, other types of T-cells such as the γδT-cell, $CD8^+$ T (Tc17) cell and natural killer T (NKT) cell also produce IL-17A and IL-17F [159], however, IL-17A and IL-17F are not always co-expressed. Among these IL-17-producing cell types, γδT-cells are well-investigated. γδT-cells account for approximately 3-5% of all lymphocytes and are highly prevalent at mucosal tissues and epithelial sites, especially the intestine, skin and lung [160]. According to the TCR usage, peripheral γδT-cells are composed of two main subsets: $Vγ1^+$ and $Vγ4^+$ T-cells. These two subsets have divergent functions during different inflammation situations, however, the $Vγ4^+$ subset is reported as the dominant source of IL-17 [161],[162]. Unlike αβT-cells, the special TCR expressed on γδT-cells appears to act more like pattern recognition receptors [163],[164]. Together with the expression of Toll-like receptors [165], γδT-cells can rapidly react to antigens and initiate an innate immune response by producing immunoregulators as the first line of defense. γδT-cells constitutively express IL-1R, IL-23R and RORγt, so that IL-23 and IL-1β can directly promote IL-17 production by γδT-cells in the absence of IL-6 and TCR ligation [130]. Besides that, IL-18 and TGF-β have been implicated in the development of IL-17-producing γδT-cells. Furthermore, activation of dendritic cell (DC) associated C-type lectin 1(dectin 1), STAT3, as well as internal receptor aryl hydrocarbon receptor (AhR) have been involved in the differentiation of IL-17-producing γδT-cells [165],[163].

In addition to IL-17-producing T-cells, innate immune cells such as neutrophils, monocytes, mast cells, natural killer cells, and lymphoid tissue inducer (LTi) like cells are other sources of IL-17A and IL-17F, and play an important role during infectious and autoimmune response [159],[166]. Most recently, B-cells have also been found to have the capability of secreting IL-17A during infection [167]. Lastly, non-immune cells such as intestinal Paneth cells and colonic epithelial cells have been shown to produce IL-17A and IL-17F, respectively

[121],[168].

IL-17-producing cells, either adaptive immune cells, innate immune cells, or non-immune cells, are responsible for the generation of IL-17 under several conditions, suggesting IL-17 as a bridge between adaptive and innate immunity to regulate and modulate immune responses.

2.3.5. Receptors for IL-17A and IL-17F

The IL-17 receptors family consists of five subunits, IL-17Ra through IL-17Re [169]. The receptors of IL-17 are widely expressed not only on non-hematopoietic cells, such as fibroblast, keratinocyte, and epithelia cells, but can also be detected on innate immune cells such as macrophages and neutrophils [160]. All IL-17 receptors have conserved structural similarity by containing two fibronectin III-like domains in the extracellular region and a SEF/IL-17R (SEFIR) domains in the intracellular part [169]. Functional receptors for IL-17 family cytokines are thought to exist in the form of homodimers or heterodimers. IL-17Ra was the first defined receptor in the family and is a common shared subunit [130],[169]. IL-17Ra paired with IL-17Rc recognizes IL-17A, IL-17F and IL-17A/F. Although IL-17Ra also binds to IL-17F and IL-17A/F, studies have shown that IL-17Ra binds with high affinity to IL-17A, medium affinity to IL-17A/F and low affinity to IL-17F [170]. Unlike IL-17Ra, IL-17Rc has been shown to have similar affinities for IL-17A, IL-17F and IL-17A/F. Together with IL-17Rb, IL-17Ra forms a receptor complex that binds IL-17E [171], whereas IL-17C has been recently reported to bind to IL-17Ra complexed with IL-17Re [172]. IL-17Rd, previously considered as a negative regulator in fibroblast growth factor mediated pathways, is associated with IL-17A signaling [173].

2.3.6. Signaling pathways of IL-17A and IL-17F

IL-17A and IL-17F signals are associated with the adaptor protein Act1 through the SEFIR domain, which is stabilized by Hsp90 [174]. Act1 is able to activate the nuclear factor Kb (NF-kB) pathway, mitogen-activated protein kinases (MAPKs) cascade, and the

CCAAT/enhancer binding proteins (C/EBPs) pathway in the presence of the tumor necrosis factor receptor associated factor 6(TRAF6) to up-regulate the expression of a variety of pro-inflammatory chemokines and cytokines [166],[175],[171]. It has been suggested that the engagement of the IL-17 to IL-17 receptor can activate other common downstream signaling pathways including Janus-activated kinase-Phosphoinositide 3-kinases (JAK-PI3K) and Janus-activated kinase-signal transducers and activators of transcription (JAK-STAT) cascades [176],[177], however the exact downstream molecular mechanisms are still elusive. Moreover, IL-17 signaling plays a major role in mRNA stability [178].

2.3.7. Function of IL-17A and IL-17F with neutrophils

The best-known function of IL-17A is described during bacterial and fungal infections. IL-17A plays protective roles in host defense against the pathogens at the epithelial and mucosal barriers. IL-17A can induce anti-microbial peptides (HBD2, S100A7 and S100A8) secreted by epithelial cells in response to invading pathogens [179]. Most importantly, IL-17A is an essential regulator of the activation and migration of neutrophils in inflammation. IL-17A mediates the release of neutrophil-activating cytokines as well as granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) from macrophages by inducing the production of IL-1β and TNF-α [180],[181]. IL-17A could stimulate the release of chemokines such as CXCL-1 that specifically attract neutrophils and monocytes to sites of inflammation [182]. In addition, if IL-17A is expressed in an unbalanced fashion, it promotes matrix metalloproteinase (MMP) production to accelerate tissue destruction [160]. Based on the structure identity, IL-17F is considered to have a similar biological function to IL-17A. IL-17F can mediate the pro-inflammatory response in host defense and inflammation and also play a role in recruiting neutrophils, fighting pathogens, and production of anti-microbial peptide. However, the function of IL-17F is more limited than IL-17A due to its weak signaling [130],[169], while the potency of IL-17A/F is significantly higher than the IL-17F homodimer with respect to inducing

neutrophil recruitment and chemokine production, but not as potent as the IL-17A homodimer [183].

2.3.8. The role of IL-17A in pemphigoid diseases

IL-17A is a multifunctional cytokine which can evoke pro-inflammatory responses in innate and adaptive immune response. It is clear that IL-17A plays an important role in skin and mucosal immune responses [184]. In addition to its role in host defense against micro-organisms, the pro-inflammatory role of IL-17A in neutrophil-mediated subepidermal blistering autoimmune diseases has previously been highlighted. High numbers of IL-17A^{+} cells were detected in skin lesions [185] from pemphigus vulgaris (PV) patients and significantly increased levels of Th17 associated cytokines IL-17A, CCL-20 in serum have been reported from pemphigus vulgaris (PV) patients [186]. In the perilesional skin of BP patients, mRNA levels of IL-17A and related mediators are up-regulated [187]. Consistent with clinical studies, IL-17A levels correlate with disease severity in the antibody-induced bullous pemphigoid mouse model [187]. Furthermore, IL-17A$^{-/-}$ mice were protected from the pathogenic effect of anti-Col17 IgG and biological inhibition of IL-17A significantly reduced anti-Col17 IgG-induced skin lesions in mice [187].

EBA also belongs to the subepidermal blistering autoimmune diseases; however, only little data is available in EBA clinical research due to the limited disease morbidity. The aberrant expression of IL-17A has also been noted in experimental EBA. Furthermore, in our preliminary research, antibody-induced EBA disease was completely abolished in IL-17A$^{-/-}$ mice[188], indicating that IL-17A is the major driver of this disease. However, the contribution of IL-17F, the cellular source, and induction requirements, as well as the target cells of IL-17A, are still unknown.

3. Aim of this study

Epidermolysis bullosa Acquisita (EBA) is a skin-specific autoimmune disorder mediated by autoantibodies against type VII collagen and the subsequent infiltration by neutrophils in the dermal-epidermal junction. Since previous studies have reported that Interleukin (IL)-17 can mediate the secretion of various chemokines and cytokines, which can promote the recruitment and activation of neutrophils involved in inflammation and immunity, it is implied that IL-17 may contribute to the pathogenesis of EBA. Consistently, our preliminary data showed that the development of antibody-induced EBA disease was completely abrogated in IL-17A-deficient ($^{-/-}$) mice [188]. However, the following questions remained unanswered and are addressed in the present thesis.

- What is the relative contribution of IL-17A and IL-17F to the pathogenesis of antibody-induced EBA?
- What is the cellular source of IL-17A in antibody-induced EBA?
- What are the possible target cells of IL-17A in the development of EBA?

4. Materials and methods

4.1. Materials

4.1.1. Equipment and consumables

Name	Manufacturer
Centrifuge	Hettich Lab Technology, Germany
Cytocentrifuge	Thermo Fisher Scientific, USA
Biological safety cabinet	Thermo Fisher Scientific, USA
Fluid aspiration system	Vacuubrand GmbH, Germany
Mini centrifuge	Carl Roth GmbH, Germany
Vortes mixer	Scientific Industries, Inc., USA
Ultrasonicator	Branson Ultrasonics, USA
Spectrophotometer	Thermo Fisher Scientific, USA
Thermocycler	Bio-Rad Laboratories GmbH, USA
Spin tissue processor	Thermo Fisher Scientific, USA
Microtome	Leica Biosystems Nussloch, Germany
Water bath	Gesellschaft für Labortechnik mbH, Germany
Heating stage	Medite GmbH, Germany
Paraffin embedding workstation	Thermo Fisher Scientific, USA
LightCycle480	Roche Molecular Systems, Inc., USA
Microscope	Olympus Corporation, Japan
Image acquisition system	Nikon Corporation, Japan
Absorbance reader	Tecan Trading AG, Switzerland
PH meter	Knick Elektronische Messgeräte GmbH, Germany
Heating plate	Heidolph Instruments, Germany
Balance	Kern&Sohn GmbH, Germany
Analytical balance	Sartorius Research GmbH, Germany

Orbital shaker	Gesellschaft für Labortechnik mbH, Germany
Pipette controller	Brand GmbH + CO KG, Germany
Antigen retrieval pot	Instant Pot Company, Germany
Multi-channel pipettes	Sartorius Research GmbH, Germany
Single channel pipettes	Brand GmbH + CO KG, Germany
Ultra-low freezer	Thermo Fisher Scientific, USA
Cell counter	Schärfe Systems GmbH, Germany
Confocal microscope SP5	Leica Microsystems, Germany
Flowcytometer LSR II	Beckton Dickinson, USA
Syringe 1ml/ 5ml	Beckton Dickinson, USA
Needles 20G/30G	Beckton Dickinson, USA
Tubes 1.5ml /2.0ml/15ml/50ml	Sarstedt, AG&Co, Germany
Serum collection tubes	Beckton Dickinson, USA
Molds for cryo-embedding	Sakura Finetek Europe, Netherlands
Neg-50 cryo-embedding medium	Thermo Fisher Scientific, USA
24 well/96 well cell culture plate	Corning Incorporated, USA
Tips for pipette	Sarstedt, AG&Co, Germany
Cassette for paraffin embedding	VWR International GmbH, Germany
Slides	R. Langenbrinck GmbH, Germany
PAP pen	Kisker Biotech GmbH & Co. KG, Germany
Microtome blade	Feather Safety Razor Co., Ltd, Japan
Multi-well plate for real-time PCR	Rohe Diagnostics GmbH, Germany
RNAse-free tubes	Sarstedt, AG&Co, Germany
Scissors and forceps	Karl Hammacher GmbH, Germany
5ml FACS tube	Beckton Dickinson, USA
Cell strainer(40um, 70um)	Beckton Dickinson, USA
Macrochemotaxis chamber 48well	Neuro Probe, UK
3 um Filter for 48well chamber,	Neuro Probe, UK

PVP surface treatment, 25X80mm	

4.1.2. Chemical substances and solutions

Name	**Manufacturer**	**Catalog number**
aluminum sulfate hydrate (Al_2 (SO4) $_3$ * H_2O)	Sigma-Aldrich	36845-8
ammonium chloride (NH_4Cl)	Merck	101145
citric monohydrate ($C_6H_8O_7$)	Merck	1.002.440.500
disodium hydrogen phosphate (Na_2HPO_4)	Merck	1.065.801.00
potassium bicarbonate ($KHCO_3$)	Merck	1048540500
potassium chloride (KCl)	Applichem	A3582.1000
potassiumdihydrogen phosphate(KH_2PO_4)	Applichem	A3620.1000
sodium azide (NaN_3)	Roth	K305
sodium chloride (NaCl)	Applichem	A3597.5000
sodium hydroxide (NaOH)	Merck	1.64.985.000
sodium iodate ($NaIO_3$)	Sigma-Aldrich	S4007-100G
natrium hydrogencarbonate ($NaHCO_3$)	Merck	1.63.291.000
tris	Roth	4855.3
ethylene diamine tetraacetic acid (EDTA)	Roth	8043.1
agarose	peqLab	35-1020
hematoxyln	Sigma-Aldrich	H3136
eosinY disodium salt	Sigma-Aldrich	E6003-100G
paraformaldehyde (PFA)	Merck	104005
H_2O	Braun	180448001
35%H_2O_2	Roth	2317650
ethanol	Merck	1.009.831.000
xylol	Sigma-Aldrich	247642
37% hydrochloric acid (HCL)	Roth	74761

ethylene glycol	Merck	1.009.492.500
10X PBS without Ca/Mg	Gibco	1945217
10X PBS without Ca/Mg	Gibco	1677485
tri-fast	PeqLab	302020
chloroform	Merck	1.024.450.25
isopropanol, reinst	Roth	2006617
Trypen Blue	VWR	0026C232
DMSO	Pierce	20684
heparin natrium	Ratiopharm	5394.02.00
10X 'Roti-Immunoblock'	Roth	T1441
bovine serum albumin (BSA)	PAA	K51-001
L-Glutamin	Biochrom	1223D
penicillin/streptomycin	Biochrom	1102E
0.25% trypsin-EDTA	Biochrom	L2153
Percoll	GE Healthcare	17-0891-02
PMA (phorbol-12-myristat-13-acetat)	Sigma-Aldrich	BCBD9264V
Golgiplug	Beckton Dickinson	4309737
Ionomycin	Sigma-Aldrich	117K4034
Cytofix/CytoPerm solution	Beckton Dickinson	5075560
perm/wash buffer	Beckton Dickinson	7316715
5X reverse transcription PCR Reactionbuffer	Fermentas	00128953
Random hexamer primer	Fermentas	
ribolock ribonuclease inhibitor (40U/ul)	Fermentas	00134886
Revert Aid HMinusM-MuLV Reverse Transcription	Fermentas	00313558
DNTP Mix 10Mm	Fermentas	
10X HBSS without Ca/Mg without phenol red	Biowest	S13678X0507
1M HEPES	PAA	S00108-2635

4.1.3. Buffers

Buffer	Reagent	Final Conc.
10X PBS, PH7.4	KCl	2.7 mM
	KH_2PO_4	1.5 mM
	NaH_2PO_4	9 mM
	NaCl	137 mM
	H_2O	1L
TBS, PH7.4	NaCl	150 mM
	Tris	50 mM
	H_2O	1L
Red Blood Cell Lysis Buffer (1L)	$KHCO_3$	100mg
	NH_4CL	830mg
	5%EDTA	180ul
	H_2O	1L
Citrate buffer	citric monohydrate ($C_6H_8O_7$)	10 mM
	H_2O	1L
FACS buffer	FCS	3%
	NaN3	0.1%
	1XPBS	1L
1% eosin solution	eosin Y disodium salt	1%
	glacial acetic acid	2-3 drops
	H_2O	1L
Hematoxylin Gill	hematoxylin	19.85 mM
	sodium iodate ($NaIO_3$)	0.03 mM
	aluminum sulfate hydrate (Al_2 (SO_4) 3 * H_2O)	52.8 g/L
	ethylene glycol	25%

	glacial acetic acid	6%
	H_2O	1L
1% paraformaldehyde,PH7.4	PFA	1 mg / ml
	1 M sodium hydroxide (NaOH)	1 drops
	PBS	1L
4% formalin	10XPBS	50ml
	30% formalin	65ml
	H_2O	385ml
Anesthesia	10% Ketamin	250ul
	2% Rompun	100ul
	0.9%NaCl	1.7ml
HBSS prepared solution	10X HBSS without Ca/Mg without Phenol red	25ml
	3,5% $NaHCO_3$	2.5ml
	FCS	1.25ml
	1M HEPES	5ml
	H_2O	216.25ml
62% Percoll	Percoll	67.2 ml
	10X PBS without Ca/Mg	7.44ml
	HBSS prepared solution	45.6ml

4.1.4. Serums, enzymes and recombinant cytokines

Name	**Manufacturer**	**Catalog number**
liberase TL	Roche	5401119001
proteinase K	PeqLab	04-1076
rat serum	PAA	B03706-1628

hamster serum	Jackson Immunoresearch	107163
mouse serum	GE Healthcare	803813-1604
recombinant mouse IL-17A	R&D systems	AEK1308061
recombinant mouse IL-1b	Biolegend	B222788
recombinant mouse IL-17	Biolegend	B231460
recombinant mouse G-CSF	Biolegend	B195832
recombinant mouse complement 3a	R&D systems	DCVY0117011

4.1.5. Medium

Name	**Manufacturer**	**Catalog number**
IMEM	Gibco	1880352
DMEM	Biochrom	0413D
RPIM 1640 (4.5g/L glucose) without phenol red	PAN Biotech	8430716

Medium	**Component**	**Concentration**
Complete medium	IMEM (4.5g/L glucose)	
	FCS	10%
	Penicillin/Streptomycin (P/S)	1%
	L-Glutamine	1%
Liberase medium	IMEM (4.5g/L glucose)	
	Penicillin/Streptomycin (P/S)	1%
	L-Glutamine	1%
	LiberaseTL	1.2mg/ml
Fibroblast and keratinocyte culture medium	DMEM (4.5g/L glucose)	
	FCS	10%
	Penicillin/Streptomycin (P/S)	1%

	L-Glutamine	1%
Neutrophil CL medium	RPIM 1640 (4.5g/L glucose) without phenol red	
	FCS	10%
	Penicillin/Streptomycin (P/S)	1%
	L-Glutamine	1%
	HEPES	1%

4.1.6. Real-time PCR Primers and UPL sondes

All primers and UPL sondes used for real-time PCR were from Roche.

Gene	**Primer**	**Sonde number**
HPRT	s: 5‘-TCC-TCC-TCA-GAC-CGC-TTT-T-3‘ as: 5’-CCT-GGT-TCA-TCA-TCG-CTA-ATC-3’	95
IL-17A	s: 5‘-TGT-GAA-GGT-CAA-CCT-CAA-AGT-CT-3‘ as:5’-GAG-GGA-TAT-CTA-TCA-GGG-TCT-TCA-T-3’	50
IL-17F	s: 5‘-CCC-AGG-AAG-ACA-TAC-TTA-GAA-GAA-A-3‘ as:5’-CAA-CAG-TAG-CAA-AGA-CTT-GAC-CA-3’	46
IL-1b	s: 5’-TGT-AAT-GAA-AGA-CGG-CAC-ACC-3’ as: 5’-TCT-TCT-TTG-GGT-ATT-GCT-TGG-3’	78
IL-6	s:5’-GTC-ACC-AAA-CTG-GAT-ATA-ATC-AGG-A-3’ as: 5’-CCA-GGT-AGC-TAT-GGT-ACT-CCA-GAA-3’	6
CXCL2	s: 5’-CCA-GCC-ACA-CTT-CAG-CCT-A-3’ as: 5’-CAG-TTC-ACT-GGC-CAC-AAC-AG-3’	49
CCL2	s: 5’-CAT-CCA-CGT-GTT-GGC-TCA-3’ as: 5’-GAT-CAT-CTT-GCT-GGT-GAA-TGA-GT-3’	62

4.1.7. FACS antibodies

Specificity	Marker	Clone	Species	Dilution	Provider
CD16/CD32	unconjugated	2.4G2	Rat IgG2b, k	1:100	Biolegend (101302)
CD3	FITC	17A2	Rat IgG 2b	1:200	BD (555274)
B220	PE	RA3-6B2	Rat IgG 2a,k	1:200	Biolegend (103208)
γδTCR	PercP-Cy5.5	GL3	Rat IgG2a	1:200	Biolegend (118117)
CD4	PE-Cy7	GK1.5	Rat IgG2b, κ	1:200	Biolegend (100421)
CD45	Pacific Blue	30-F11	Rat IgG2b, κ	1:200	Biolegend (101223)
Ly6G	BV711	1A8	Rat IgG2a	1:200	Biolegend (127643)
IL-17A	Alexa 647	TC11-18H10.1	Rat IgG1, κ	1:150	Biolegend (506911)
CD4	PE	RM 4-5	Rat IgG2a, κ	1:200	BD (563048)
CD8	PE	53-6.8	Rat IgG2a, κ	1:200	BD (553033)
CD11b	PE	M1/70	Rat IgG2b, κ	1:200	BD (557397)
NK1.1	PE	PK136	Mouse IgG2b, κ	1:200	BD (553033)
Live/Dead	PI	-	-	1:1000	Miltenyi Biotec

					(5161017 251)
Live/Dead	DAPI	-	-	1:1000	Invitrogen (L23105)

4.1.8. IF and ICH antibodies

Name	**Company**	**Catalog number**
Alex546 goat anti-rat IgG	Invitrogen, USA	1744742
Alexa488 goat anti-rabbit IgG	Invitrogen, USA	514957
Rat anti-mouse complement 3	Cedarlane laboratories corporation, Canada	RMC11H9
Rat anti-mouse neutrophil	Cedarlane laboratories corporation, Canada	CL8993AP
Prolong™ Gold antifade reagent with DAPI	Invitrogen, USA	1876264

4.1.9. Pharmacological antibodies

Name	**Clone**	**Provider**
Anti-IL-17A antibody	17F3	BioXCell, West Lebanon, USA
Isotype antibody IgG1	MOPC-21	BioXCell, West Lebanon, USA

4.1.10. Kits

Name	Company	Catalog number
ABC kit	Vector Laboratory, USA	PK-6100
Biotin-avidin blocking kit	Vector Laboratory, USA	SP-2001
DAB kit	Vector Laboratory, USA	SK-4100

4.2. Methods

4.2.1. Experimental animals

Brief descriptions of the mice strains used in this thesis are given below (Table 1). All animals were treated for between 8-16 weeks in the trials. All mice were housed under specified pathogen-free conditions with 12-hour light/darkness cycles at the animal facility at the Borstel Research Center. All animal studies have been reviewed and approved by the Animal Research Ethics Board of the Ministry of the Environment, Kiel, Germany. All clinical examinations, biopsies and bleedings were performed under anesthesia using intraperitoneal (i.p.) administration of a mixture of ketamine and xylazine.

Table 1. Mouse strains used in the thesis

Mouse strain	Genetic background	Provider/Breeding
C57BL/6	-	Charles River Laboratories
IL-17A$^{-/-}$	C57BL/6	Animal facility in Kiel
IL-17Ra$^{-/-}$	C57BL/6	Animal facility in Kiel
IL-17F$^{-/-}$	C57BL/6	Animal facility in Kiel
IL-17A/F$^{-/-}$	C57BL/6	Animal facility in Kiel
γδTCR$^{-/-}$	C57BL/6	Institute of Immunology, Hannover
γδTCR-eGFP	C57BL/6	Institute of Immunology, Hannover
129	-	Animal facility in Xiamen University

4.2.2. Induction of experimental EBA

The antibody used to establish the experimental EBA mouse model (anti-mCol7-IgG and normal rabbit IgG) were obtained from the group of dermatology in Lübeck. The procedure to produce anti-mCol7 IgG was according to Sitaru et al. 2005 [95].

For local EBA induction, mice were treated as described earlier[189],[190] with minor modifications. In brief, female mice with an age of 8-16 weeks of the indicated strains received once 0.42 mg IgG by intracutaneous (i.d.) injection in the base of the ear (Figure 5). Pathogenic anti-mCol7 IgG was injected into the right, normal rabbit (NR) IgG into the left ear.

For induction of antibody-transfer (passive) systemic EBA, studies that had published protocols with minor modifications were used for this thesis [95]. Briefly, female mice with an age of 8-16 weeks of the indicated strains received a total of 6 times subcutaneous (s.c.) injections of 2-3mg rabbit anti-mCol7 IgG (exact amount of IgG used is indicated in the experiment protocols) or corresponding amounts of NR IgG into the abdomen every second day (days 0, 2, 4, 6, 8 and 10) (Figure 5). Disease severity was expressed as percentage of body surface area affected by skin lesions and determined at 3 time points (days 4, 8 and 12).

In the pharmacological inhibition of IL-17A experiment, the anti-IL-17A antibody (clone 17F3, BioXCell, West Lebanon, USA) and isotype antibody (IgG1, clone MOPC-21, BioXCell) were injected at a dose of 200µg/mouse on day -2, 0, 2, 4, 6, 8 and 10[187].

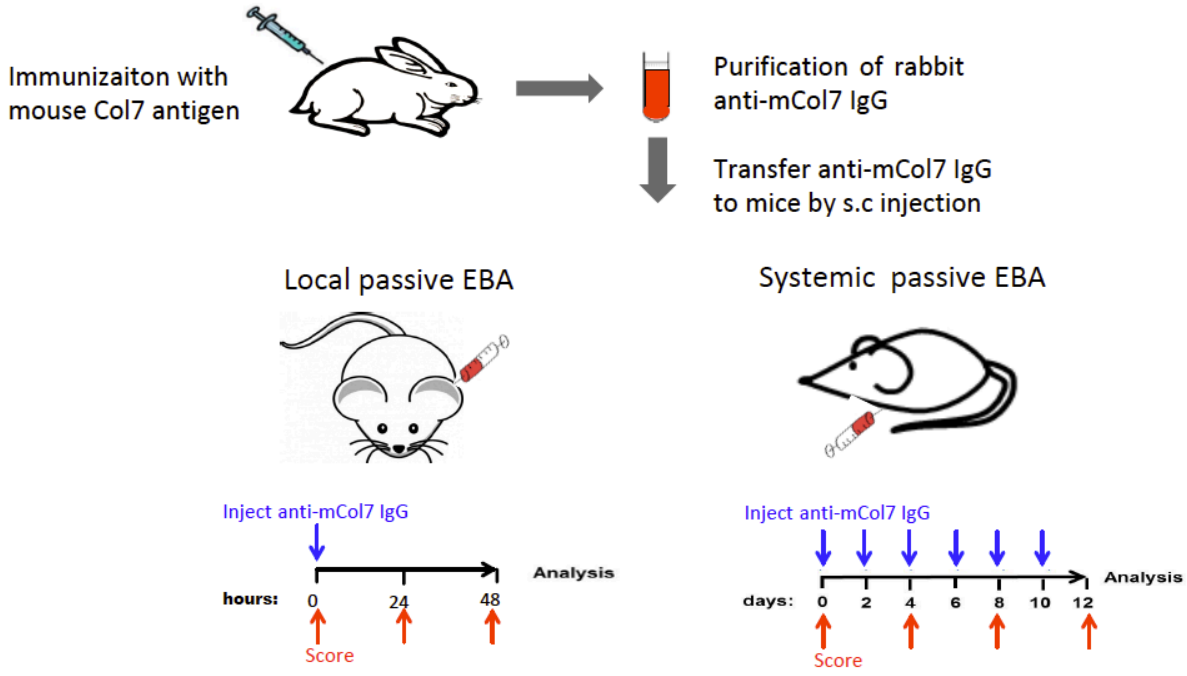

Figure 5. Schematic representation of transfer of rabbit anti-mCol7 IgG-induced local and systemic BEA mouse model. In the passive transfer model, rabbits were immunized with recombinant murine type VII collagen protein (mCol7c or vWFA2) together with adjuvant. Then the rabbit IgG was purified. This purified anti-mCol7 IgG was subcutaneously injected into mice to induce EBA. In the local EBA model, anti-mCol7 IgG was injected into the mouse ear. In contrast, anti-mCol7 IgG was administered into the mouse abdomen in systemic model. Normal rabbit (NR)IgG was used as control.

4.2.3. Calculation of EBA severity

In local EBA, the surface area of the ear covered by lesions (blisters, crust, erythema) was scored 24h and 48h later. For systemic EBA, disease severity was evaluated according to the area affected. EBA-typical skin changes including redness, scabs, hair loss and wet spots. Here the percentages of affected skin areas in the various parts were according to Table 2. The affected entire body surface area was calculated by allotting individual fractions to each part of the body. The percentage of affected skin areas in the various parts were multiplied

according to their weight of total body skin (Table 3) and then added together: this gave the percent of the affected skin surface of the entire body.

Table 2. Percentage of skin areas in various parts

body part	area	Proportion %
ear	front	50
	rear	50
eye	top lid	50
	lower lid	50
snout	left	50
	right	50
oral mucosa	up	50
	down	50
head&neck	up	50
	down	50
leg	paw up	10
	paw down	10
	leg up	40
	leg down	40
tail	up	50
	down	50
trunk	back	50
	abdomen	50

Table 3. Percentage of affected skin surface of entire body

mouse C2N1	percentage of affected area in body parts (%)	weight of total body skin area (%)
Ear (left)		2.5
Ear (right)		2.5
Eye (right)		0.5
Eye (left)		0.5
Snout		2.5
oral mucosa		2.5
head&neck		9
front leg (left)		5
front leg (right)		5
rear leg (left)		10
rear leg (right)		10
tail		10
trunk		40
overall score		

4.2.4. Histological assessment

The skin samples were fixed in 4% formalin for 24h. After dehydration and paraffinization (Microm STP120 Spin Tissue Processor, Table 4), the tissue samples were embedded in paraffin (Thermo Scientific) and sectioned at a thickness of 3 μm (microtome RM2155 Leica). Then the paraffin sections were first deparaffinized in xylene and rehydrated in gradient ethanol solutions, then stained with hematoxylin and eosin solutions (H&E, Table 5). After dehydration and clearing, the stained sections were mounted.

Table 4. Procedure of tissue dehydration and paraffinization

Step	Reagent	Incubation time (min)
1	4% formalin	60
2	70% ethanol	60
3	80% ethanol	60
4	90% ethanol	60
5	96% ethanol	60
6	absolute ethanol	60
7	absolute ethanol	60
8	absolute ethanol	60
9	xylene	60
10	xylene	60
11	paraffin	90
12	paraffin	90

Table 5. Procedure of hematoxylin-eosin staining

Step	Reagent	Incubation time (min)
Deparaffinization	Xylene I	5
	Xylene II	5
	Xylene III	5
Re-hydration	Absolute ethanol I	5
	Absolute ethanol II	5
	Ethanol 96%	5
	ddH_2O	5
Staining	Gill's hematoxylin solution	20
	Wash in running tap water (blueing)	10
	Eosin (1%, acidic) counterstain	3
	ddH_2O	2 dip

Dehydration	Ethanol 70%	2 dip
	Ethanol 96% I	2 dip
	Ethanol 96% II	2 dip
	Absolute ethanol I	2 dip
	Absolute ethanol II	3
	Xylene I	5
	Xylene II	5
	Xylene III	5
Mounting	Entellan (Merck)	

4.2.5. Immunohistochemistry

Immunohistochemistry staining was performed on skin sections after fixation with formalin followed by paraffin embedding. Tissue sections were deparaffinized in xylol and rehydrated in gradient ethanol. Antigen retrieval was performed by heating slides at 121 °C in a 10 mM citrate buffer (PH 6.0) for 1 hour (Retriever2100, Prestige Medical). Endogenous peroxidase, endogenous biotin and unspecific binding was blocked with 3% H_2O_2, biotin blocking solution (Vector, USA) and 5% BSA solution, respectively. Then, sections were incubated overnight at 4°C with primary antibodies against the murine neutrophil marker (07-APR, Cedarlane, Canada). Incubation of the biotinylated secondary antibody (Polyclone, Jackson Immunoresearch, USA) was conducted for 45 minutes at room temperature, followed by incubation with avidin biotinylated HRP solution (Vector, USA) for 20 minutes. Diaminobenzidine (Vector Laboratories, USA) was applied to visualize immunoreactivity. Afterwards, the sections were counter-stained with hematoxylin for 5 minutes. Images were taken using bright-field microscopy (Nikon, Japan). Table 6 summarizes the detailed procedure of the immunohistochemistry staining.

Table 6. Procedure of immunohistochemistry staining for neutrophils

Step	Reagent	Incubation
Deparaffinization	Xylene I	5 min
	Xylene II	5 min
	Xylene III	5 min
Re-hydration	Absolute ethanol I	5 min
	Absolute ethanol II	5 min
	Ethanol 96%	5 min
	Ethanol 70%	5 min
	Ethanol 40%	5 min
	Deionized water	5 min
Antigen retrieval	10Mm citrate buffer (PH＝6.0)	121°C, (50 min in antigen retrieval pot)
Cool down	10Mm citrate buffer (PH＝6.0)	20 min
Blocking endogenous peroxidase	3% H_2O_2 solution	15 min
	PBS	5 min, three times
Blocking endogenous biotin	Avidin solution	15 min
	PBS	5 min
	Biotin solution	15 min
	PBS	5 min, three times
Blocking unspecific binding	5% BSA	50 min
Incubation of primary antibodies	Primary antibodies diluted in PBS	4°C , overnight
	PBS	5 min, three times
Incubation of biotinylated secondary	Biotinylated secondary antibodies diluted in PBS	50 min, RT
	PBS	5 min, three times

Preparation of avidin and biotinylated HRP complex (ABC solution)	100 ul of avidin solution and 100 ul of biotinylated HRP solution in 5 ml of PBS	30 min, RT
Incubation of ABC solution	ABC solution	30 min, RT
	PBS	5 min, three times
Incubation of DAB solution	84 ul of buffer stock solution, 100 ul of DAB reagent and 80 ul of H2O2 in 5 ml of deionized water	2 min, RT
	Tap water	5 min, three times
Counterstaining	Gill's hematoxylin solution (No.2)	1 min
Blueing	Running tap water	5 min
Dehydration	Ethanol 70%	10 seconds
	Ethanol 96% I	10 seconds
	Ethanol 96% II	3 min
	Absolute ethanol I	3 min
	Absolute ethanol II	3 min
Clearing	Xylene I	5 min
	Xylene II	5 min
	Xylene III	5 min
Mounting	Entellan (Merck)	

4.2.6. Immunofluorescence staining

Immunofluorescence staining was used to detect the IgG and complement deposition on the 5 µm cryosections prepared from murine skin. After blocking with Roti-Immunoblock (Roth), IgG deposition in the skin tissue of mice was detected using the Alexa488 goat anti-rabbit IgG antibody (Invitrogen), and deposition of complement component 3 was detected using indirect immunofluorescence staining with the primary rat anti-mouse complement 3 antibody (Cedarlane) and Alexa546 goat anti-rat IgG (Invitrogen) as the detecting reagent. Coverslips

were mounted with Gold mounting agent (Thermo Fisher). Fluorescence was determined using confocal microscopy (Leica SP5).

4.2.7. RT-PCR

4.2.7.1. RNA isolation

After the mice were sacrificed using CO_2, the skin homogenates were stored in Tri-fast. 200 µl chloroform was added to 1ml homogenate. The samples were briefly shaved and incubated for 10 min at room temperature. This was followed by centrifugation at 10400 rpm at 4°C for 5 min. The upper aqueous phase was collected in a new 1.5 ml RNAse-free tube (Biosphere) and 500 µl of ice-cold isopropanol added, well-shaken and incubated for 15 min on ice. The samples were centrifuged at 10400 rpm and 4°C for 10 min. The white RNA pellets were visible and washed with 1 ml of ice-cold 75% ethanol twice. The ethanol was completely removed with a pipette and the RNA was dried at room temperature for about 5 minutes. Then RNA pellets were resuspended with 30ul ddH2O. The RNA concentration and quantity were measured using the NanoDrop 1000 spectrophotometer (Thermo Scientific). The RNA solutions were stored at -80°C until transcription.

4.2.7.2. Reverse transcription

According to the concentration of RNA, 1.5 ug RNA was diluted into ddH2O at 11.5 ul respectively. 1ul Random hexamer primer (0.2ug) was put into each reaction tube. The samples were briefly vortexed at 65°C for 5 min. Then the samples were placed on ice for cooling. Subsequently ,7.5 µl of the RT mixture (20% 5Xreaction buffer, 1 mM dNTPs, 20 U RiboLock, and 200 U of RevertAid in ddH2O) was added to each sample and incubated for 10 min at 25°C, then kept for 1h at 42°C. To stop the reaction, the samples were heated to 70°C for 10 min and cooled on ice. Each cDNA sample contributed 5-8 ul for the standard. The remaining samples were diluted 1:10 with ddH_2O. These cDNAs were stored at 4°C or at

-80°C for longer storage.

4.2.7.3. Quantification by real-time PCR

The gene expression was quantified using the LightCycler480 (Roche). For each cDNA sample and the standard, 1 ul was mixed with 9ul master mix (50% 2x LC480 Probes Master, 6.25 uM sense and antisense primer, 1% UPL probe in ddH2O) respectively. Primers and probes were used according to different targets. The Lightcycler software LCS480 1.5.0.39 (Roche) was used for analysis.

4.2.8. Cell isolation from mice skin

After the mice were sacrificed using CO_2, the crushed skin pieces were digested with 38ul liberase TL (25mg/ml Roche) in a 3ml ice-cold IMDM medium (No FCS) for 1.5h with 80-200 rpm in a 37 °C water bath. To stop the enzyme reaction, 3 ml of FCS-containing IMDM medium was added. Then tissue pieces were dissociated by gently meshing in cold IMDM medium, and the dissociated solution was filtered by passing through a 70 µm cell strainer. Single cell suspensions passed through the cell strainer were centrifuged at 1200 rpm 4 °C for 8 min. After being washed with cold IMDM medium twice, cells were resuspended in 1 ml of cold IMDM medium. The numbers of cells were measured; 1 x 10^6 cells were used for flow cytometric analysis.

4.2.9. Cell isolation from mice spleen

Spleen from γδTCR-eGFP mice was collected into a 15 ml tube with 5 ml cold IMDM medium. The spleen was dissociated by gently meshing in cold IMDM medium, and the dissociated spleen solution was filtered by passing through a 70 µm cell strainer. The single cell suspension passed through the cell strainer was centrifuged at 300g for 5 min, 4°C, then the cell pellets were incubated with RBC lysis buffer to remove the erythrocytes. After being

washed with cold IMDM complete medium twice, the cells were resuspended in an IMDM complete medium and counted: all cells were used for flow cytometric sorting.

4.2.10. Intracellular staining for cytokines

1-2x10^6 cells were stimulated using 1ul PMA (store at 500ug/ml, 1:1000), 1ul ionomycin (stored at 500 ug/ml, 1:1000) and 1 µl Golgi-PlugTM (BD) for 4.5 h at 37 °C with 5%CO_2. The control group only had 1ul Golgi-PlugTM. After incubation, the cells were transferred into FACS tubes and washed with PBS. 100ul DAPI live/dead staining solution was added to the cells for 10 min at 4°C in the dark. Then the cells were washed with 1 ml FACS buffer and blocked with 100 ul blocking solution (mouse serum, rat serum, hamster serum and anti-CD16/CD32 rat IgG in FACS buffer) for 30 min at 4°C. After washing the cells with 1 ml FACS buffer, they were incubated with 100ul corresponding fluorescent antibody mixture (CD45-Pacific Blue, CD3-FITC, CD4-PE-Cy7, Ly6G-BV711, γδTCR-PerCP-Cy5.5, B220-PE) for 30 min at 4°C in the dark. After incubation, the cells were washed with 1 ml FACS buffer and fixed with 250 µl Cytofix/Cytoperm ™ Buffer (BD) for 20 min at 4°C in the dark. Subsequently, the cells were washed with 1 ml Perm/Wash ™ buffer and were stained with 100 ul intracellular antibody (IL-17A-Alex647) for 45 min at 4°C in the dark. Finally, the cells were washed with 1 ml FACS buffer twice and were resuspended in 200ul FACS buffer and stored at 4°C in dark. The cells were measured using an LSR II flow cytometer (BD, USA) within three days, and the data were analyzed using FACS Express software (De Novo Software, USA, version 5).

4.2.11. FCSA sorting of γδT-cells

The γδT-cells were sorted by dumb staining. Prior to staining with fluorescent antibodies, spleen cells from γδTCR-eGFP mice were washed once with 4 ml FACS buffer and centrifuged at 1200 rpm and 4°C for 8 min. Then the cells were incubated with 200ul surface fluorescent antibody mixture including PE dye containing CD4, CD8, CD11b, B220 and

NK1.1 (6ul of each antibody). This coloring step was maintained for 30 min at 4°C in the dark. Then the cells were washed with 2 ml FACS buffer twice and were resuspended in 10 ml FACS buffer. 3ul PI dye, which was used for live/dead staining, was added before sorting. The γδT-cells were sorted using FACS Array, and the data were analyzed using FACS Express software (De Novo Software, USA, version 5).

4.2.12. γδT-cells assay

The sorted γδT-cells were distributed in a 96-well plate (0.2 x10^6/well) and stimulated by different compounds. To mimic the IgG and complement deposition in EBA, mouse collagen type 7（mCol7） antigen (2ug/well) and anti-mCol7 antibody (200ug/well), as well as complement 3a (20ng/well), were coated to form an immune complex one day before. With regard to the classical IL-17A-producing mediators, IL-23 (20ng/ml) and IL-1b (20ng/ml) were also chosen as stimulators. All these compounds were used as single-stimulation or co-stimulation for 48h and 72h. IL-17A concentrations in the supernatants were detected using ELISA according to the manufacturer's instructions (R&D).

4.2.13. Fibroblast and keratinocyte assay

Murine keratinocyte (C5N) and murine fibroblast (L929) were grown in 48-well plates at a concentration of 10x10^6/ml and treated with 100 ng/ml IL-17A, respectively. PBS was used as a control. The supernatants were collected 24h after stimulation and used to conduct the neutrophils trans-well experiment (Figure 6). The fibroblasts and keratinocytes were used for Q-PCR to analyze chemokine mRNA expression.

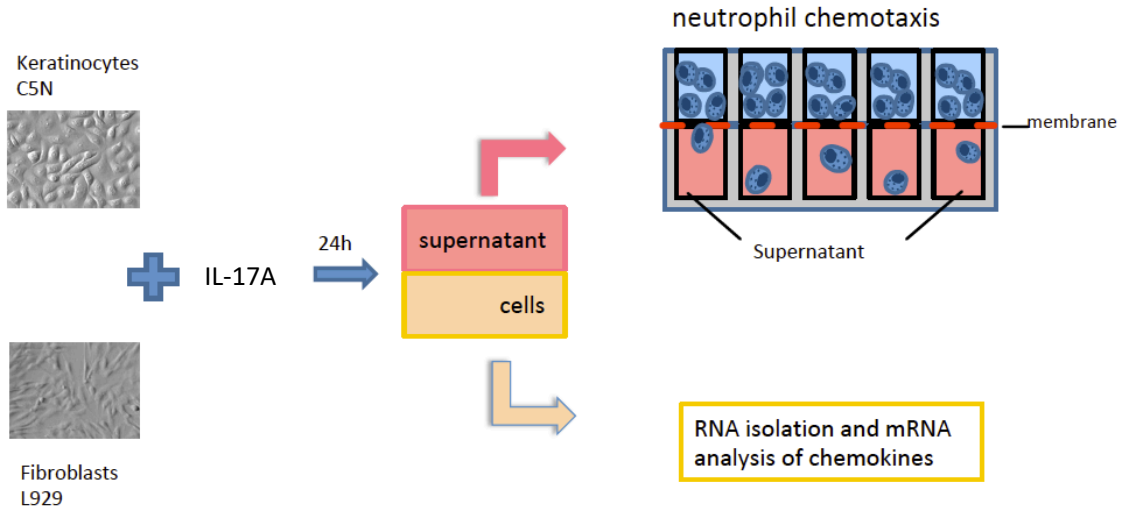

Figure 6. Schematic representation of skin cell assay and neutrophil chemotaxis experiment. Keratinocyte and fibroblast were stimulated with IL-17A for 24h. Then supernatants were collected to conduct the neutrophil chemotaxis experiment in which neutrophils were migrated through the membrane from up to down in the chamber. Besides the supernatants, fibroblasts and keratinocyte were used for Q-PCR to analyze the mRNA expression of chemokines.

4.2.14. Isolation of neutrophils

Neutrophils were isolated from mice bone marrow. Bones were cut with scissors and flushed with 2-5 ml cold HBSS bone. The cells were resuspended in the well to dissolve cell clumps, then centrifuged at 1500rpm for 4min at room temperature. Supernatants were discarded and pellets resuspended in a 5ml lysis buffer per bone for 5min for analysis off red cells. 20ml HBSS was added to wash the pellets and the cell solution passed through a 70um cell strainer to filter then the cell solutions were centrifuged at 1500rpm for 4min at room temperature. Supernatants were discarded and pellets resuspended in PBS. The cell number with was counted using Trypan Blue, and the cells resuspended at a density of $4x10^6$ cells/ml in a complete medium with 10 ng/ml G-CSF and incubated at 37°C in 5%CO_2 for 48h. This was followed by 62% pancoll (PanBiotech, Aidenbach, Germany) density centrifugation for 30min at 1000g, 20°C with acceleration 3 and brake 2. More than 98% of the cells were viable as assessed by Trypan Blue exclusion and the percentage of neutrophils exceeded 97% in all experiments as determined by hematoxylin staining, with 1–3% remaining eosinophils as the major contaminant. The neutrophils were suspended in neutrophil culture medium

(RPMI 1640 buffered with 25 mM HEPES without phenol red; Biochrom, Berlin, Germany) before use.

4.2.15. Neutrophil chemotaxis assay

Neutrophil migration was measured using a 48-well macrochemotaxis chamber (Neuro Probe) with nitrocellulose filters (Neuro Probe) (Figure 6). Briefly, a chemotactic solution was diluted in PBS and added to the bottom wells (30ul) of a 48-well chamber. PVP-treated nitrocellulose filters with 3 um pore size were placed between the bottom plate and top plate of the chamber assembly. Neutrophils (30ul) were added to the top wells at a cell density of $2X10^6$ cell/ml. The chamber was incubated at 37°C in 5% CO_2 for 1.5h. After incubation, the percentage of migrated neutrophils in the bottom was calculated.

4.2.16. Whole-genome SNPs genotyping

DNA was extracted from mice tail biopsies using the DNeasy® Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. 300ng of the DNA of each mouse was sent to Neogen Europe for whole-SNP genotyping using a GigaMUGA array. The whole-genome genotyping data were analyzed using Excel.

4.3. Statistical analysis

The statistical analyses were conducted using GraphPad Prism 6.0 (GraphPad Software). Unless otherwise noted, data is presented as mean ± standard error (SD). For comparisons of two groups, a t-test or Mann-Whitney Rank Sum Test was used, whenever appropriate. For comparison of more than two groups, ANOVA was used. For equally distributed data One-Way ANOVA, followed by a Bonferroni t-test for multiple comparisons was used; if data was non-parametric, ANOVA on ranks (Kruskal-Wallis) was applied, followed by a Bonferroni t-test for multiple comparisons. The classification of the significances was as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

5. Results

5.1. The pathogenic role of IL-17A and IL-17F in antibody-induced EBA

5.1.1. Antibody-induced EBA attenuated in IL-17Ra deficient ($^{-/-}$) mice

Previous studies in the Infection Immunology research group had found that IL-17A$^{-/-}$ mice were completely protected from antibody-induced EBA, indicating that IL-17A plays an important role in the pathogenesis of experimental EBA [188]. Given that the IL-17A signal is transmitted by IL-17 receptors and IL-17A has the highest affinity to IL-17Ra, IL-17Ra$^{-/-}$ mice were initially used in the present thesis to characterize the pathway of IL-17A in the development of EBA and to confirm the essential role of IL-17A in EBA.

To this aim, experimental EBA was induced in C57BL/6, IL-17Ra$^{-/-}$, and IL-17A$^{-/-}$ mice by an s.c. injection of anti-mCol7 IgG on day 0, 2, 4, 6, 8 and 10, while normal rabbit IgG (NR IgG) was used as a control. After receiving anti-mCol7 IgG, C57BL/6 and IL-17Ra$^{-/-}$ mice developed EBA disease features, including hair loss, redness, crust and skin lesions, and the disease progressed over time (Figure 7A). To evaluate EBA severity, the percentage of affected skin area on the body surface area was calculated 4, 8 and 12 days after the initial injection. However, the affected body surface area in IL-17Ra$^{-/-}$ mice was significantly lower as compared to C57BL/6 mice. In contrast, IL-17A$^{-/-}$ mice showed no signs of disease (Figure 7B).

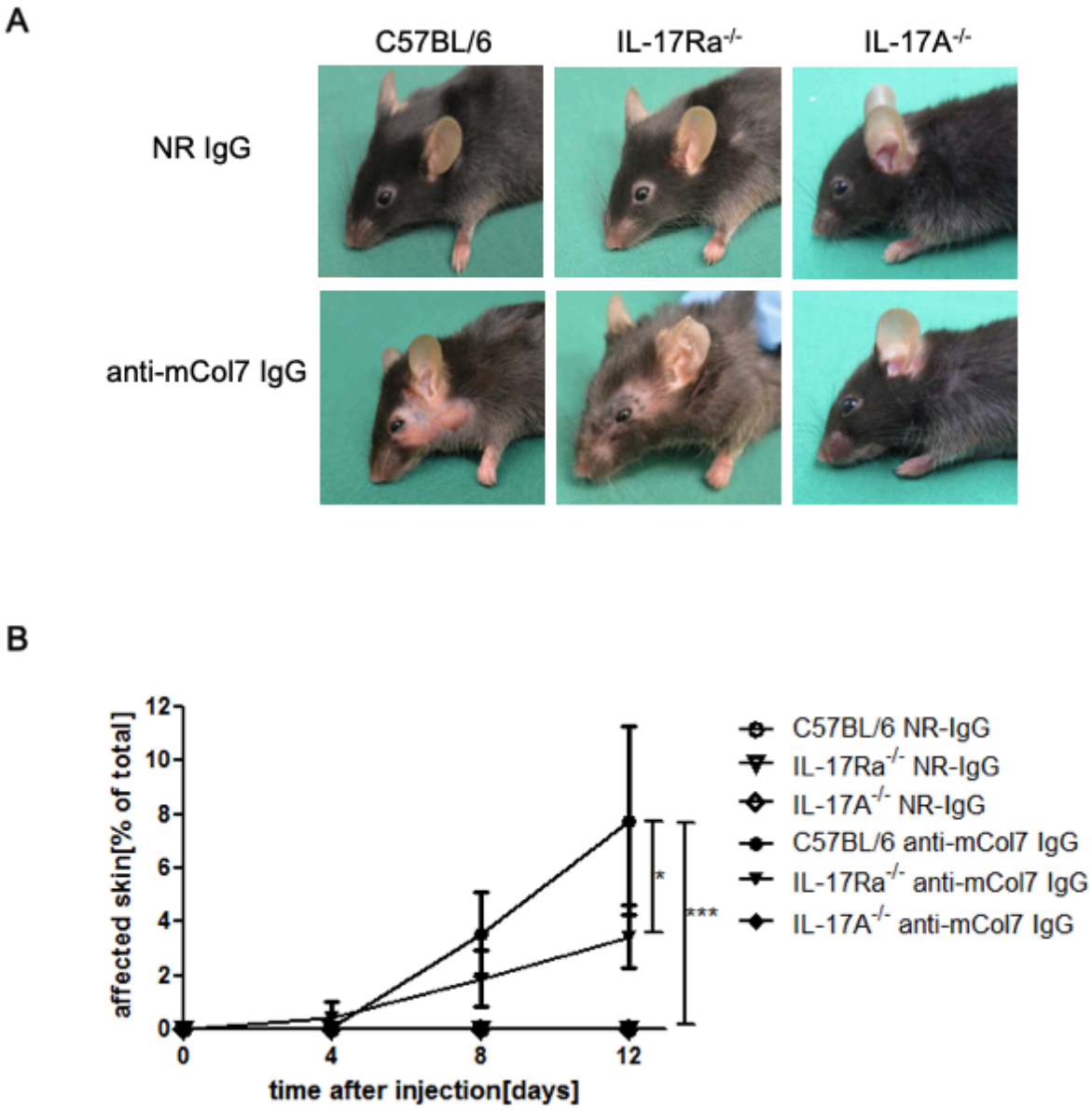

Figure 7. Attenuated EBA in IL-17Ra$^{-/-}$ mice after injection of anti-mCol7 IgG. C57BL/6, IL-17Ra$^{-/-}$, and IL-17A$^{-/-}$ mice received 18 mg anti-mCol7 IgG or normal rabbit IgG (NR IgG) on day 0, 2, 4, 6, 8, and 10 by subcutaneous injection. (A) Representative clinical pictures of experimental EBA in C57BL/6, IL-17Ra$^{-/-}$, and IL-17A$^{-/-}$ mice 12 day after initial s.c. injection with anti-mCol7 IgG or NR IgG. (B) EBA score assessed by the percentage of affected body surface area on 4, 8 and 12 days after initial s.c. injection of NR IgG or anti-mCol7 IgG. Data are shown as mean±SD (n=5 mice/group, * $p<0.05$, ***$p<0.001$, ANOVA Test).

Autoantibody binding and complement deposition in the DEJ are typical diagnostic methods of EBA disease. Therefore, deposition of rabbit anti-mCol7 IgG and C3 was detected by immunofluorescence staining (Figure 8). IgG binding and C3 deposition were detectable in

the dermal-epidermal junction of skin from the anti-mCol7 IgG injected C57BL/6, IL-17Ra$^{-/-}$ and IL-17A$^{-/-}$ mice. However, no IgG binding and C3 deposition were observed in the NR IgG injected groups.

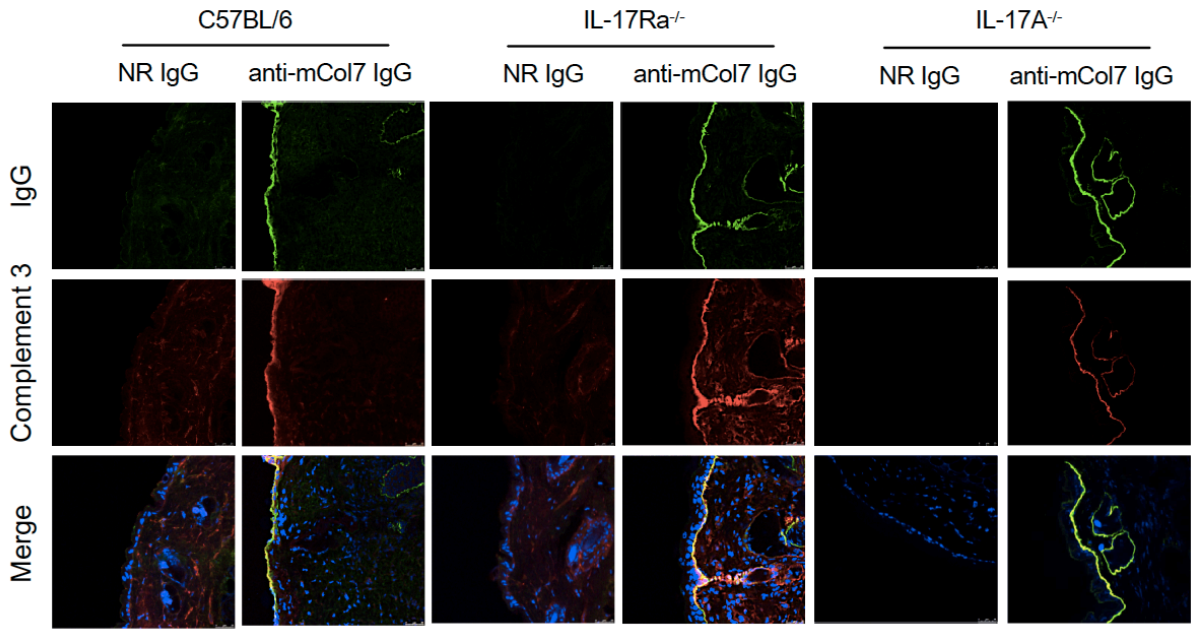

Figure 8. IgG binding and C3 deposition at the DEJ in C57BL/6, IL-17Ra$^{-/-}$ and IL-17A$^{-/-}$ mice. Representative micrographs of the immunofluorescence (630X). Cryosections of the skin tissue from C57BL/6, IL-17Ra$^{-/-}$, and IL-17A$^{-/-}$ mice were prepared on day 12 after initial s.c. injection with anti-mCol7 IgG or NR IgG and incubated with rat anti-mouse C3 antibody, then further stained with Alexa-546 labeled goat anti-rat IgG and Alex-488 conjugated goat anti-rabbit IgG to detect the IgG (green) and C3 deposition (red), respectively (n=5 mice/group, representative of 1 mouse/group).

Histological analysis was performed to evaluate the inflammation and disease features of EBA. IL-17Ra$^{-/-}$ mice exhibited a decreased number of infiltrating neutrophils in the skin as compared with C57BL/6 mice, whereas no cell infiltration was observed in IL-17A$^{-/-}$ mice (Figure 9). Since EBA is histologically characterized by neutrophil infiltration, immuno-histochemical staining of neutrophils was performed. The perivascular infiltration in diseased skin from both C57BL/6 and IL-17Ra$^{-/-}$ mice was dominated by neutrophils, whereas no neutrophil infiltration was detected in IL-17A$^{-/-}$ mice (Figure 9).

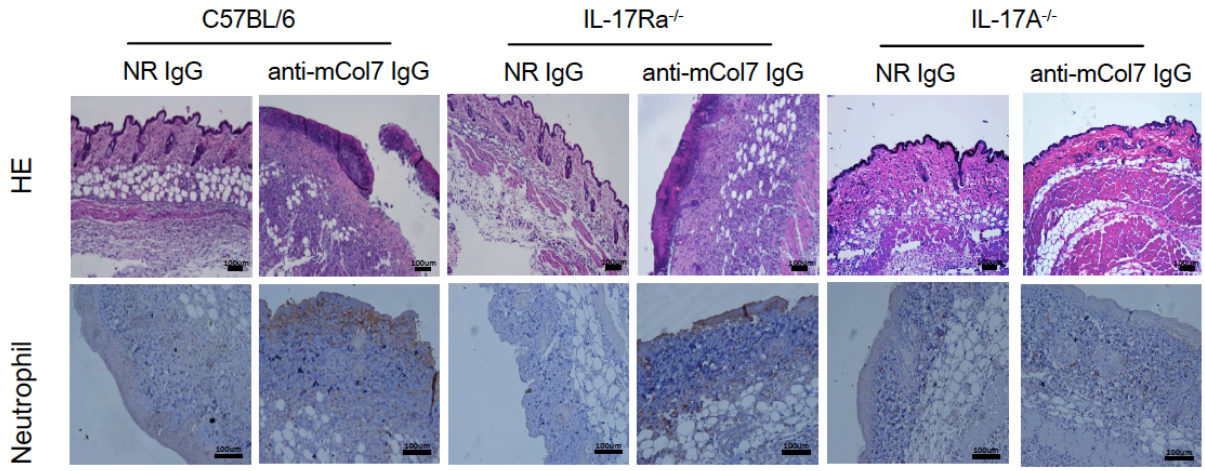

Figure 9. Decreased inflammatory infiltration in IL-17Ra$^{-/-}$ mice after injection of anti-mCol7 IgG. Photomicrograph of HE stained and immuno-histochemical stained sections of skin from C57BL/6, IL-17Ra$^{-/-}$, and IL-17A$^{-/-}$ mice 12 days after s.c. injection of NR IgG or anti-mCol7 IgG (100X). Infiltrated neutrophils were detected by immunohistochemistry staining with rat anti-mouse neutrophil antibodies in dark brown (n=5 mice/group, representative of 2 mice/group).

To further evaluate the inflammatory production in experimental EBA, gene expressions of cytokines and chemokines in the skin were measured. The induction of experimental EBA in both C57BL/6 and IL-17Ra$^{-/-}$ mice led to a pronounced increase in inflammatory cytokine and chemokine mRNA expression. However, as compared to C57BL/6 mice, mRNA expressions of IL-6 and CCL-2 significantly decreased in anti-mCol7 IgG injected IL-17Ra$^{-/-}$ mice (Figure 10). In IL-17A$^{-/-}$ mice, which were completely protected from EBA, the gene expressions of IL-6 and CCL-2 were diminished.

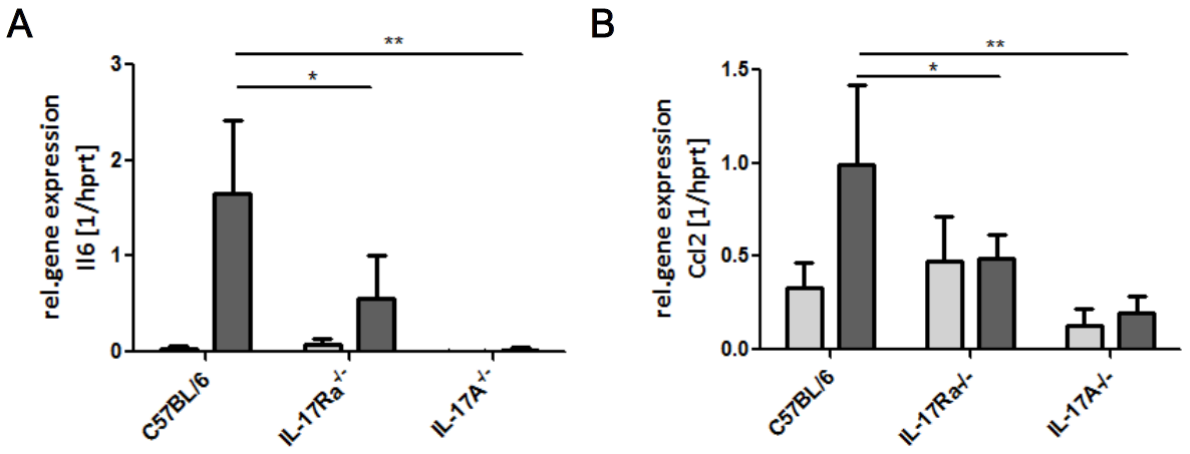

Figure 10. Declined inflammatory cytokines and chemokines expression in IL-17Ra$^{-/-}$ mice. Gene expressions of cytokines and chemokines in skin from C57BL/6, IL-17Ra$^{-/-}$, and IL-17A$^{-/-}$ mice 12 days after initial s.c. injection with NR IgG (gray) or anti-mCol7 IgG (black) by Q-PCR. Data are shown as mean±SD (n=5 mice/group, * $p<0.05$, ** $p<0.01$, ANOVA test).

5.1.2. Decreased skin blistering in IL-17A/F$^{-/-}$ mice in antibody-induced EBA

As shown above, IL-17Ra$^{-/-}$ mice developed milder EBA disease than C57BL/6 mice, whereas IL-17A$^{-/-}$ mice were completely resistant to EBA. Since IL-17R is not only the receptor for IL-17A, but can also be bound by IL-17E and IL-17F [169], this implied that IL-17E or IL-17F regulates the development of EBA. Therefore, the role of IL-17E and IL-17F were investigated in the present thesis. Firstly, a preliminary study was performed to clarify the role of IL-17E in the pathogenesis of EBA and the disease progress in IL-17A/F$^{-/-}$ and IL-17A$^{-/-}$ mice were compared.

C57BL/6, IL-17A/F$^{-/-}$ and IL-17A$^{-/-}$ mice were subcutaneously administered with pathogenic anti-mCol7 IgG or NR IgG on day 0, 2, 4, 6, 8 and 10 respectively. After receiving anti-mCol7 IgG, C57BL/6 mice developed severe EBA, whereas IL-17A/F$^{-/-}$ mice developed milder EBA (Figure 11A). No sign of disease was observed in IL-17A$^{-/-}$ mice (Figure 11A). To evaluate the disease progression, the mice were scored at 4, 8 and 12 days after the initial anti-mCol7 IgG injection. Of note, the overall extent of skin blistering in IL-17A/F$^{-/-}$ was significantly reduced compared to C57BL/6 mice in the anti-mCol7 IgG receiving group, whereas no disease was present in IL-17A$^{-/-}$ mice (Figure 11B).

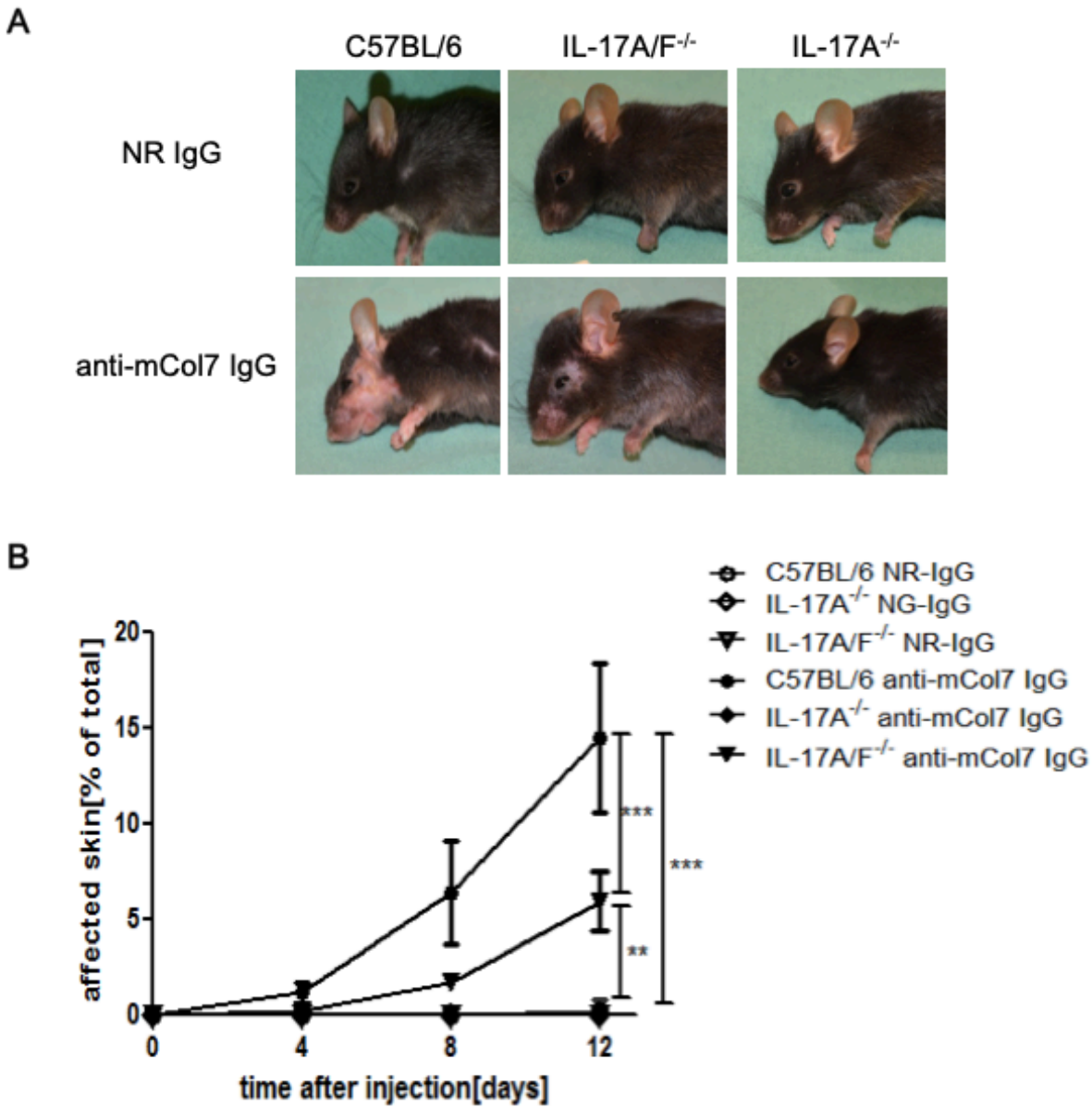

Figure 11. Reduced blister formation in IL-17A/F$^{-/-}$ mice with anti-mCol7 IgG-induced EBA. C57BL/6, IL-17A$^{-/-}$, and IL-17A/F$^{-/-}$ mice were subcutaneously injected with 18mg pathogenic anti-mCol7 IgG or NR IgG on day 0, 2, 4, 6, 8 and 10 respectively to induce experimental EBA. (A) Representative clinical pictures of experimental EBA in C57BL/6, IL-17A $^{-/-}$ and IL-17A/F$^{-/-}$ mice 12 days 12 after initial s.c. injection with NR IgG or anti-mCol7 IgG. (B) Percentage of affected skin area on body surface area was calculated on 4, 8 and 12 days after the initial injection of anti-mCol7 IgG or NR IgG (n=5 mice/group, ** $p<0.01$, *** $p<0.001$, ANOVA Test).

The decreased degree of EBA in IL-17A/F$^{-/-}$ mice was similar to the reduced disease in IL-17Ra$^{-/-}$ mice. The results using the IL-17Ra$^{-/-}$ and IL-17A/F$^{-/-}$ mice were evaluated in two

different experiments. In order to compare EBA severity between these two mice strains, a standardized score analysis was performed by calculating the ratio of affected skin area in IL-17Ra$^{-/-}$ and IL-17A/F$^{-/-}$ mice to the affected skin area in C57BL/6 mice. No significant difference in the standardized score between IL-17A/F$^{-/-}$ mice and IL-17Ra$^{-/-}$ mice in antibody-induced EBA was found (Figure 12). This result implied that IL-17E has no effect on the pathogenesis of antibody-induced EBA.

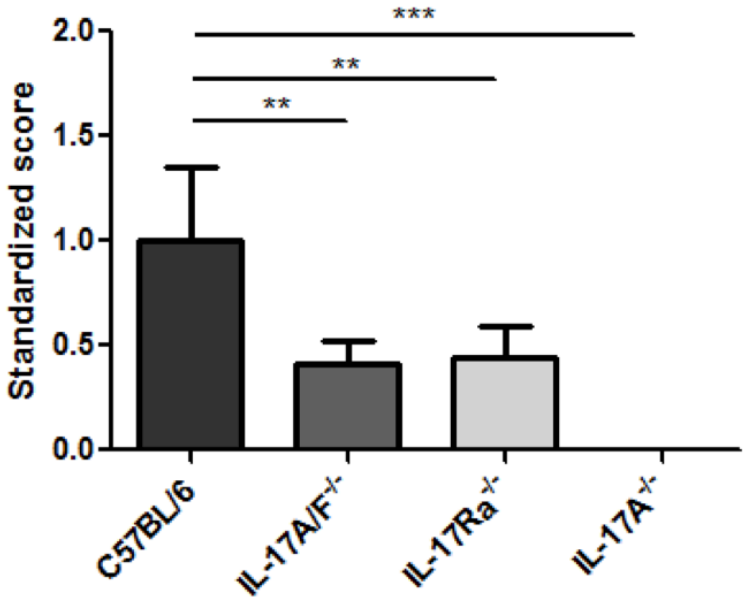

Figure 12. Reduced anti-mCol7 IgG-induced blister formation in IL-17A/F$^{-/-}$ and IL-17Ra$^{-/-}$ mice. Standardized EBA score in IL-17A/F $^{-/-}$, IL-17Ra$^{-/-}$, and IL-17A$^{-/-}$ mice 12 days after the initial injection of anti-mCol7 IgG. The standardized score was calculated as the ratio of the affected skin area in IL-17A/F $^{-/-}$, IL-17Ra$^{-/-}$, and IL-17A$^{-/-}$ mice to the affected skin area in C57BL/6 mice. Data are shown as mean±SD (n=5 mice/group, ** $p<0.01$, *** $p<0.001$, ANOVA Test).

Furthermore, IgG binding and C3 deposition in the skin of C57BL/6, IL-17A/F$^{-/-}$ and IL-17A$^{-/-}$ mice were detected by immunofluorescence staining on day 12 after the initial injection of anti-mCol7 IgG or NR IgG. IgG binding and C3 deposition were visualized in the DEJ from C57BL/6, IL-17A/F$^{-/-}$ and IL-17A$^{-/-}$ mice, which were injected with anti-mCol7 IgG, but no IgG binding and C3 deposition was detectable in the skin of mice which were injected with NR IgG (Figure 13).

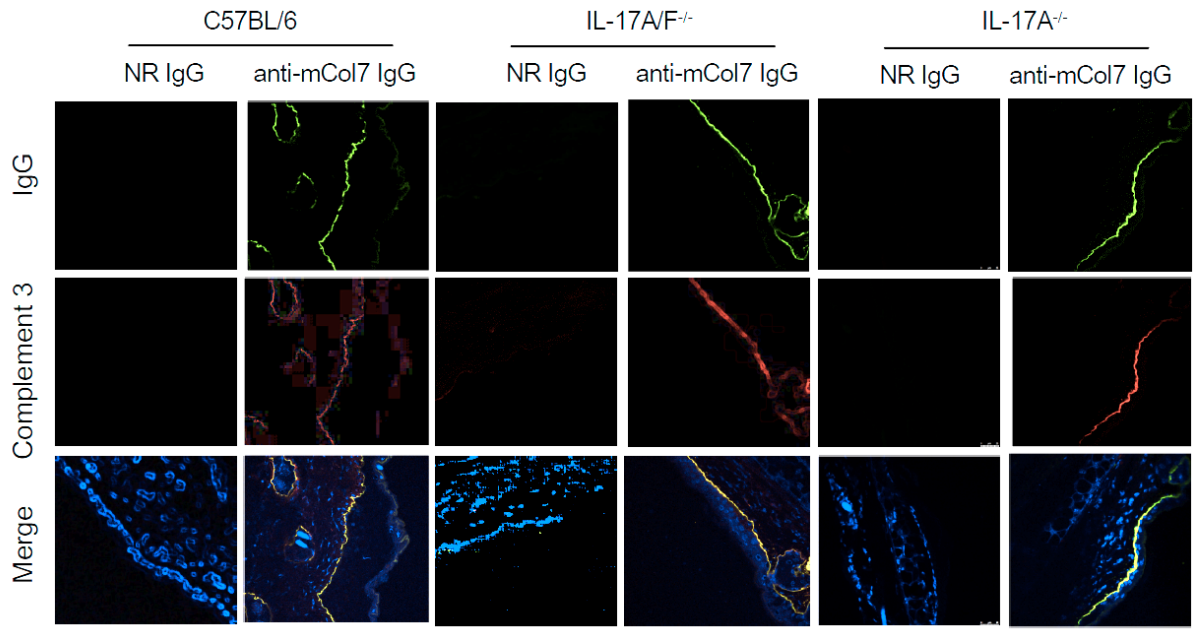

Figure 13. IgG binding and complement (C3) deposition at the DEJ in C57BL/6, IL-17A/F$^{-/-}$ and IL-17A$^{-/-}$ mice. Representative micrographs of the immunofluorescence are shown (630X). Cryosections of the skin tissue from C57BL/6, IL-17A/F$^{-/-}$, and IL-17A$^{-/-}$ mice were prepared on day 12 after initial s.c. injection with anti-mCol7 IgG or NR IgG and incubated with rat anti-mouse C3 antibody, then further stained with Alexa-546 labeled goat anti-rat IgG and Alex-488 conjugated goat anti-rabbit IgG to detect the IgG (green) and C3 deposition (red), respectively (n=5 mice/group, representative of 1 mouse/group).

Consistent with the clinical score, the H&E stained sections presented that fewer cells infiltrated the DEJ of IL-17A/F$^{-/-}$ mice compared with C57BL/6 mice after receiving the anti-mCol7 IgG (Figure 14). Furthermore, neutrophils were proved to be the predominant infiltrating cell type in the lesional skin from C57BL/6 and IL-17A/F$^{-/-}$ mice. (Figure 14). No cellular infiltration was observed in the skin from IL-17A $^{-/-}$ mice and mice injected with NR IgG.

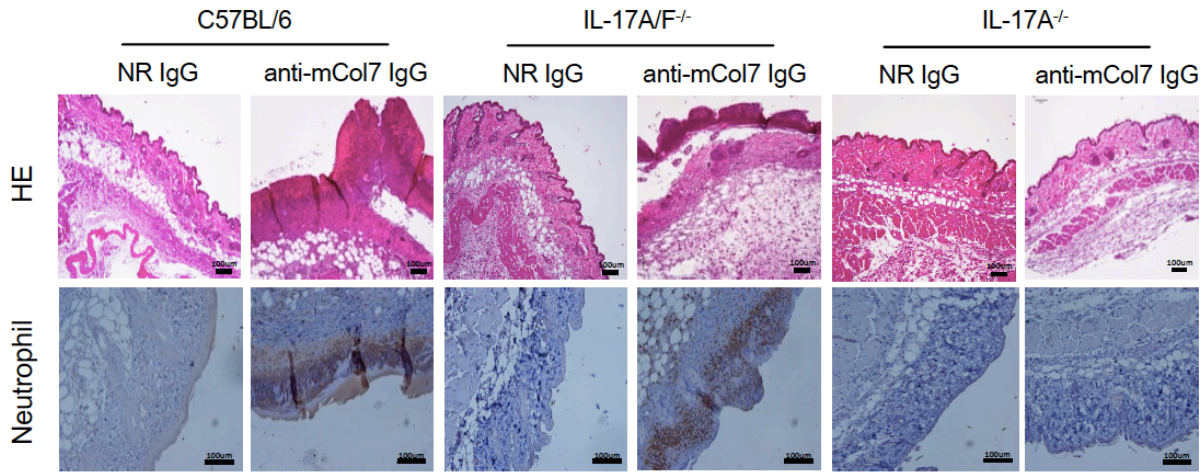

Figure 14. Reduced cell infiltration in IL-17A/F$^{-/-}$ mice with anti-mCol7 induced EBA. Photomicrograph of HE stained and immunohistochemically stained sections of skin from C57BL/6, IL-17A/F$^{-/-}$, and IL-17A$^{-/-}$ mice 12 days after s.c. injection of NR IgG or anti-mCol7 IgG (100X). Neutrophils were stained by rat anti-neutrophil antibody and shown in dark (n=5 mice/group, representative of 2 mice/group).

In order to further compare the inflammation among these mice, gene expressions of inflammatory cytokines and chemokines in the skin from C57BL/6, IL-17A/F$^{-/-}$ and IL-17A$^{-/-}$ mice were analyzed using Q-PCR on day 12. Consistent with the reduced inflammation in lesional skin from IL-17A/F$^{-/-}$ mice, mRNA expression of IL-6 and CCL-2 was significantly reduced in IL-17A/F$^{-/-}$ mice compared to C57BL/6 mice which were injected with anti-mcol7 IgG (Figure 15). IL-6 and CCL-2 was apparently not induced in anti-mCol7 injected IL-17A$^{-/-}$ mice.

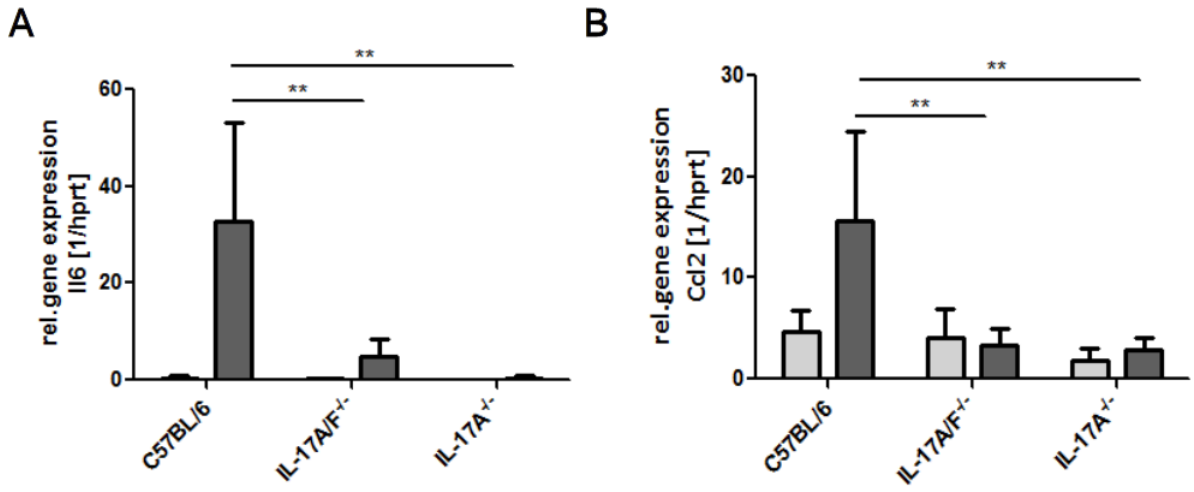

Figure 15. Decreased inflammatory cytokines and chemokines expression in IL-17A/F$^{-/-}$ mice. IL-6 (A) and CCL-2 (B) gene expressions in skin from C57BL/6, IL-17A$^{-/-}$, and IL-17A/F$^{-/-}$ mice 12 days after initial s.c. injection with NR IgG (gray) or anti-mCol7 IgG (black) by Q-PCR. Data are shown as mean±SD (n=5 mice/group, ** $p<0.01$, ANOVA Test).

5.1.3. IL-17F plays a pro-inflammatory role in antibody-induced EBA

Since IL-17E was suggested to play no role in the pathogenesis of EBA, the different outcome of the experimental EBA in IL-17A$^{-/-}$ and IL-17A/F$^{-/-}$ mice suggested a protective role of IL-17F in the development of the disease. Therefore, in the next step, EBA was induced in IL-17F$^{-/-}$ mice to validate this hypothesis.

After receiving these pathogenic anti-mCol7 antibodies, the C57BL/6 mice developed a EBA-like disease, including subepidermal blisters, skin crust and erosion, as well as neutrophil infiltration in the DEJ, and the disease severity increased over time (Figure 16). By comparison, IL-17F$^{-/-}$ mice exhibited a milder disease, and the total affected skin percentage was significantly decreased on day 8 and day 12 compared with that of C57BL/6 mice (Figure 16).

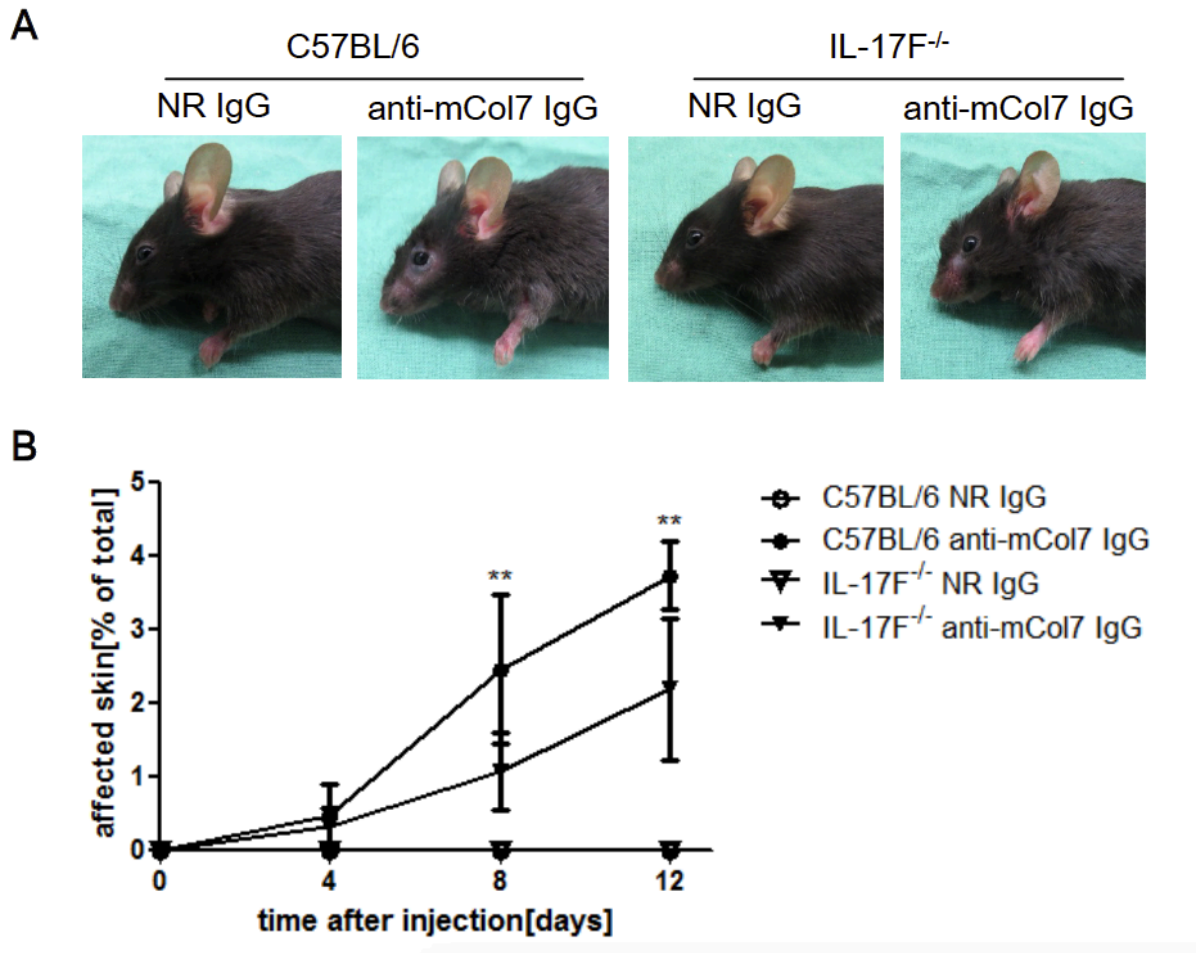

Figure 16. Anti-mCol7 IgG-induced EBA eased in IL-17F$^{-/-}$ mice. C57BL/6 and IL-17F$^{-/-}$ mice were subcutaneously injected with 18mg pathogenic anti-mCol7 IgG or NR IgG on day 0, 2, 4, 6, 8 and 10 respectively to induce experimental EBA. (A) Representative clinical pictures of experimental EBA in C57BL/6 and IL-17F$^{-/-}$ mice 12 days after initial injection of anti-mCol7 IgG or NR IgG. (B) Percentage of affected skin area on body surface area was calculated on 4, 8 and 12 days after initial injection of anti-mCol7 IgG or NR IgG. Data are shown as mean±SD (n=5 mice/group, ** $p<0.01$, Mann-Whitney Rank Sum Test).

Rabbit anti-mCol7 IgG binding and C3 deposition in the DEJ in skin from C57BL/6 and IL-17F$^{-/-}$ mice were analyzed using immunofluorescence staining on day 12 after injection of anti-mCol7 IgG or NR IgG. As shown in Figure 17, IgG binding and C3 deposition were only detectable in anti-mCol7 IgG injected groups, including both C57BL/6 and IL-17F$^{-/-}$ mice, but not in NR IgG injected groups.

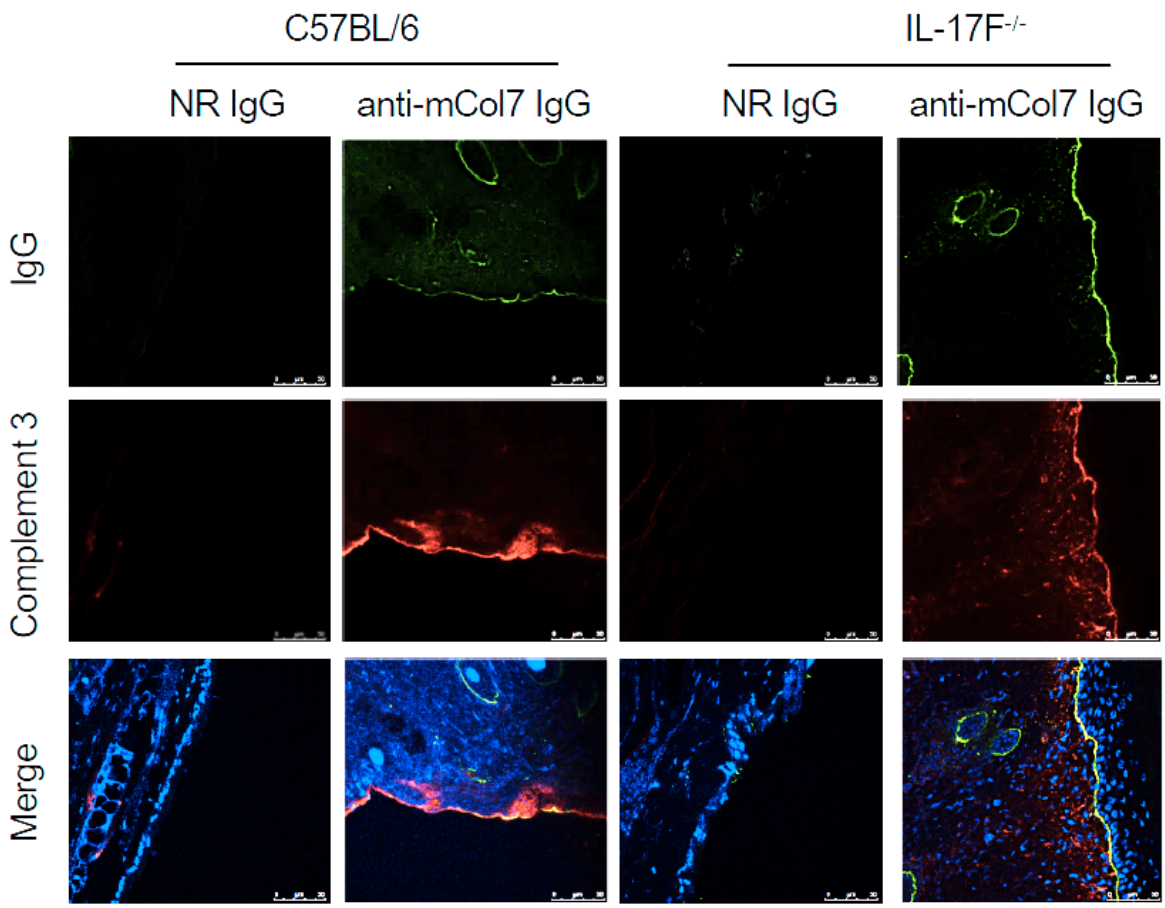

Figure 17. IgG binding and C3 deposition at the DEJ in C57BL/6 and IL-17F$^{-/-}$ mice. Representative micrographs of the immunofluorescence (630X). Cryosections of the skin tissue from C57BL/6 and IL-17F$^{-/-}$ mice were prepared on day 12 after initial s.c. injection with anti-mCol7 IgG or NR IgG and incubated with rat anti-mouse C3 antibody, then further stained with Alexa-546 labeled goat anti-rat IgG and Alex-488 conjugated goat anti-rabbit IgG to detect the IgG (green) and C3 deposition (red), respectively (n=5 mice/group, representative of 1 mouse/group).

H&E staining of skin sections revealed cell infiltration in the EDJ of IL-17F$^{-/-}$ mice and C57BL/6 mice after injection of anti-mCol7 IgG (Figure 18). All mice which received NR IgG showed a normal skin structure.

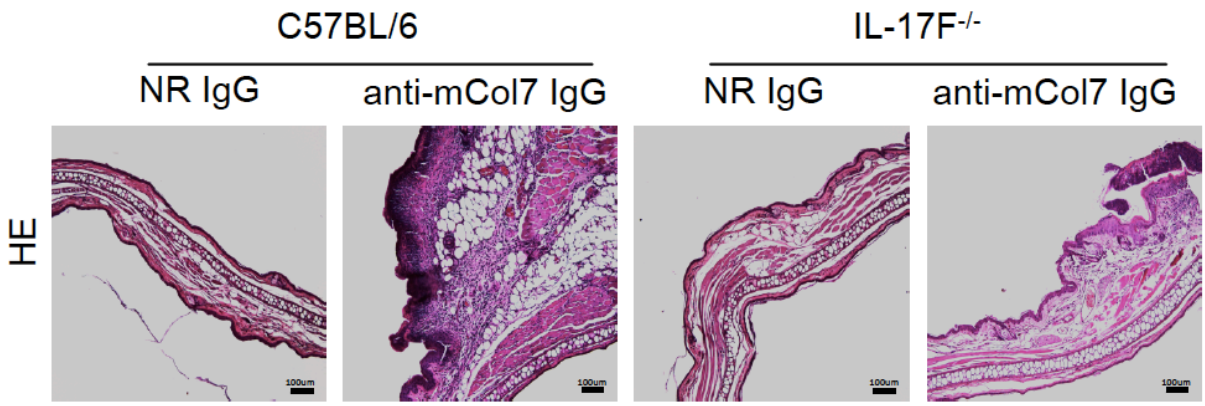

Figure 18. Histological sections of skin from C57BL/6 mice and IL-17F$^{-/-}$ mice with anti-mCol7 induced EBA. Photograph of HE stained sections of skin from C57BL/6 and IL-17F$^{-/-}$ mice, 12 days after s.c. administered with NR IgG or anti-mCol7 IgG (100X) (n=5 mice/group, representative of 4 mice).

In further analysis, mRNA expressions of inflammatory cytokines and chemokines in the skin from both C57BL/6 and IL-17F$^{-/-}$ mice were measured by Q-PCR on day 12. Consistent with the reduced inflammation in IL-17F$^{-/-}$ mice, the mRNA expression of CCL-2 was remarkably decreased in IL-17F$^{-/-}$ mice as compared to C57BL/6 mice (Figure 19). However, the mRNA level of IL-6 was not significantly different between both strains after receiving anti-mCol7 antibodies (Figure 19).

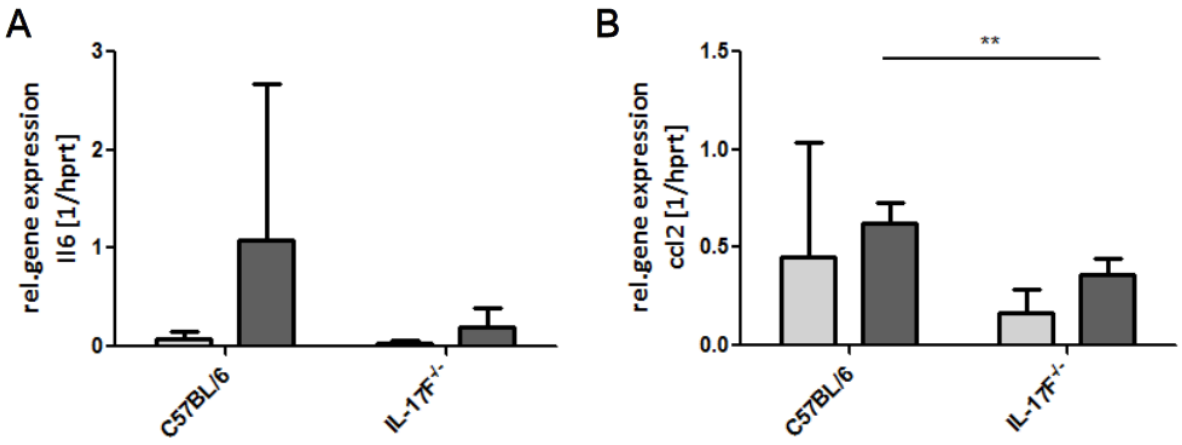

Figure 19. Reduced inflammatory cytokine expression in IL-17F$^{-/-}$ mice. IL-6 (A) and CCL-2 (B) gene expressions in lesional skin from C57BL/6 and IL-17F$^{-/-}$ mice, 12 days after injection of NR IgG (gray) or anti-mCol7 IgG (black), were detected by Q-PCR. Data are shown as mean±SD (n=5 mice/group, ** $p<0.01$, Mann-Whitney Rank Sum Test).

5.1.4. Genetic background contributes to resistance to EBA in IL-17A$^{-/-}$ mice

Because IL-17A$^{-/-}$ mice were resistant to EBA, whereas IL-17A/F$^{-/-}$ mice developed mild disease, it was speculated in the present thesis that IL-17F played a protective role in the development of EBA. However, the reduced disease developed in IL-17F$^{-/-}$ mice revealed that IL-17F is in fact pro-inflammatory in EBA. Together, this evidence suggested that other factors may be critical for the disease resistance of IL-17A$^{-/-}$ mice. Since IL-17A gene targeting was performed in the embryonic stem cell from 129 mice, and chimeric mice were subsequently backcrossed onto C57BL/6 mice[191], some 129 mice-derived genes might remain in IL-17A$^{-/-}$ mice and contribute to the disease resistance. To validate this hypothesis, EBA was firstly induced in 129 mice to determine the disease resistance of mice with this genetic background.

To examine the susceptibility of 129 mice to EBA, a local EBA disease was induced, which was an efficient way to test disease susceptibility. Briefly, anti-mCol7 IgG was injected once into the ear of the mice. 24h after injection of anti-mCol7 IgG, C57BL/6 mice developed EBA-like disease, including skin erosion on the ears and neutrophil infiltration, and the disease severity increased over time. However, the 129 mice were completely resistant to the disease (Figure 20).

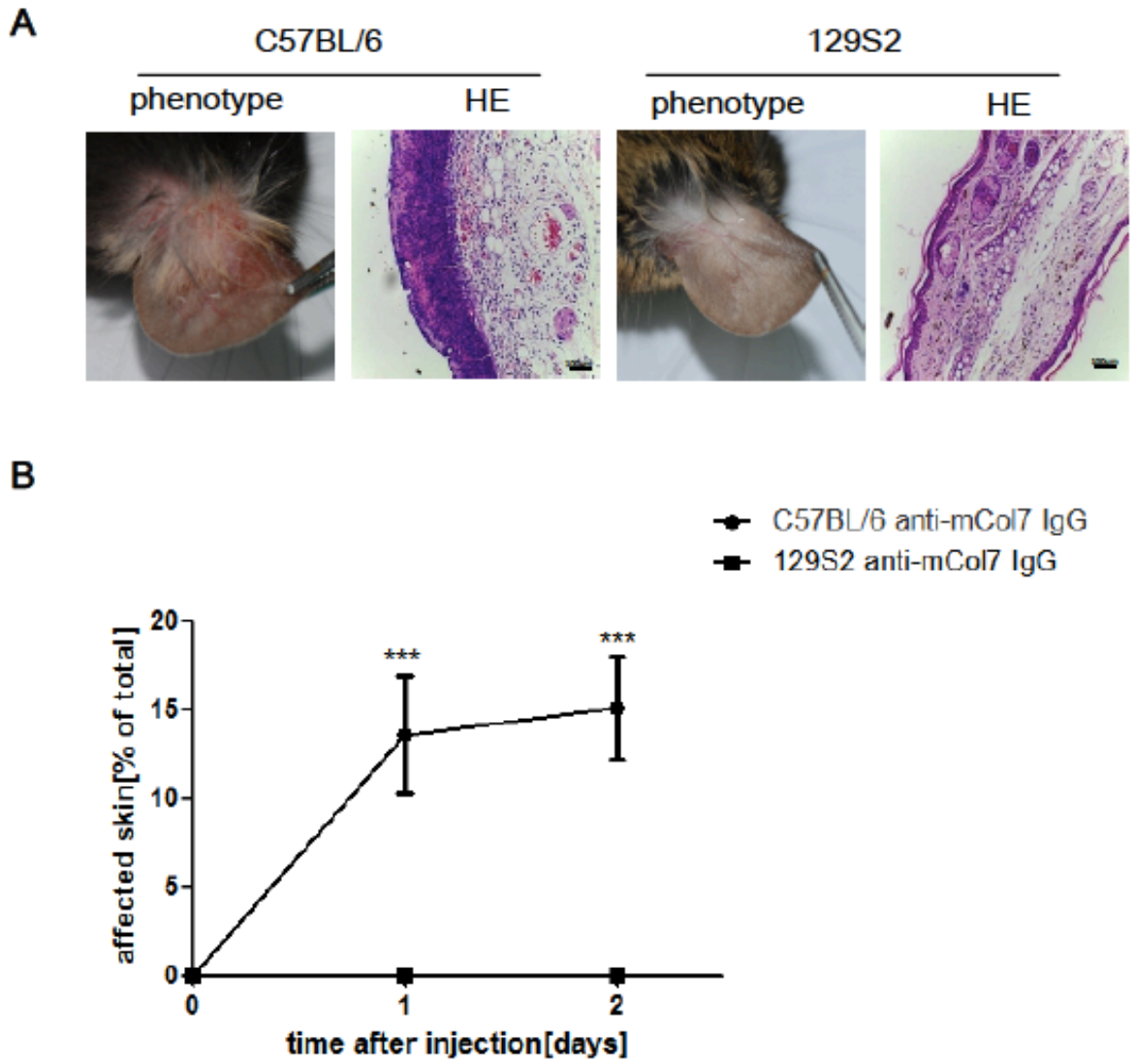

Figure 20. 129 mice were resistant to local EBA after injection of anti-mCol7 IgG to mice ear. 129 and C57BL/6 mice received once 0.42 mg anti-mCol7 IgG i.d. in the base of the ear. (A) Representative clinical pictures and HE staining pictures of skin from C57BL/6 and 129 mice on 48h after anti-mCol7 IgG administered (100X). (B) Percentage of affected area in ear (blisters, crust, erythema) was calculated 24 and 48h after the initial anti-mCol7 IgG injection. Data are shown as mean±SD (n=6 mice/group, *** $p<0.001$, Mann-Whitney Rank Sum Test).

The resistant phenotype of 129 mice indicated that a genetic contamination of the 129 genome linked to the IL-17A locus was selected during backcrossing to the C57BL/6 genetic background. To further validate this hypothesis, whole-genome analysis was performed on C57BL/6, 129, and IL-17A$^{-/-}$ mice. In total, 143,249 signal nucleotide polymorphisms (SNPs) were sequenced. Among the total SNPs, 34,298 informative SNPs were selected, at which the nucleotides of the IL-17A$^{-/-}$ strain were the same as the 129 strain, but different from the

C57BL/6 strain. By this method, the 129-derived genes were identified IL-17A$^{-/-}$ mice. As shown in Figure 21, the majority of these informative SNPs clustered into 8 fragments, which were distributed on chromosome 1, 2, 3, 10, 13 and 16 of IL-17A$^{-/-}$ mice. The largest fragments were located on chromosomes 1 and 2 (Table 5).

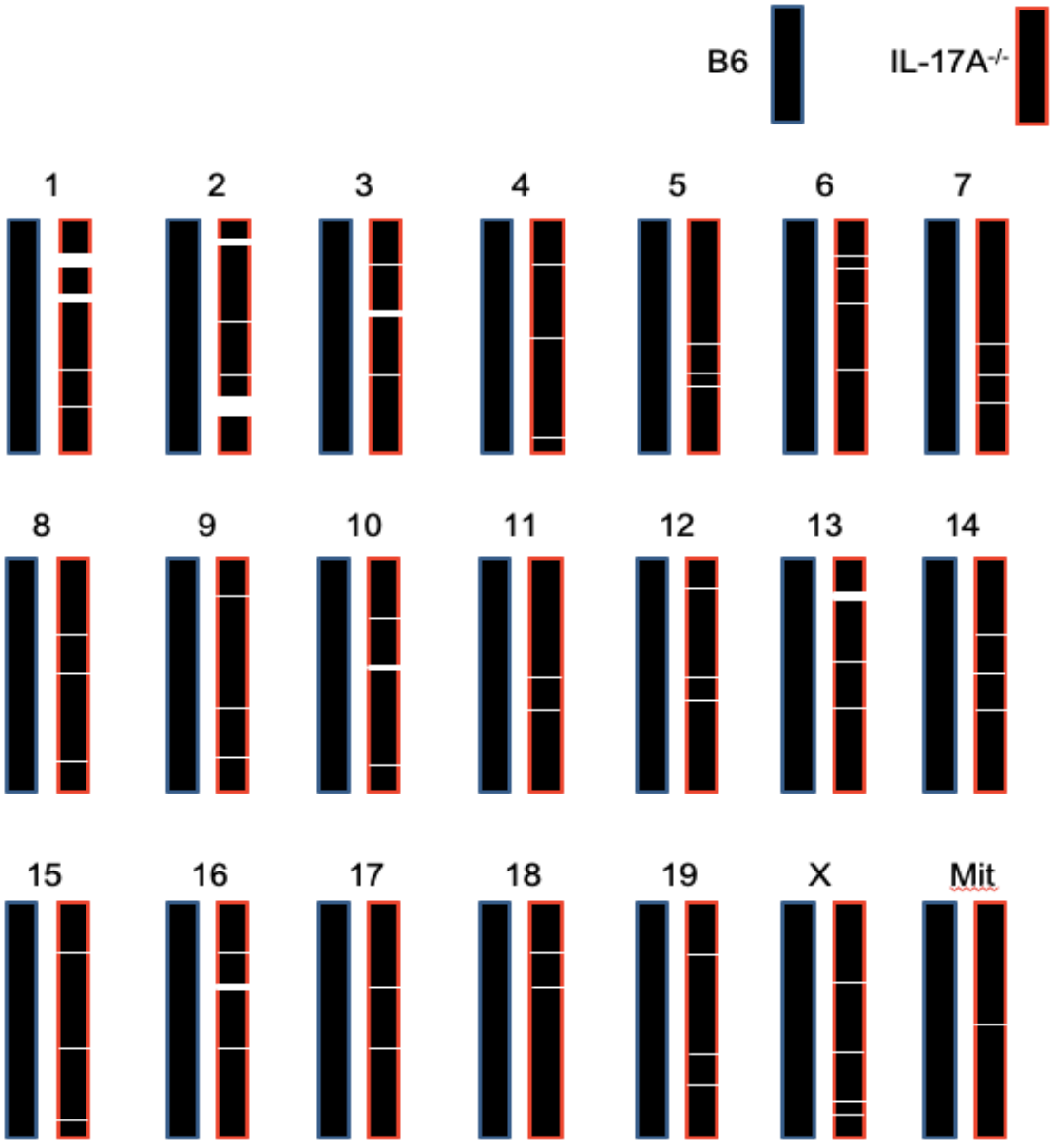

Figure 21. Schematic diagram of 129 mice background genes remaining in IL-17A$^{-/-}$ mice. DNA isolated from C57BL/6, 129 and IL-17A$^{-/-}$ mice, was sent for genotyping by GigaMUGA. Informative SNPs were distributed on all 21 chromosomes of IL-17A$^{-/-}$ mice. Black bars represent chromosomes of C57BL/6 mice, black bars with a red frame represent chromosomes of IL-17A$^{-/-}$ mice. White bars represent genes from 129 mice in IL-17A$^{-/-}$ mice.

Table 5. 129 mice-derived background genes remaining in IL-17A$^{-/-}$ mice

Chromosome	position	size	gene
1	15110051-38275608	23.17Mbp	270gene IL-17A,IL-17F,
1	73455399-76026538	2.57Mbp	75gene CXCR2，CXCR1，IL-1r1
2	12196989-19473747	7.28Mbp	82gene
2	80713295-93310921	12.6Mbp	477gene
3	54581274-56511763	1.93Mbp	31gene
10	53489079-53729209	0.24Mbp	7gene
13	24749072-27129019	2.38Mbp	26gene
16	41688007-42174802	0.49Mbp	2gene

5.1.5. Biological inhibition of IL-17A reduces antibody-induced EBA

Based on previous studies in the Infection Immunology research group of antibody-induced EBA in IL-17A$^{-/-}$ mice, a pathogenic role of IL-17A had been suggested. However, in the present thesis the identified genetic contamination of IL-17A$^{-/-}$ mice with genes of EBA-resistant 129 mice may have accounted for this result. Therefore, anti-IL17A antibody was used to prove the role of IL-17A in the pathogenesis of EBA. Avoiding the effect of a different genetic background, experimental EBA was induced in C57BL/6 mice by repetitive anti-mCol7 IgG or NRS injections on day 0, 2, 4, 6, 8, and 10. To block IL-17A function, on day -2, 0, 2, 4, 6, 8 and 10, mice were treated with an anti-IL-17A antibody or isotype antibody.

EBA manifestation, such as erosion and blister formation, was observed in both the isotype

antibody and anti-IL-17A antibody treatment groups after transferring anti-mCol7 IgG (Figure 22 A). However, as compared with mice treated with the isotype antibody, significantly ameliorated EBA disease was observed in mice treated with anti-IL-17A antibody, characterized by a decreased area of affected skin (Figure 22 B).

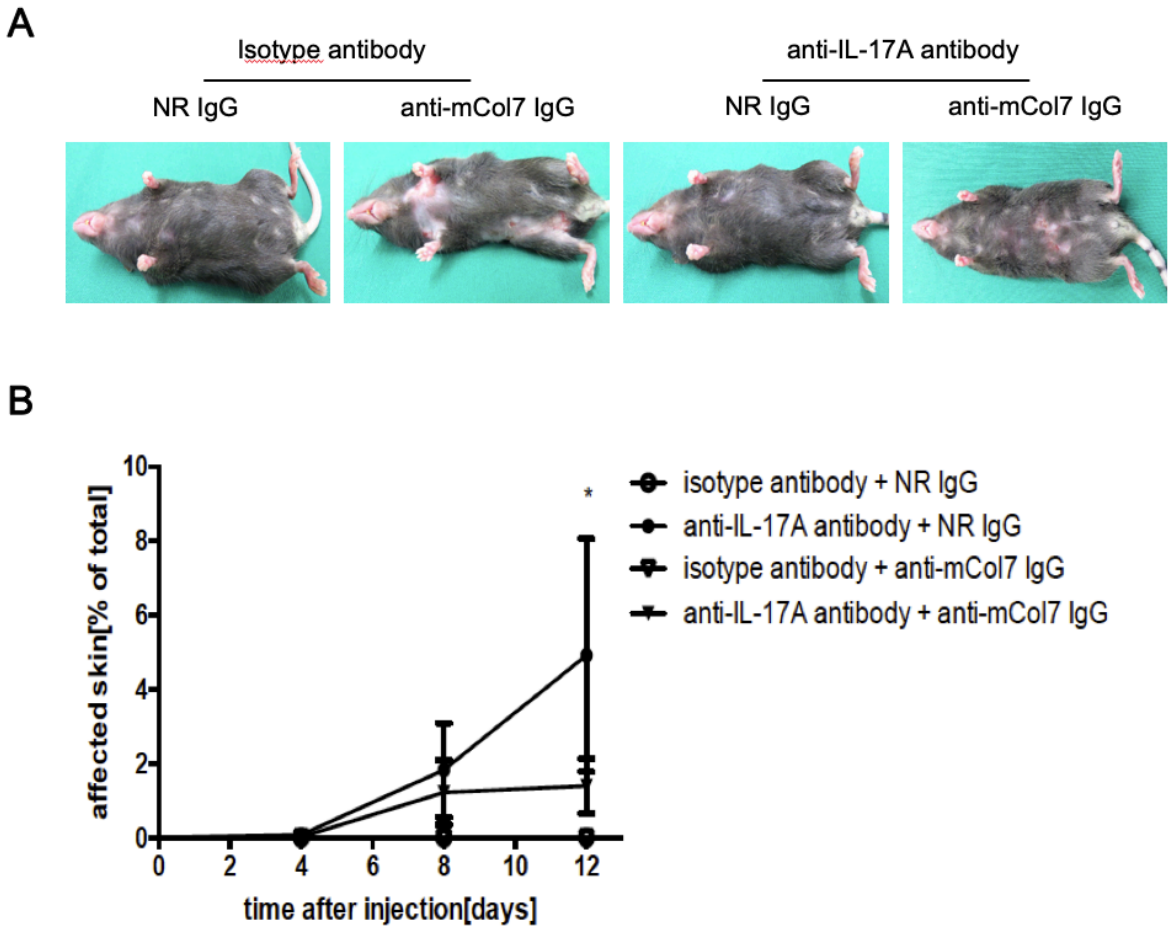

Figure 22. Decreased blister formation in experimental EBA after anti-IL-17A antibody treatment. Experimental EBA was induced in C57BL/6 mice by subcutaneous injection a total of 12 mg anti-mCol7 IgG or normal rabbit IgG (NR IgG) on day 0, 2, 4, 6, 8, and 10. To block IL-17A function, on day -2, 0, 2, 4, 6, and 8, mice were treated with anti-IL-17A antibody or isotype antibody respectively. (A) Representative clinical pictures of C57BL/6 mice with anti-IL-17A antibody or isotype antibody treatment, 12 days after NR IgG or anti-mCol7 IgG injection. (B) EBA severity assessed by the percentage of body surface area covered by skin lesions on 4, 8 and 12 days after the initial injection of anti-mCol7 IgG or NR IgG. Data are shown as mean±SD (n=3-7 mice/group, ** $p<0.01$, Mann-Whitney Rank Sum Test).

To confirm the autoantibody binding and complement deposition in the DEJ, immunofluorescence staining was performed on cryosections of skin from mice on day 12 after injection of anti-mCol7 IgG or NR IgG. As shown in Figure 23, IgG binding and C3 deposition were observed in the anti-mCol7 IgG injected groups, but not in the NR IgG injected groups.

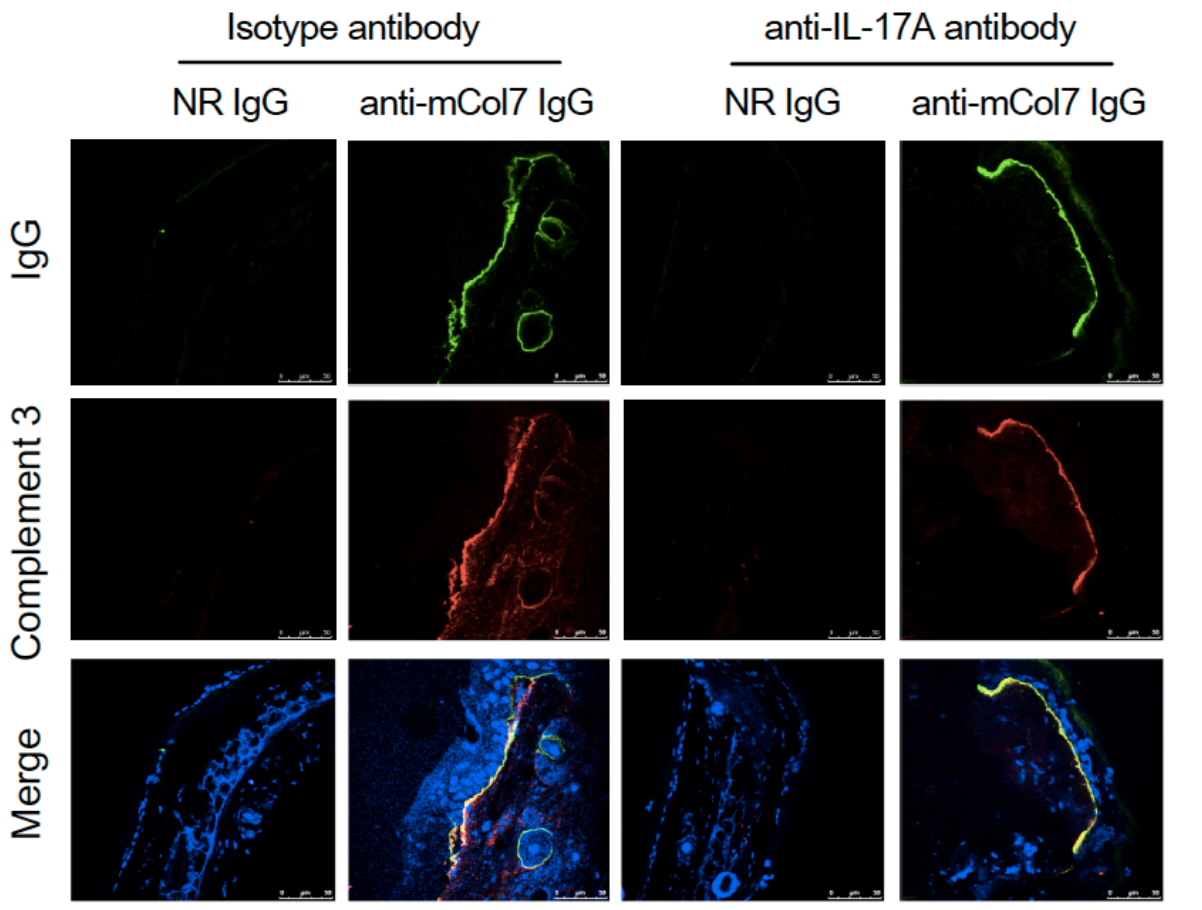

Figure 23. IgG binding and complement (C3) deposition at the DEJ in mice with biological IL-17A inhibition. Representative micrographs of the immunofluorescence (630X). Cryosections of the skin tissue from C57BL/6 with anti-IL-17A antibody or isotype antibody treatment were prepared on day 12 after initial s.c. injection with anti-mCol7 IgG or NR IgG and incubated with rat anti-mouse C3 antibody, then further stained with Alexa-546 labeled goat anti-rat IgG and Alex-488 conjugated goat anti-rabbit IgG to detect the IgG (green) and C3 deposition (red), respectively (n=3-7 mice/group, representative of 1 mouse/group).

Histological analysis was performed to evaluate the inflammation and disease features of EBA. Histopathological examination of paraffin skin sections revealed reduced inflammatory infiltration as well as impaired dermal-epidermal separation in anti-IL-17A antibody treated mice compared with mice that received the isotype antibody (Figure 24). There was no cell infiltration in mice received that NR IgG.

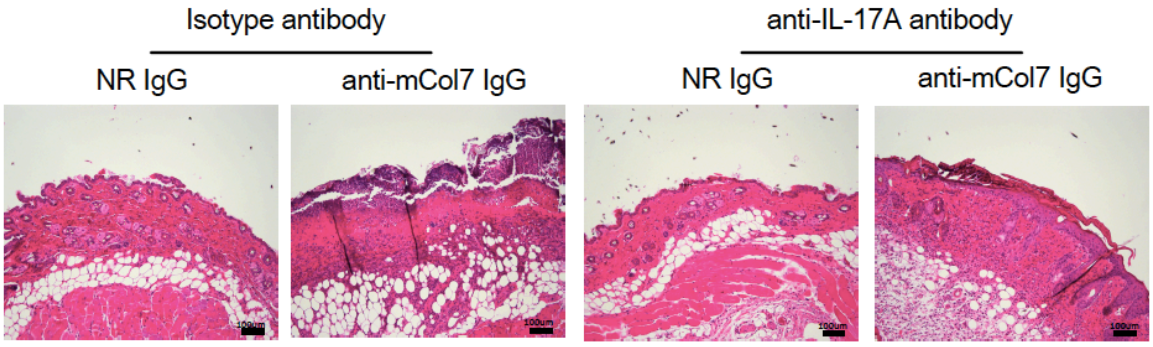

Figure 24. H&E staining of skin sections from C57BL/6 mice with anti-mCol7 induced EBA after biological inhibition of IL-17A. Photomicrograph of HE stained sections of skin from C57BL/6 mice under anti-IL-17A antibody treatment or isotype antibody treatment 12 days after injection of NR IgG or anti-mCol7 IgG (X100) (n=3-7 mice/group, representative of 1 mouse/group).

5.2. The cellular source of IL-17A during the course of EBA

Since the previous findings and current results suggested that IL-17A plays a pathogenic role in EBA, the cellular source and the contribution of these cells to the disease activity were investigated.

5.2.1. γδT-cells are the main source of IL-17A in antibody-induced EBA

In order to distinguish the cellular source of IL-17A in EBA, IL-17A-eGFP mice were used to induce EBA by anti-mCol7 IgG injection. After receiving the anti-mCol7 IgG, IL-17A-eGFP mice developed EBA disease similar to C57BL/6 mice, showing erosion, redness and hair loss on the ears and body surface (Figure 25A). Histologically, cell infiltration was clearly

observed in both C57BL/6 and IL-17A-eGFP mice after anti-mCol7 IgG transfer (Figure 25A). Calculation of the overall disease score according to the affected area in mice revealed no difference between IL-17A-eGFP and C57BL/6 mice (Figure 25B).

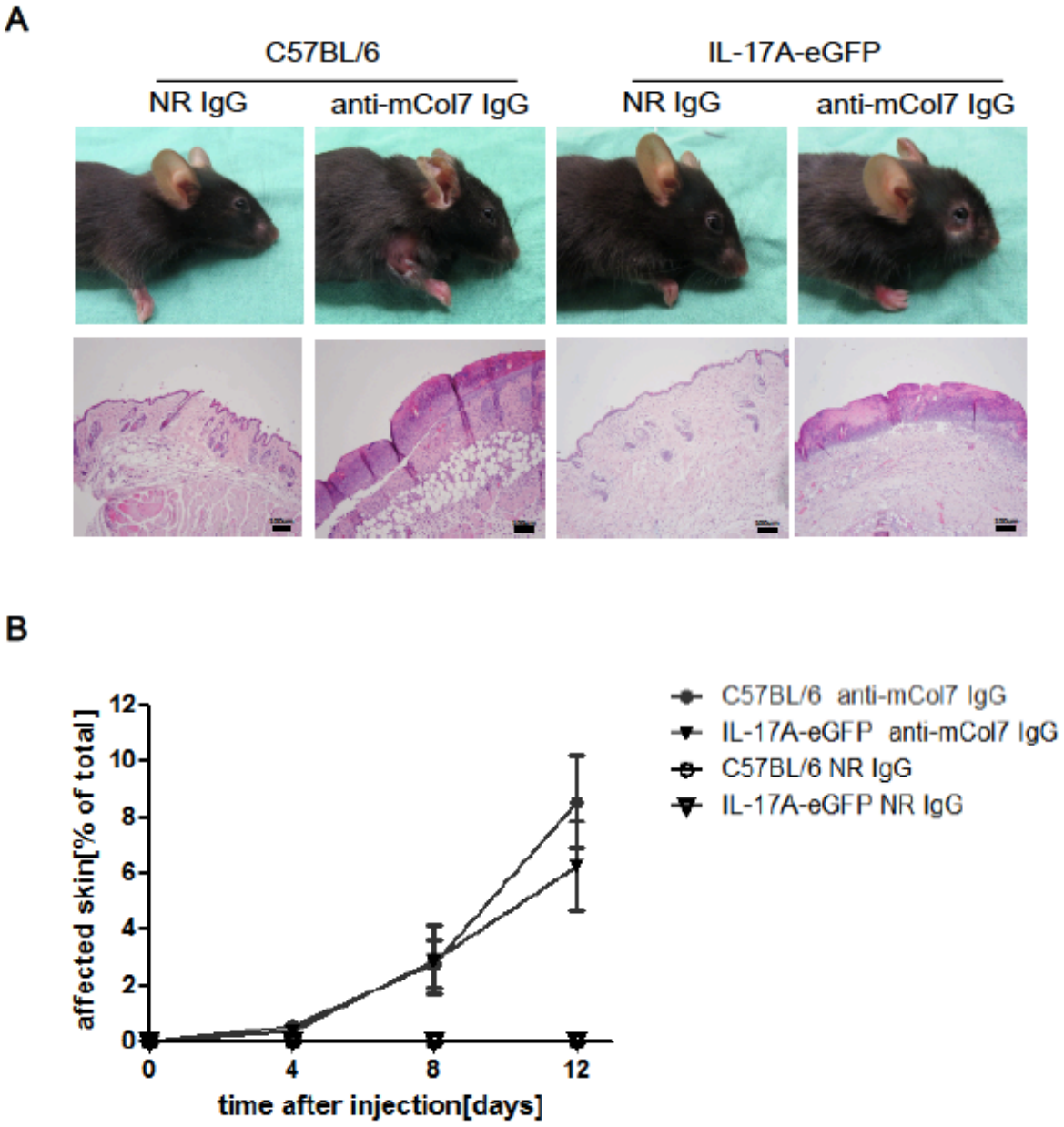

Figure 25. IL-17A-eGFP mice developed EBA after anti-mCol7 IgG injection. C57BL/6 and IL-17A-eGFP mice were injected with 18 mg anti-mCol7 IgG or normal rabbit IgG (NR IgG) on day 0, 2, 4, 6, 8, and 10 by subcutaneous injection. (A) Representative clinical pictures and HE staining pictures of C57BL/6 and IL-17A-eGFP mice 12 days after s.c. injection of NR IgG or anti-mCol7 IgG. (B) EBA severity assessed by the percentage of body surface area covered by skin lesions on 4, 8 and 12 days after the initial s.c. injection of NR IgG or anti-mCol7 IgG. Data are shown as mean±SD (n=5 mice/group, Mann-Whitney Rank Sum Test).

To clarify the exact IL-17A-producing cells in experimental EBA, cells were isolated from the lesional skin of IL-17A-eGFP mice, stained with different cell surface markers and analyzed using flow cytometry. Since Th17-cells and γδT-cells are the most-reported IL-17A producing cells in autoimmune disorders, and neutrophil is the predominant infiltrating cell type in EBA, skin cells were incubated with antibodies against the surface epitopes of these cells: anti-CD45, anti-CD4, anti-Ly6G and anti-γδTCR antibodies. Live/dead staining was added to exclude dead cells from tissue digestion. The live $CD45^+$ cell population was investigated for IL-17A-producing cell types. Furthermore, $IL\text{-}17A^+$ cells were classified into different cell types. As shown in Figure 26, γδT-cells (62.98±12.3251%) were identified as the major cellular source of IL-17A in experimental EBA, followed by $CD4^+$ T-cells (29.954±11.4235%) and $Ly6G^+$ neutrophils (9.364±6.8831%).

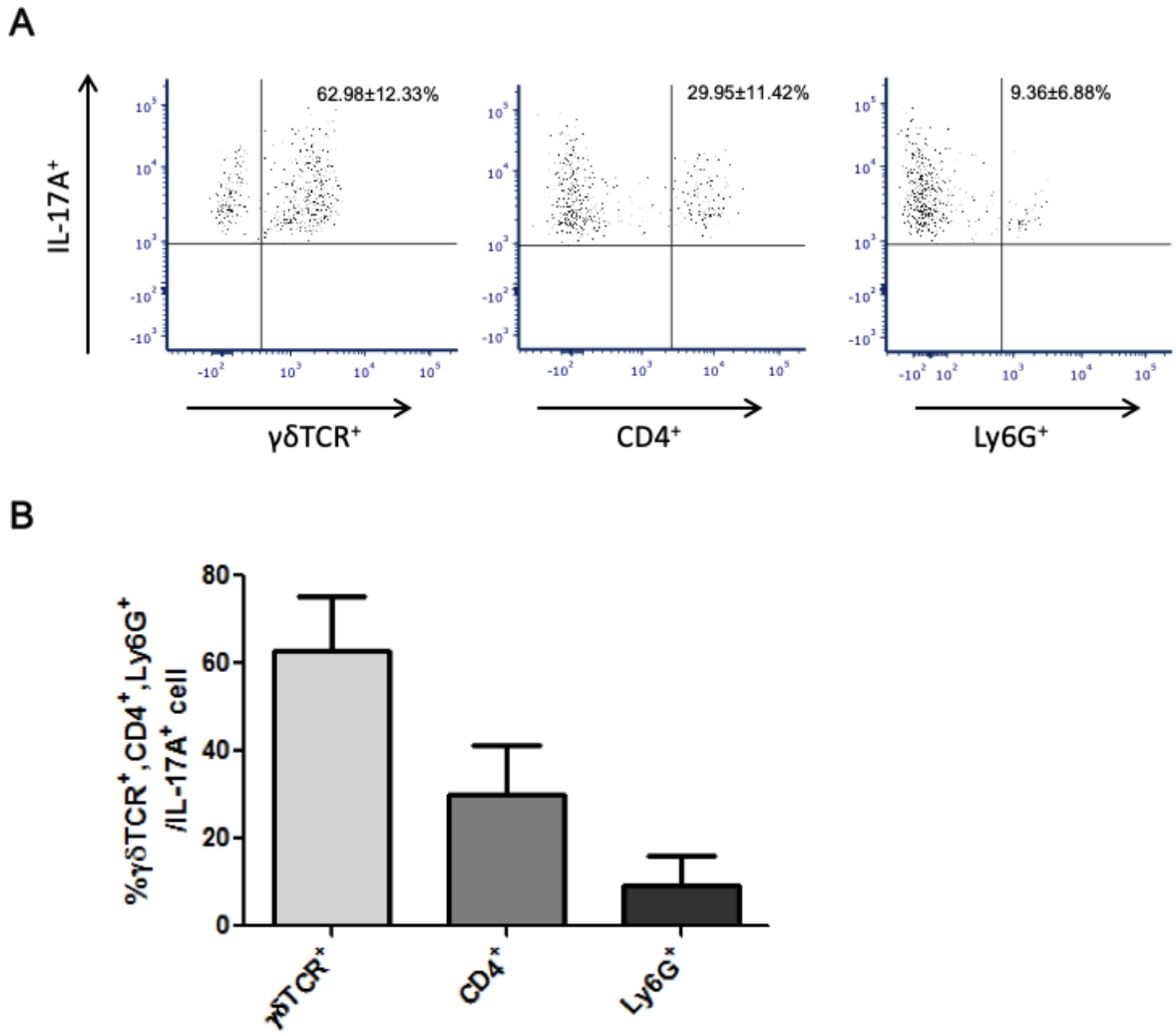

Figure 26. γδT-cells are the major IL-17A-producing cells in passive EBA. IL-17A-eGFP mice were injected with 18 mg anti-mCol7 IgG or normal rabbit IgG (NR IgG) on day 0, 2, 4, 6, 8, and 10 by subcutaneous injection. Cells were isolated from the lesional skin of IL-17A-eGFP mice 12 days after s.c. injection of NR IgG or anti-mCol7 IgG, and stained with anti-CD45, anti-CD4, anti-γδTCR and anti-Ly6G antibodies followed by live/dead staining, then analyzed using flow cytometry. (A) Representative FACS analysis were performed using isolated skin cells from IL-17A-eGFP mice 12 days after s.c. injection of anti-mCol7 IgG. (B) Gated live single IL-17A$^+$ cells were analyzed for the producing cell types (CD4, γδTCR and Ly6G). Data were shown as mean±SD (n=5 mice/group).

5.2.2. γδTCR$^{-/-}$ mice are protected from antibody-induced EBA

Together, these results suggested that the IL-17A is primarily produced by γδT-cells in the development of EBA. By using γδTCR-eGFP reporter mice, the presence of resident γδT-cells was determined in skin tissue from untreated mice. As shown in Figure 27A, resident γδT-cells were observed in the skin, the majority of them located in the dermis and

hypodermis. To investigate the kinetics of γδT-cell infiltration during the development of EBA, populations of γδT-cells in the skin of anti-mCol7 IgG or NR IgG injected mice were measured by flow cytometry. After the injection of anti-mCol7 IgG, the population of γδT-cells increased in lesional skin (Figure 27B).

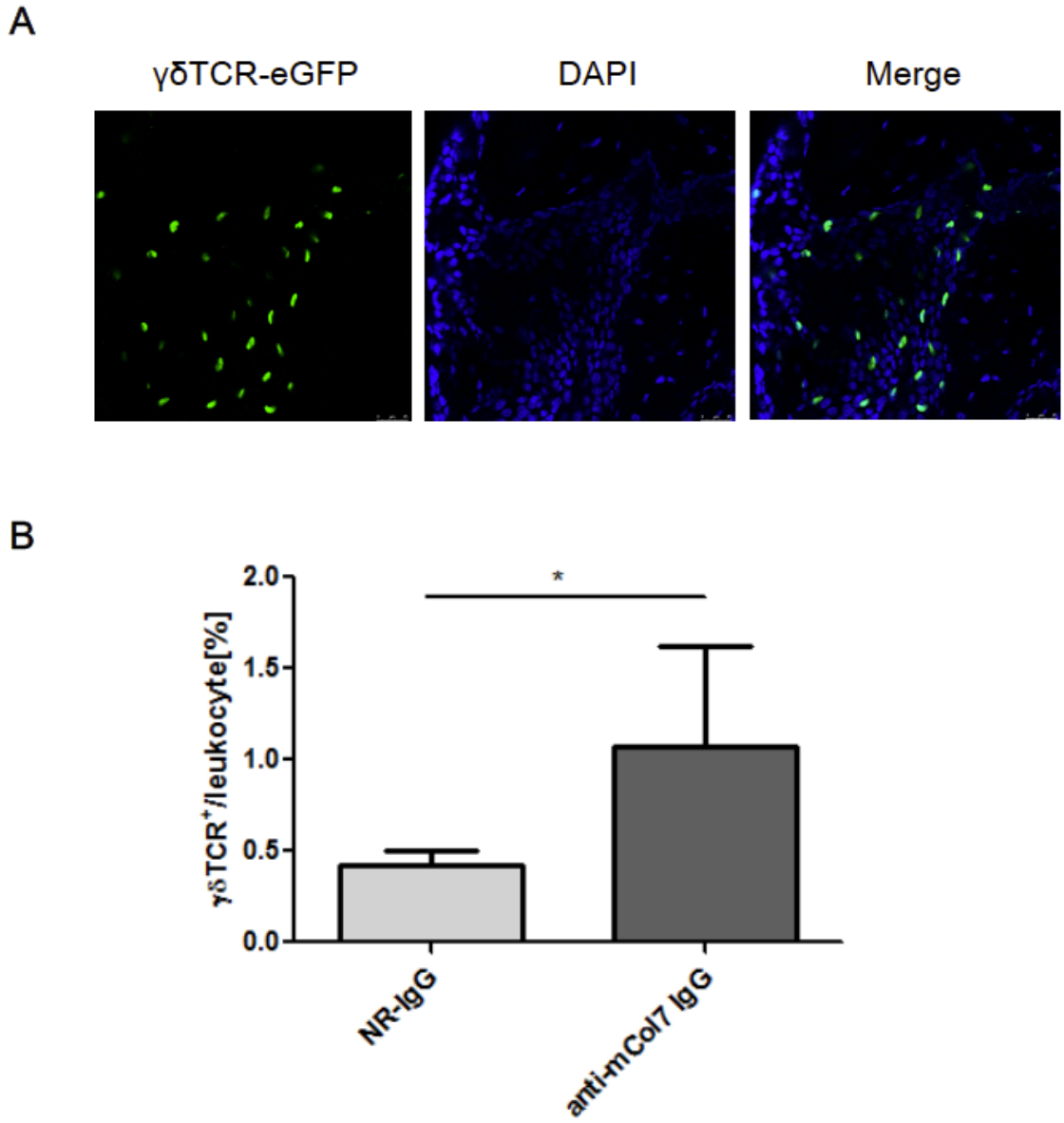

Figure 27. γδT-cells increased in lesional skin after receiving anti-mCol7 IgG transfer. (A) Representative micrographs of the immunofluorescence (630x). Cryosections of the healthy skin tissue of γδTCR-eGFP mice were used to detect γδT (green). (B) Cells were isolated from the lesion skin of C57BL/6 mice 12 days after injection of anti-mCol7 IgG and stimulated with PMA/ionomycin and GolgiPlug for 4.5h. The harvested cells were stained with surface marker antibodies (anti-CD45, anti-CD3, anti-CD4, anti-Ly6G, anti-γδTCR, anti-B220 antibodies) and measured for LSR II. The percentage of γδTCR$^+$-cells in skin from C57BL/6 mice which received NR IgG or anti-mCol7 IgG is shown. Data are shown as mean±SD (n=5 mice/group, *$p<0.05$, Mann-Whitney Rank Sum Test).

To prove the contribution of IL-17A producing γδT-cells to EBA, γδTCR$^{-/-}$ mice were used to induce experimental EBA, and the levels of IL-17A and disease severity were evaluated. The levels of IL-17A producing cells were measured in C57BL/6 and γδTCR$^{-/-}$ mice. EBA was induced in C57BL/6 mice and γδTCR$^{-/-}$ mice by 6 repetitive injections of 18 mg anti-mCol7 IgG. The same dose NR IgG was given to C57BL/6 and γδTCR$^{-/-}$ mice as a control. After injection of anti-mCol7 IgG, numerous IL-17A^{+} cells were detected in the skin of C57BL/6 mice, however, the levels of IL-17^{+} cells were significantly decreased in γδTCR$^{-/-}$ mice (Figure 28A). Consistently, the mRNA levels of IL-17A and IL-17F in the affected skin were also significantly reduced in γδTCR$^{-/-}$ mice as compared to C57BL/6 mice (Figure 28B, C).

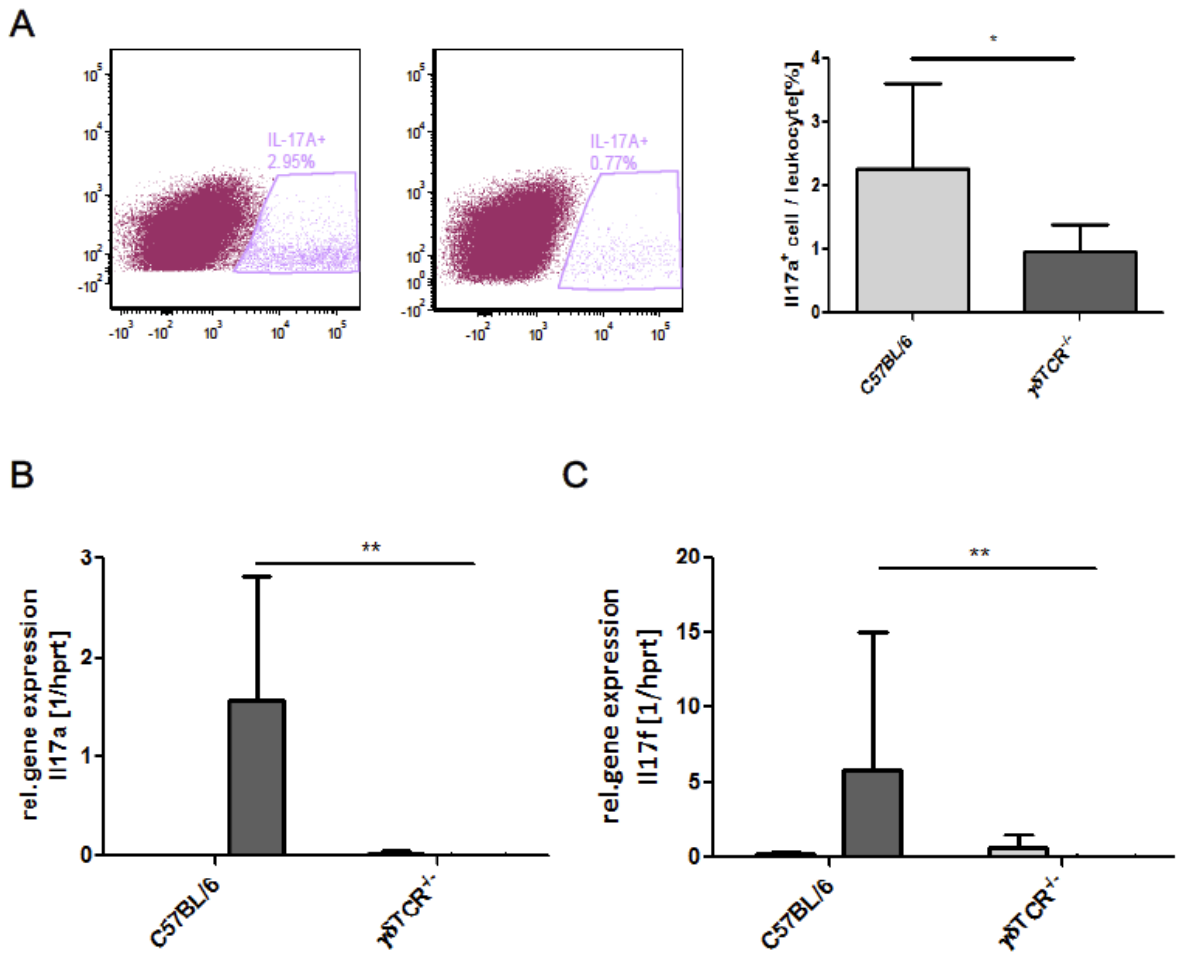

Figure 28. Reduced levels of IL-17A in γδTCR$^{-/-}$ mice. Cells were isolated from the lesional skin of C57BL/6 and γδTCR$^{-/-}$ mice 12 days after injection of anti-mCol7 IgG and stimulated with PMA/ionomycin and GolgiPlug for 4.5h. Harvested cells were firstly stained with surface marker antibodies (CD45, CD3, CD4, Ly6G, γδTCR, B220) and then incubated with the IL-17A antibody. After intracellular staining, cells were measured for LSR II. (A) Representative gating of IL-17A^{+} cell populations and the percentage of IL-17A producing cells in lesional skin from C57BL/6 and γδTCR$^{-/-}$ mice. mRNA expression of IL-17A (B) and IL-17F (C) in skin from C57BL/6 and γδTCR$^{-/-}$ mice on day 12 after injection of NR IgG (gray) or anti-mCol7 IgG (black) were evaluated using Q-PCR. Data are shown as mean±SD (n=5 mice/group, *p<0.05, Mann-Whitney Rank Sum Test).

Furthermore, after injection of anti-mCol7 IgG, C57BL/6 mice showed severe skin blister formation, whereas γδTCR$^{-/-}$ mice developed milder EBA (Figure 29A). EBA severity was assessed by determining the percentage of the affected body surface area covered by skin lesions after 4, 8 and 12 days. Compared to C57BL/6 mice, the disease severity was significantly ameliorated in γδTCR$^{-/-}$ mice (Figure 29B).

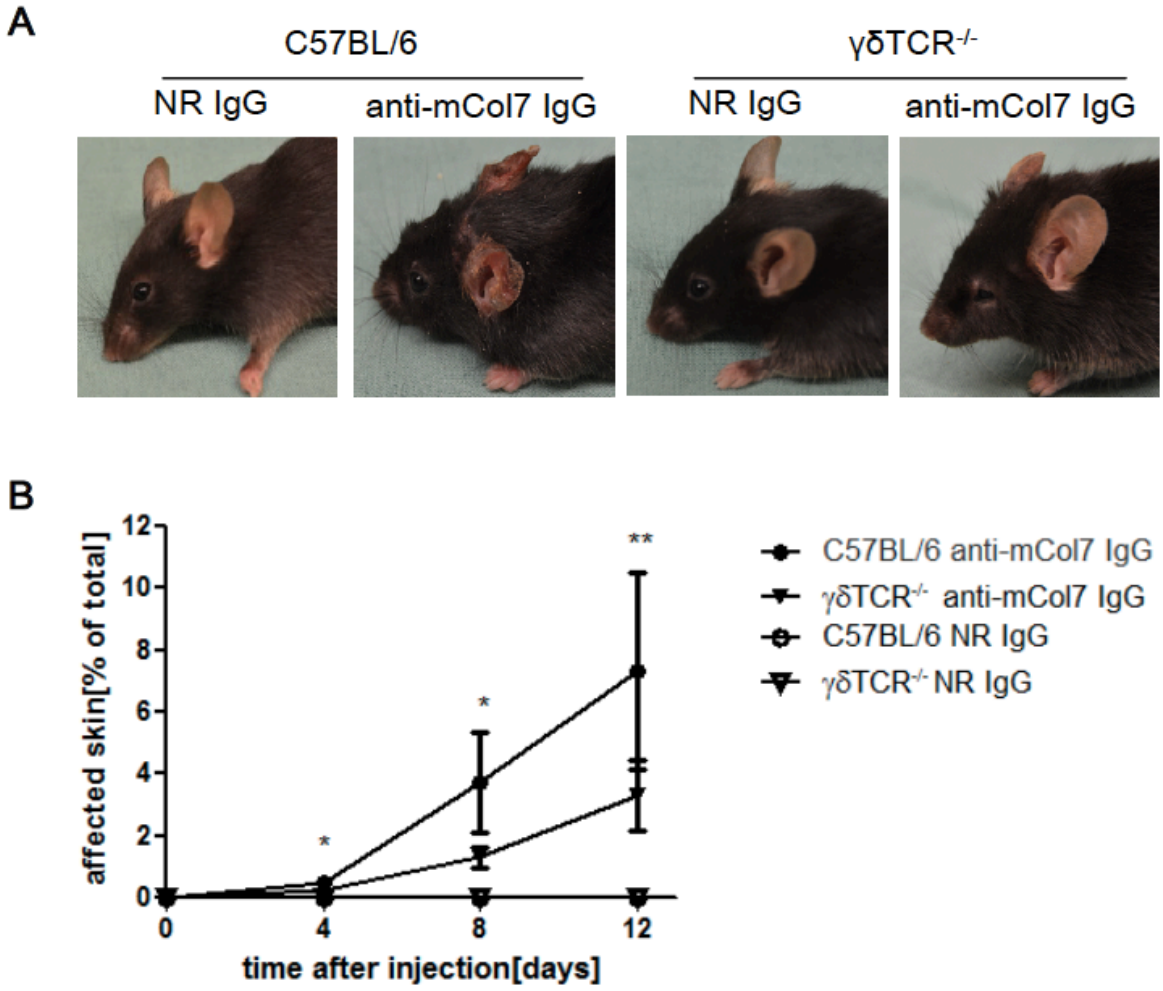

Figure 29. γδTCR$^{-/-}$ mice were protected from antibody-induced EBA. C57BL/6 and γδTCR$^{-/-}$ mice were injected with 18 mg anti-mCol7 IgG or normal rabbit IgG (NR IgG) on day 0, 2, 4, 6, 8, and 10 by subcutaneous injection. (A) Representative clinical pictures of C57BL/6 and γδTCR$^{-/-}$ mice 12 days after s.c. injection of NR IgG or anti-mCol7 IgG. (B) EBA severity assessed by the percentage of body surface area covered by skin lesions on 4, 8 and 12 days after initial injection of the NR IgG or anti-mCol7 IgG. Data are shown as mean±SD (n=5 mice/group, * $p<0.05$, ** $p<0.01$, Mann-Whitney Rank Sum Test).

In addition, rabbit anti-mCol7 IgG binding and C3 deposition in the DEJ in skin from C57BL/6 and γδTCR$^{-/-}$ mice were analyzed by immunofluorescence staining on day 12 after injection of anti-mCol7 IgG or NR IgG. As shown in Figure 30, IgG binding and C3 deposition were observed in both C57BL/6 and γδTCR$^{-/-}$ mice after injection with anti-mCol7 IgG, but no IgG binding and C3 deposition were found in the NR IgG injected groups.

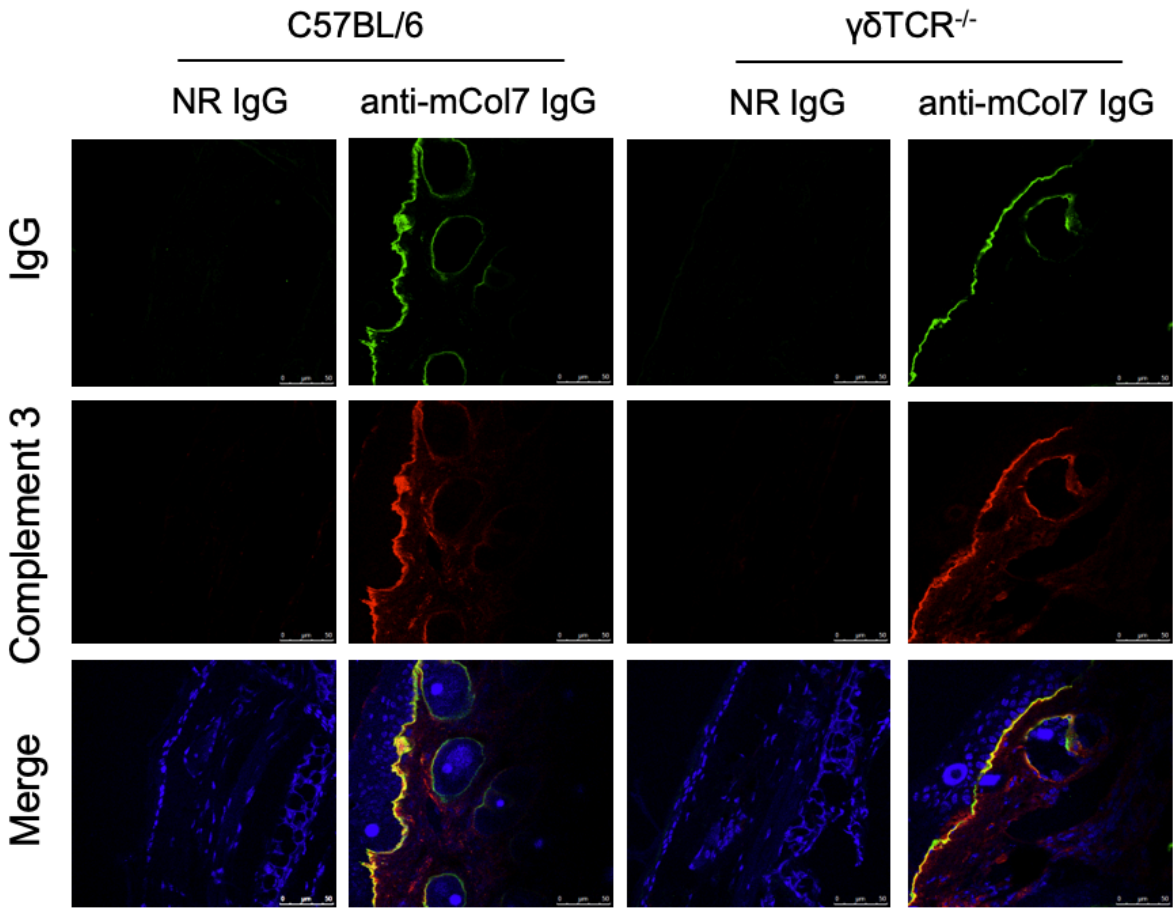

Figure 30. IgG binding and C3 deposition at the DEJ in C57BL/6 and γδTCR $^{-/-}$ mice. Representative micrographs of the immunofluorescence (630X). Cryosections of skin tissue from C57BL/6 and γδTCR$^{-/-}$ mice were prepared on day 12 after initial s.c. injection with anti-mCol7 IgG or NR IgG and incubated with rat anti-mouse C3 antibody, then further stained with Alexa-546 labeled goat anti-rat IgG and Alex-488 conjugated goat anti-rabbit IgG to detect the IgG (green) and C3 deposition (red), respectively (n=5 mice/group, representative of 1 mouse/group).

Histologically, numerous inflammatory infiltrating cells in the skin and separation of the dermis and epidermis were observed in anti-mCol7 IgG treated C57BL/6 and γδTCR$^{-/-}$ mice (Figure 31). However, the severity of cell infiltration and blister formation was remarkably eased in γδTCR$^{-/-}$ mice in contrast to C57BL/6 mice. There was no sign of skin damage in NR IgG injected mice.

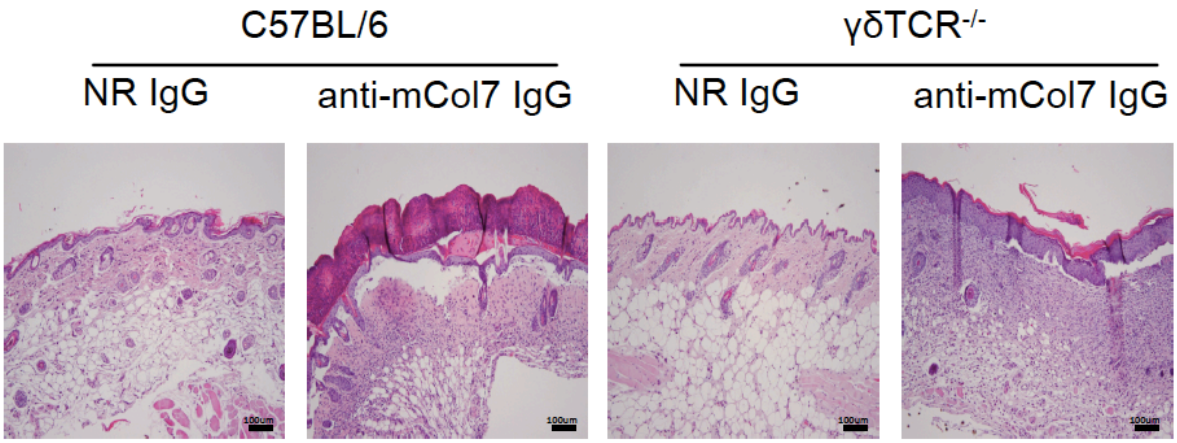

Figure 31. Histological photomicrographs of antibody-induced EBA in C57BL/6 and γδTCR$^{-/-}$ mice. Photograph of HE stained sections from C57BL/6 and γδTCR$^{-/-}$ mice administered 12 days after s.c. injection of NR IgG or anti-mCol7 IgG (100X) (n=5 mice/group, representative of 1 mouse/group).

Accordingly, the mRNA level of the pro-inflammatory cytokine, IL-1β, was decreased in γδTCR$^{-/-}$ mice in contrast to C57BL/6 mice after injection of anti-mCol7 IgG (Figure 32). The mRNA level of neutrophil chemokine CCL-2 was also analyzed, and also found to be reduced, yet not statistically significantly different compared to C57BL/6 mice.

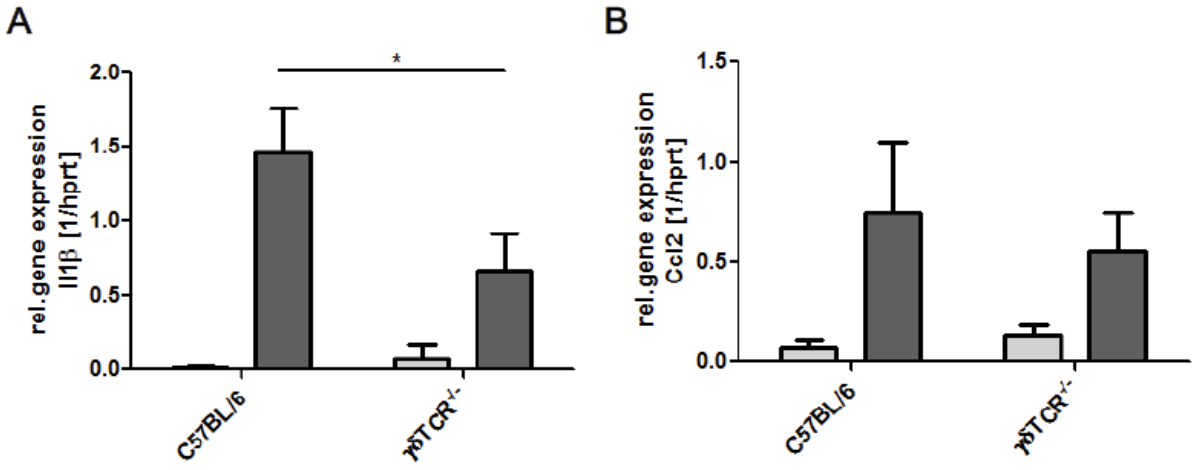

Figure 32. mRNA expression of IL-1β and CCL-2 in γδTCR$^{-/-}$ mice. mRNA expression of IL-1β (A) and CCL-2 (B) in skin from C57BL/6 mice and γδTCR$^{-/-}$ mice, 12 days after s.c. injection of NR IgG (gray) or anti-mCol7 IgG (black), were evaluated using Q-PCR. Data are shown as mean±SD

(n=5 mice/group, *p<0.05, Mann-Whitney Rank Sum Test).

5.3. IL-1β promotes γδT cells to release IL-17A

To further dissect the production of IL-17A by γδT-cells, an *in vitro* assay was performed to determine the inducing mechanism of IL-17A secretion in γδT-cells. After γδT-cells were isolated from the skin tissue of γδTCR-eGFP mice, an immune complex of anti-Col7 antibody and C3 was added to the cells to mimic the pathological conditions in the skin during EBA. Since it has been reported that IL-23 and IL-1β are major mediators of IL-17A secretion in several diseases [192], these cytokines were used to evaluate their capacity to induce the production of IL-17A in γδT-cells in the development of EBA.

The γδT-cells did not produce IL-17A in response to the immune complex of anti-Col7 antibody and C3a. However, upon co-stimulation with IL-1β, significantly increased levels of IL-17A were produced by γδT-cells (Figure 33). When γδT-cells were stimulated with IL-23 and immune complexes, the level of IL-17A was only slightly elevated in the supernatant of the γδT-cells (Figure 33). Interestingly, even without treatment with the immune complex, IL-1β dramatically promoted the production of IL-17A by γδT-cells, whereas IL-23 did not (Figure 33).

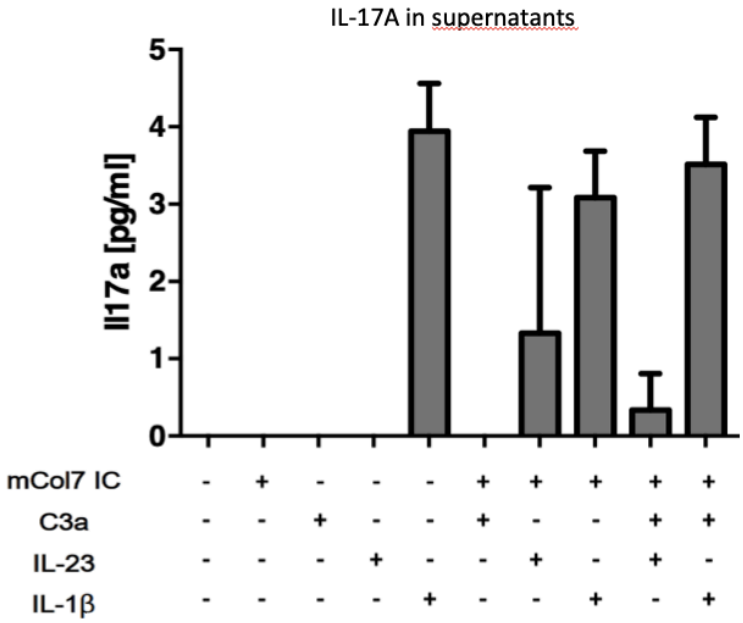

Figure 33. IL-1β mediates IL-17A production by γδT-cells. γδT-cells from the spleen of 5 γδTCR-eGFP mice were sorted after dumb staining with surface marker antibodies (anti-CD11b, anti-CD3, anti-B220, anti-Ly6G and anti-NK1.1 antibodies) on FACS Array. γδT-cells were stimulated with mCol7 immune complex, C3a, IL-23 and IL-1β as indicated for 72h. The IL-17 concentrations in supernatants were quantified using ELISA. Data are shown as mean±SD (each test had two duplicates and was repeated three times).

5.4. IL-17A promotes the migration of neutrophils by stimulating skin-resident cells

Finally, the mechanism by which IL-17A may mediate pathological changes in EBA, was studied, since resident keratinocytes and fibroblasts express IL-17Ra and are involved in mediating cell infiltration in several IL-17A-dependent diseases [193]. Therefore, it was hypothesized that keratinocytes and fibroblasts may be activated by IL-17A and subsequently contribute to the development of EBA by recruiting neutrophils. To validate this hypothesis, a neutrophil chemotaxis assay was performed *in vitro*. As shown in Figure 25, the supernatants collected from fibroblasts and keratinocytes that had been incubated with IL-17A recruited a higher number of neutrophils as compared to those from unstimulated cells (Figure 34A, B). Since CXCl-1 is one of the main neutrophil-attracting chemokines, its expressions in

IL-17A-stimulated fibroblasts and keratinocytes were analyzed to determine whether the IL-17A-promoted neutrophil migration was associated with the production of CXCl-1. The mRNA levels of CXCl-1 were in fact significantly increased in fibroblasts and keratinocytes after treatment with IL-17A as compared to the unstimulated cells (Figure 34C, D).

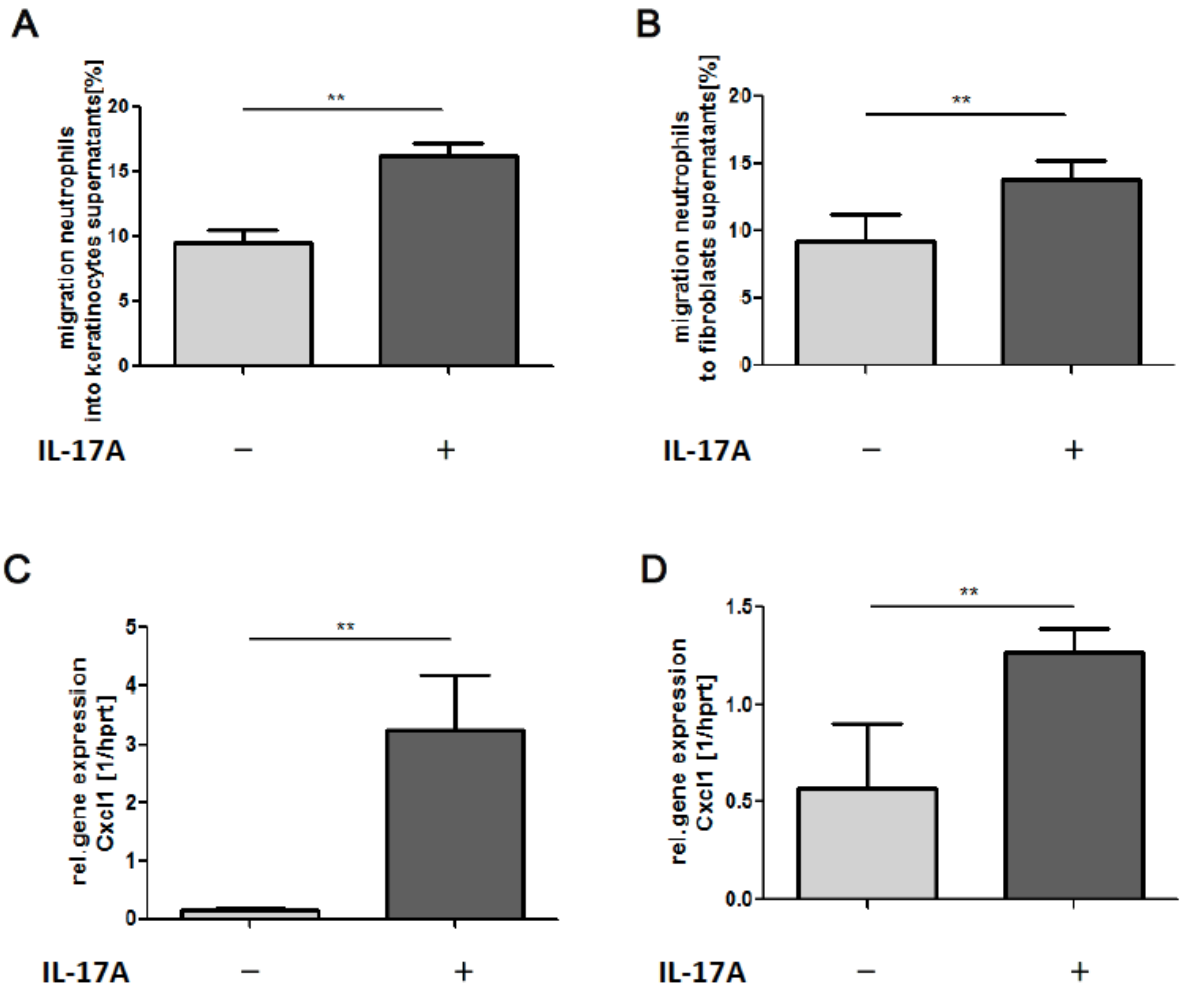

Figure 34. IL-17A promotes the production of chemokines in fibroblasts and keratinocytes to accelerate neutrophil migration. Fibroblasts and keratinocytes were incubated with and without IL-17A for 24hr. Supernatants were collected to perform neutrophil trans-well experiment as described. The percentage of migrated neutrophils toward the supernatant of keratinocytes (A) and fibroblast (B) was measured after 1hr incubation at 37°C. mRNA expression of CXCL-1 in keratinocytes (C) and fibroblast (D) with and without IL-17A was quantified using real-time Q-PCR. Data are shown as mean±SD (each test had three duplicates and was repeated twice, **$p<0.01$, t-test).

6. Discussion

The main aim of the current thesis was to clarify the relative contribution and mechanism of IL-17A and IL-17F to the pathogenesis of antibody-induced EBA and investigate the cellular source and target cells of IL-17A in the development of EBA.

Our preliminary data showed that antibody-induced EBA was completely abrogated in IL-17A$^{-/-}$ mice [188]. However, EBA severity was only partially reduced in a similar way in IL-17Ra$^{-/-}$ and IL-17A/F$^{-/-}$ mice. Clusters of genes in IL-17A$^{-/-}$ mice were found to originate from 129, which is resistant to EBA, implying that the gene derived from 129 may contribute to EBA resistance in IL-17A$^{-/-}$ mice. However, the pathogenic role of IL-17A was confirmed by neutralization of IL-17A which inhibited the development of EBA in C57BL/6 mice. Furthermore, the present thesis also revealed a pro-inflammatory function of IL-17F in the development of EBA by IL-17F deficiency ameliorating the development of antibody-induced EBA.

Mechanistically, flow cytometric analysis of skin cells from IL-17A-eGFP mice during EBA revealed that γδT-cells were the major cellular source of IL-17A, which was further confirmed by disease remission in γδTCR$^{-/-}$ mice. Besides γδT-cells, CD4^{+}-cells and neutrophils also produce IL-17A in experimental EBA. Moreover, the present thesis demonstrated that IL-1β is an important mediator of IL-17A production by γδT-cells. Lastly, keratinocytes and fibroblasts were identified as target cells for IL-17A.

In total, the present thesis for the first time revealed a similar pathogenic role of IL-17A and IL-17F in the pathogenesis of antibody-induced EBA. Furthermore, it confirmed γδT-cells as the main IL-17A producing cell type which plays an essential role in experimental EBA by inducing the release of chemokine in keratinocytes and fibroblasts to promote neutrophil infiltration. These results extend our understanding of the role of IL-17A and IL-17F in the pathogenesis of EBA and enable the development of novel therapeutics for the treatment of blister autoimmune disorders.

6.1. Adjustment of the pathogenic role of IL-17A in EBA

IL-17 family cytokines have been reported to play important roles in several autoimmune diseases [194], but their contribution to the pathogenesis of EBA is not well-understood. In previous studies, IL-17A$^{-/-}$ mice have been shown to be fully resistant to the development of experimental EBA [188] and BP [187]. So far these results indicate IL-17A to be absolutely essential in mediating subepidermal bullous skin disorders. However, in the current thesis, the disease severity was only partially reduced in IL-17Ra$^{-/-}$ and IL-17A/F$^{-/-}$ mice. Since IL-17R is not only the receptor for IL-17A, but can also be bound to IL-17E and IL-17F [169], these results initially implied that the contribution of IL-17E in experimental EBA is limited, whereas IL-17F may play a protective role in EBA. To the contrary, however, the present thesis eventually revealed a pathogenic role of IL-17F in the development of EBA, because genetic ablation of IL-17F ameliorated the development of antibody-induced EBA. The area of lesional skin and the inflammatory infiltration were reduced in IL-17F$^{-/-}$ mice compared with C57BL/6 mice, suggesting a pathogenic pro-inflammatory role of IL-17F in EBA. The apparently inconsistent results obtained in IL-17A$^{-/-}$, IL-17F$^{-/-}$, IL-17A/F$^{-/-}$, and IL-17Ra$^{-/-}$ mice suggested that other factors may have contributed to the resistant phenotype of IL-17A$^{-/-}$ animals. Whereas IL-17A$^{-/-}$ mice were generated by gene targeting in the embryonic stem cells of a 129 genetic background and subsequent microinjection of these cells into C57BL/6 blastocysts [191], all other mutant mouse strains used in the present thesis were produced from a pure C57BL/6 genetic background [195]. Hence, genes close to the IL-17A locus may have been co-selected during backcrossing of IL-17A$^{-/-}$ with C57BL/6 mice. In fact, comparative genetic analysis in the present thesis disclosed several clusters of genes in IL-17A$^{-/-}$ mice to originate from the 129 strain. Because subsequent induction of EBA in 129 mice identified this strain to be resistant to EBA, 129 genes co-selected with the IL-17A locus during the backcross process may have contributed to the resistance of IL-17A$^{-/-}$ animals to the development of EBA.

Among the genes that affect susceptibility to autoimmune diseases, MHC II has a profound impact [196]. Clinical studies have reported that human leucocyte antigen (HLA)-DR2 is more frequent in EBA patients and EBA is associated with DRB1*15 in African patients [197].

These clinical findings are paralleled by similar observations in experimental EBA. In immunization-induced EBA, susceptibility to developing EBA disease is associated with the MHC locus[198]. However, although C57Bl/6 and 129 carry the same H2b haplotype, they exhibited a completely different susceptibility to antibody-induced EBA in the present thesis. The different susceptibility in immunization-induced EBA may be attributed to the involvement of the antigen-presenting process. However, antigen presentation is less important in antibody-induced EBA. Accordingly, disease severity in antibody-induced EBA is not affected by the MHC II haplotype of different mouse strains [199]. These findings imply that the resistance of 129 mice to antibody-induced EBA is independent of MHC locus.

Besides MHC II, several other factors may also contribute to the resistance of 129 mice to EBA. The levels of plasmacytoid dendritic cells (pDC), which play an important role in inflammation, are increased in 129 compared to C57BL/6 mice. Increased pDCs in 129 mice lead to enhanced IFN-γ expression in response to infection with human influenza virus compared to C57BL/6 mice [200]. Furthermore, the activity of NK cells in 129 and C57BL/6 mice is different. Since killer-cell lectin-like receptors (also called Ly49) are expressed on NK cells in 129 mice but not in C57BL/6 mice, 129 mice were resistant to tumor induction and had a lower tumor incidence than C57BL/6 mice [201]. In addition, 129 mice carry the r (resistance) allele in the Slc11a1 gene, which is associated with phagolysosomes in neutrophils and monocytic phagocytic cells [202],[203]. This mutation in the Slc11a1 gene resulted in a more infection-resistant phenotype of the 129 mice strain. These differences in cellular functions result in different immune responses in 129 and C57BL/6 mice and may account for the different susceptibility of 129 and C57BL/6 mice to developing antibody-induced EBA.

Because numerous genes in IL-17A$^{-/-}$ mice were shown to be of 129 genetic origin, many of them on chromosome 1, on which IL-17A is located, a preliminary analysis of this fragment was performed in the present thesis to gain better insight into the IL-17A-independent mechanism leading to the disease resistance of the mice. Among the 129-derived genes on chromosome 1 of IL-17A$^{-/-}$ mice, CXCR1, CXCR2 and IL-1r1 are of major interest, because these genes are critical for the function of neutrophils [204],[205], which are essential effector

cells for antibody-induced EBA. CXCR1 and CXCR2 are chemokine receptors, which are highly expressed on neutrophils [206]. Upon binding of their ligands CXCL-8, CXCL-1, these receptors can promote the migration of neutrophils in a number of pathological conditions [204],[205],[207]. Accordingly, administration of antagonists to CXCR1 and CXCR2 could significantly improve the disease outcome of antibody-induced EBA by reducing IL-8 production and ROS release from neutrophils [208]. IL-1r1 is a receptor for IL-1α, IL-1β, and IL-1RA, is abundantly expressed on neutrophils and also accounts for the activation of these cells [207]. Hence, administration of the IL-1 receptor antagonist could also inhibit blister formation and inflammatory infiltration in mice during antibody-induced EBA[209]. So far, the present thesis has revealed that the genetic contamination in IL-17A$^{-/-}$ mice with 129 genes that is responsible for neutrophil activation may account for the IL-17A-independent resistance of these mice to EBA.

Finally, despite the fact that the fully resistant phenotype of IL-17A$^{-/-}$ mice in EBA was mediated to a significant extent by a genetic contamination, the pathogenic role of IL-17A was confirmed in the present thesis by the antibody-mediated neutralization of IL-17A in C57BL/6 mice. In summary, the date presented here have adjusted the degree of contribution of IL-17A to the pathogenesis of EBA.

6.2. The pathogenic role of IL-17F in antibody-induced EBA

IL-17A has been reported to play important roles in several autoimmune diseases [184],[194]. However, the role of other IL-17 cytokines in EBA remained elusive. In the present thesis, the significance of the IL-17 family in the pathogenesis of EBA was expanded by demonstrating the pathogenic role of IL-17F in EBA. During the course of EBA, IL-17F$^{-/-}$ mice developed milder symptoms compared with C57BL/6 mice, characterized by a decreased disease score and less cell infiltration into the DEJ. Hence, the results presented here clearly indicate that, in addition to IL-17A, IL-17F also contributes to the development of EBA.

IL-17F is a member of the IL-17 family, is structurally homologous to IL-17A, and can bind to the same receptor complexes [129]. Although IL-17A has been extensively studied in

numerous autoimmune diseases [187],[210],[126], the role of IL-17F is far less clear. Since IL-17F binds to receptor complexes with a lower affinity than IL-17A, it is believed that IL-17F may only play a limited role in the pathogenesis of diseases [130],[169]. Consistently, a study on EAE reported that IL-17F deficiency was not able to ameliorate disease severity, but could only slightly delay disease onset [136]. However, the present thesis highlighted the pathogenic role of IL-17F in the development of experimental EBA. Because the EBA model used in the current study is primarily mediated by neutrophils, unlike other animal models of autoimmune diseases that are driven by several kinds of immune cells, IL-17F may play an important role in the pathological process by promoting the development, recruitment, and activation of neutrophils. Accordingly, the migration and infiltration of neutrophils were found to be decreased during the development of EBA in IL-17F$^{-/-}$ animals when compared to C57Bl/6 mice. Because IL-17F has been shown to mediate the migration and activation of neutrophils by inducing the production of chemokines from epithelial cells [211], the results of the present thesis suggest that IL-17F is also critical for the chemotaxis of neutrophils in EBA.

In total, this thesis demonstrates for the first time the role of IL-17F in the pathogenesis of EBA, and further extends the significance of IL-17 family members for antibody-induced EBA. In addition, this work also provides evidence for the involvement of IL-17F in the development of experimental EBA that is also mediated by neutrophils.

IL-17A and IL-17F, as two highly related cytokines, were reported to be redundant in promoting inflammation. IL-17A/F$^{-/-}$ mice were more susceptible to spontaneous *Staphylococcus aureus* infections compared with IL-17A$^{-/-}$ or IL-17F$^{-/-}$ mice, and IL-17RA$^{-/-}$ mice had higher susceptibility to *Klebsiella pneumoniae* infection than IL-17A$^{-/-}$ mice, suggesting that IL-17A and IL-17F have overlapping roles in these models [212]. This comparable function was observed consistently in IL-17F$^{-/-}$ and IL-17A/F$^{-/-}$ mice in antibody-induced EBA. EBA severity reduced in IL-17A/F$^{-/-}$ mice by 59%, while it decreased in IL-17F$^{-/-}$ mice by 40% compared with C57BL/6 mice.

In contrast with their coincident roles in host defense, IL-17A and IL-17F can even have opposite effects in certain cases. IL-17A promotes inflammation in an asthma model, with a

reduction of eosinophil infiltration into the airway of IL-17A$^{-/-}$ mice [136]. In contrast, IL-17F$^{-/-}$ animals exhibit higher Th2 cytokine expression and eosinophil infiltration, suggesting a suppressive function of IL-17F in asthma [136]. Whereas IL-17A plays a protective role in dextran sulfate sodium-induced acute colitis (DSS-induced colitis), IL-17F deficiency results in reduced colitis induced by DSS [136].

However, the present thesis provided strong evidence that IL-17A and IL-17F have a similar pathogenic contribution to antibody-induced EBA. Furthermore, EBA is the first disease in which IL-17F is significantly pathogenic.

In addition, the similar suppressed EBA severity in IL-17Ra$^{-/-}$ and IL-17A/F$^{-/-}$ mice implied that the contribution of IL-17E to the development of experimental EBA is limited. Although IL-17E belongs to the IL-17 family, IL-17E is the most distant cytokine. Correspondingly, unlike IL-17A and IL-17F, IL-17E can enhance Th2 immune response by inducing the production of Th2 cytokines in allergic and infective inflammation [137],[138]. Since EBA is mediated by autoantibody-induced neutrophil infiltration, IL-17E appears to have no impact on the development of EBA.

6.3. IL-1β-induced IL-17A production by γδT-cells mediates antibody-induced EBA

A distinctive subset of T-cells, which is mainly present in the lung, gut mucosa, and skin, is the γδT-cells [213]. Previous data have suggested that γδT-cells play an important role in the development of skin inflammation [214]. In IL-23–mediated psoriasiform dermatitis, γδTCR$^{-/-}$ mice exhibited decreased ear swelling and down-regulated expression of IL-22 and IL-17A[215]. γδTCR$^{-/-}$ mice were found to develop larger skin lesions with higher bacterial counts and impaired neutrophil recruitment upon cutaneous infection with *Staphylococcus aureus* [216]. However, the role of γδT-cells in EBA is unknown. Furthermore, the present thesis suggests that γδT-cells contribute to the development of EBA by generating IL-17A, and the pathogenic function of these cells was elucidated in γδTCR$^{-/-}$ mice. After induction of EBA, disease severity and inflammatory infiltration were decreased in γδTCR$^{-/-}$ mice as

compared to C57BL/6 mice, suggesting for the first time that γδT-cells in fact play a pathogenic role in the development of experimental EBA. Furthermore, the present thesis also indicates that γδT-cells contribute to the pathogenesis of EBA by generating IL-17A. Upon induction of experimental EBA, increased numbers of γδT-cells were observed in the skin of C57BL/6 mice, and more than half of γδT-cells produced IL-17A. Accordingly, in the absence of γδT-cells the decreased disease severity was accompanied by an impaired gene expression of IL-17A.

In most inflammatory settings, TH17 cells are the main IL-17A-producing cells [151]. However, the present thesis identified γδT-cells as dominant IL-17A producers in experimental EBA. This observation may be attributed to the different distribution of γδT-cells in the affected organs. For example, in autoimmune diseases, such as EAE, in which only small numbers of γδT-cells are observed in the affected organs, IL-17A is primarily generated by $CD4^+$ T-cells [217], while in skin autoimmune diseases, such as psoriasis, the main cellular source of IL-17A is γδT-cells that are abundantly distributed in the skin [162]. This finding is in stark contrast to BP, which is a skin autoimmune disease that shares several similarities with EBA. Here, γδT-cells are not the main IL-17A producers in the skin lesions of BP patients [218]. Hence, the presence of a specific subset of γδT-cells may also be of importance [187]. According to the variable domain of γδT-cell receptors, skin-resident γδT-cells can be divided into several subsets [203]. In this context, γδT-cells expressing Vγ4 TCR are very potent IL-17A producers [162], whereas γδT-cells with Vγ5 TCR are not [219]. Therefore, further investigation into the involvement of specific subsets of γδT-cells in the pathogenesis of EBA may be of interest.

The production of IL-17A by T-cells is induced by several cytokines, among which IL-23 is regarded as the critical factor for the production of IL-17 in autoimmune diseases [220]. For instance, in EAE, the disease is largely ameliorated in IL-23p19$^{-/-}$ and IL-23R$^{-/-}$ mice as compared to control mice, accompanied by decreased levels of IL-17A [221],[222]. However, our previous data revealed that the production of IL-17A is independent of IL-23 in EBA [188]. After treatment with anti-Col7 antibodies, disease severity was comparable in IL-23p19$^{-/-}$ and C57Bl/6 mice. Accordingly, no difference in the degree of IL-17A expression

was found in both groups. Hence, other factors than IL-23 must be involved in the induction of IL-17A during EBA.

Besides IL-23, IL-1β is also capable of promoting IL-17A production [223]. An IL-1 signal appears to be critical for the differentiation of TH17 cells by regulating the expression of the transcription factor RORr, which is essential for IL-17A production [224]. In anti-cancer chemotherapy, activation of γδT-cells and the release of IL-17A is dependent on IL-1β but not on IL-23 [225]. Consistently, the results in the present thesis revealed that IL-1β could activate γδT-cells to produce IL-17A. In response to both IL-1β and IL-23, murine γδT-cells could release IL-17A; however, more IL-17A was detected in the supernatants of γδT-cells stimulated by IL-1β than under IL-23 stimulation. In addition, the effect of IL-1β on IL-17A production could be enhanced by the addition of IL-23. These lines of evidence suggest that IL-1β may be the main stimulator for the production of IL-17A in γδT-cells during EBA. This finding is in line with observations in EAE that γδT-cells from IL-1R$^{-/-}$ mice failed to secrete IL-17A in response to IL-23 [226]. Importantly, in antibody-induced EBA, IL-1R$^{-/-}$ mice are protected from the development of EBA [209] indicating that the defective signaling of the main mediator for IL-17A production in the γδT-cells accounts for the decreased disease severity.

In summary, the present thesis demonstrated that during EBA the production of IL-17A by γδT-cells is induced by IL-1β rather than by IL-23.

6.4. The mechanism of IL-17A-mediated pathogenesis in experimental EBA

IL-17A plays a critical role not only in protective immunity against pathogens, but also in the pathogenesis of various autoimmune diseases [227]. In recent studies, it has been reported that levels of IL-17A increased in patients with BP as compared with healthy donors, and the blockade of IL-17A with the anti-IL-17A antibody could reduce the disease severity at a later phase of experimental BP in mice [187]. Moreover, increased levels of IL-17A were found in the skin lesion of psoriasis patients [135], which contributed to the onset of the disease by

promoting the inflammatory infiltration of immune cells [193]. Although the role of IL-17A in several autoimmune diseases has been highlighted, the role of IL-17A in EBA still remains unclear. In our preliminary study, it was shown that the development of experimental EBA was completely abrogated in IL-17A$^{-/-}$ mice as compared to the control mice [188]. However, as shown in the present thesis, the genes of the 129 ES cell background appear to have affected the development of experimental EBA in these mice. Nevertheless, because C57BL/6 mice treated with anti-IL-17A antibody exhibited ameliorated skin lesions and reduced inflammatory infiltration after the induction of experimental EBA, the present thesis still confirms the pathogenic role of IL-17A in EBA.

Many immune and non-immune cells may be activated by IL-17A. After stimulation with IL-17, macrophages can produce pro-inflammatory cytokines such as IL-1β, IL-6 and TNF [228], which may impact on the development of EBA. In addition, IL-17A is able to directly stimulate neutrophils [229] and enhance the production of reactive oxygen species (ROS) [230], shown to induce tissue damage during EBA. However, a direct impact on neutrophils is controversially discussed, as neutrophils have previously been reported not to exhibit the IL-17RC subunit on their surface [231].

In addition to immune cells, resident non-immune cells in the skin have been shown to secrete cytokines and chemokines, such as IL-6, IL-8 and CCL-2 [128],[232], in response to IL-17A, leading to the recruitment of neutrophils. This indirect effect of IL-17A on the infiltration of neutrophils may promote the development of EBA. In the present thesis therefore, the contribution of skin-resident cells was analyzed. Fibroblast and keratinocytes are two major skin cell types which not only play critical roles in maintaining the skin structure and physical barrier but are also involved in immune responses [224],[233]. Upon exposure to stimuli, fibroblast and keratinocytes are able to secrete chemokines such as CXCL-1, CXCL-8 and cytokines such as IL-1b, which promote the migration and activation of immune cells [234],[235]. In response to IL-10, keratinocyte releases IL-1b and plays a putative role in the pathogenesis of psoriasis [236]. Furthermore, under the stimulation of BP patient IgG, keratinocyte secretes IL-8 [237], which is a strong chemokine that triggers neutrophil migration and exacerbates inflammation. By this mechanism, skin-resident cells could

contribute to the development of inflammation. Consistently, the present thesis revealed that upon stimulation with IL-17A, both fibroblasts and keratinocytes could augment the chemotaxis of neutrophils by secreting neutrophil-attracting chemokines. This evidence helps to characterize the mechanism by which resident cells promote the migration of neutrophils in EBA. Hence, IL-17A appears to indirectly contribute to the pathogenesis of EBA by triggering chemokine production in the skin.

In summary, the results of the present thesis not only confirm the pathogenic role of IL-17A for the development of EBA, but the underlying mechanism mediated by IL-17A in the development of experimental EBA is also characterized as the induction of neutrophil-attracting chemokines in skin-resident keratinocytes and fibroblasts.

6.5. IL-17A and IL-17F are central mediators of the effector phase of EBA

In the present thesis, IL-17A and IL-17F were identified as essential pro-inflammatory cytokines in the pathogenesis of experimental EBA *in vivo*. After autoantibodies bind to Col7 at the dermal-epidermal junction, a complement cascade is activated and generates the cleavage products C3a and C5a [84],[85]. These products have strong a chemotactic property, and can recruit and activate immune cells (neutrophils, macrophages, mast cells) into the BMZ [82]. Subsequently, cytokines and chemokines are released from yet-to-be-defined cells and contribute to the tissue damage. IL-1β binds to the IL-1R expressed on γδT-cells and promotes IL-17A and IL-17F production. Since numerous resident γδT-cells are present in normal skin, γδT-cells can rapidly react to be a fast and major source of IL-17A in EBA. IL-17A and IL-17F regulate fibroblasts and keratinocytes to release chemokines, which in turn accelerate neutrophil infiltration and EBA blister formation (Figure 35). The results of this study suggest a pathogenic role of IL-17A and IL-17F in EBA, which sheds new light on our understanding of the disease pathogenesis and may enable the development of novel therapeutics.

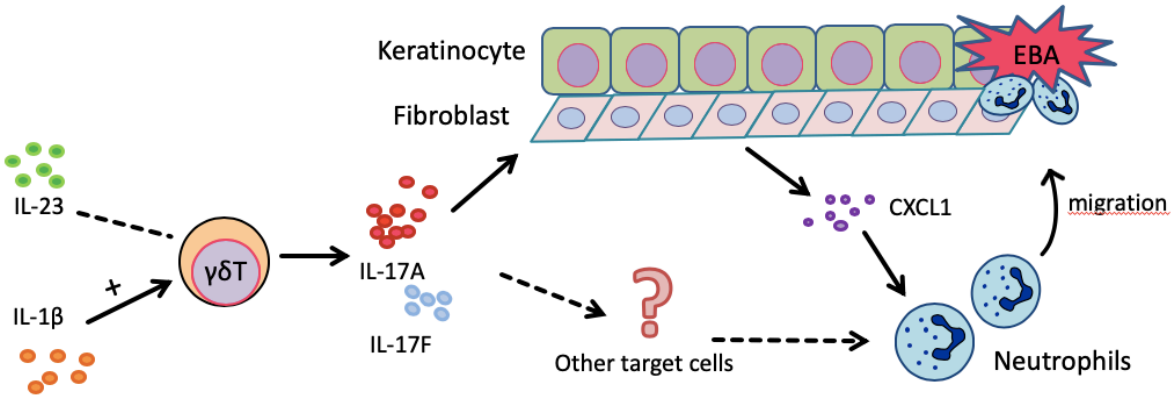

Figure 35. Schematic diagram of IL-17A and IL-17F to the pathogenesis of EBA. IL-17A and IL-17F, derived from γδT-cells in response to IL-1b, mediate chemokine CXCL-1 release in keratinocytes and fibroblasts to promote neutrophil migration to the DEJ and aggravate EBA.

Although the pro-inflammatory role of IL-17F has been demonstrated in the present thesis, the exact mechanism of IL-17F production in experimental EBA is still elusive. The gene expression of IL-17F significantly decreased in γδTCR$^{-/-}$ mice, suggesting that γδT-cells could also be the major IL-17F producing cell type. In further studies, the cellular source of IL-17F should be investigated by using IL-17F reporter mice with antibody-induced EBA. Besides IL-17F, reduced EBA in IL-17A/F$^{-/-}$ mice and IL-17Ra$^{-/-}$ mice implied that the contribution of IL-17E in antibody-induced EBA was limited. Therefore, it would be interesting to confirm the role of IL-17E in experimental EBA using IL-17E$^{-/-}$ mice. Furthermore, the present thesis identified fibroblasts and keratinocytes as target cells of IL-17A *in vitro*. However, it is still necessary to elaborate the different target cell types of IL-17A and elucidate the cell-type-specific effects mediated by IL-17 receptors *in vivo*.

6.6. Treatment of blistering diseases by targeting IL-17A and IL-17F

Because of the low prevalence and fewer clinical trials on the treatment of EBA, current

recommendations for EBA treatment are largely based on the clinical practices of clinicians specializing in autoimmune bullous dermatoses [76],[238]. Due to limited knowledge of disease mechanisms, the treatment of EBA is challenging. Currently, drugs applied in EBA treatments can be divided into three classifications: anti-inflammatory agents (Dapsone and Colchicine), immunosuppressive agents (Corticosteroid and Cyclosporine), and biological agents (Rituximab and intravenous immunoglobulin) [238]. These drugs can ameliorate the development of EBA, however, strong side effects often accompany them [238].

Most experts recommend colchicine as a first-line treatment for EBA, as it has relatively fewer adverse events than most of the other medications [239],[240]. It can be applied as monotherapy as well as in combination with Prednisone or other adjuvants, such as Azathioprine [240]. By reducing antibody production and inhibiting antigen presentation to T-cells, Colchicine usually takes effect quickly [241]. However, it often leads to nausea, vomiting and even excessive diarrhea, which makes continuous treatment very difficult [241]. Dapsone is also a good choice for treating EBA, as it inhibits neutrophil adherence to autoantibodies and IL-8 release [90],[242]. The most common adverse effects of Dapsone are hematological problems, such as methemoglobinemia and hemolytic anemia [243]. Since inflammation is the major pathological change in EBA, glucocorticoids are widely used to treat the blistering disease [244]. They can interrupt inflammation by moving into cells and suppressing the production of inflammatory proteins, thus strongly alleviating the development of EBA [245]. However, long-term treatment with glucocorticoids should be monitored carefully by physicians, because they can widely suppress the immune system, which often leads to side effects such as severe infections, Cushing's syndrome, diabetes mellitus, osteoporosis, cataracts, peptic ulcers, and hypertension [245],[246]. Cyclosporine, a strong T-cell inhibitor, is also used to treat EBA [247]. However, due to the toxic side effects such as pancreatitis, hives, diarrhea, abdominal effusion, and kidney damage in long-term therapy, it is only used if patients are refractory to other therapies [76],[247].

In recent years, new therapeutic approaches, such as Rituximab and the administration of intravenous immunoglobulins (IVIG), have also been used to treat EBA [248],[249]. Rituximab is a chimeric monoclonal antibody against the surface marker CD20 of B-cells, which can

decrease the circulating B-cells and inhibit the production of pathogenic autoantibodies (REF.). In several cases, this treatment has proved to be effective [249]. The side effects of Rituximab mainly include sepsis and fatal pneumonia [250],[251]. Intravenous immunoglobulin (IVIG) is also clinically used to treat EBA: it can ameliorate the disease by several mechanisms, such as neutralizing circulating autoantibodies, saturating FcRn to accelerate the degradation of pathogenic IgG, preventing the up-regulation of pro-inflammatory FcrRs, and activating inhibitory FcrRIIB [252],[253]. IVIG has also been reported to be effective in patients with severe refractory EBA [246],[248]. However, this therapy is very expensive and is accompanied with several side effects, such as changes in heartbeat, high blood pressure, headache, and muscle pain [238].

Above all, although current treatment for EBA can alleviate EBA, the outcome is limited. Therefore, novel therapies are needed. The present thesis has shown that the neutralization of IL -17A largely inhibits the development of EBA, which suggests that the blockade of IL-17A by specific antibodies or antagonists could be an effective treatment for EBA. In addition, clinical practice with the anti-IL-17A antibody in psoriasis has provided further evidence supporting the outcome and safety of the treatment: 83% of psoriasis patients treated with Secukinumab (an anti-IL-17A antibody) experienced reduced symptoms [254]. Another anti-IL-17A antibody, Ixekizumab, has also been tested and shown great results [255]. After Ixekizumab treatment, psoriasis symptoms were improved in more than 90% of cases [255],[256]. Generally, the most common adverse events in biological anti-IL-17A treatment (including both the group receiving the active drug and the placebo group) were worsening of disease, nasopharyngitis, upper-respiratory-tract infection, arthralgia, injection-site erythema, pain in the extremities, nausea, headache, and pruritus [255]. Most of the adverse effects are not due to the biological therapy itself but happen by chance. All the side effects can resolve by themselves or be treated with standard therapy [255]. Recently, Ixekizumab has been in clinical trial Phase III [257] and Secukinumab has already been recommended as a first-line therapy for psoriasis in 2015 [258]. These two biological anti-IL-17A antibodies could be a rational and promising therapeutic approach in patients with EBA.

Furthermore, IL-17F has also been identified in the present thesis as contributing to the pathogenesis of EBA. Therefore, neutralization of IL-17F, and not only IL-17A, could be more effective in controlling EBA, but it might also be associated with an increased risk of infection [259],[260]. In addition, the present thesis also demonstrates that skin-resident γδT-cells are the major cellular source of IL-17A, and γδT-cells play an important role during EBA. Therefore, skin γδT-cells could be another specific target in a novel EBA therapy.

7. Reference

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8. Scientific achievements

8.1. Publications

Yin J*, Zheng J*, ***Deng F****, Zhao W, Chen Y, Huang Q, Huang R, Wen L, Geng G, Zhang Z, Yue X, Petersen F, Yu X. Gene expression profiling of lacrimal glands identifies the ectopic expression of MHC II on glandular cells as a presymptomatic feature in a mouse model of primary sjögren`s syndrome. Front Immunol 2018. (first-coauthor)

Deng F1, Chen Y1, Zheng J2, Huang Q1, Cao X3, Zillikens D4, Petersen F5, Yu X1,5. CD11b-deficient mice exhibit an increased severity in the late phase of antibody transfer-induced experimental epidermolysis bullosa acquisita. Exp Dermatol. 2017 Dec;26(12):1175-1178.

Deng F1, Chen J2, Zheng J1, Chen Y1, Huang R1, Yin J1, Gao X3, Lin Q2, Huang C2, Gao Y2, Yu X1,4, Liu Z5. Association of BAFF and IL-17A with subphenotypes of primary Sjögren's syndrome. Int J Rheum Dis. 2016 Jul;19(7):715-20.

Holz K1, Prinz M2,3, Brendecke SM2, Hölscher A1, ***Deng F1***, Mitrücker HW4, Rose-John S5,6, Hölscher C1,6,7. Differing Outcome of Experimental Autoimmune Encephalitis in Macrophage/Neutrophil- and T Cell-Specific gp130-Deficient Mice. Front Immunol. 2018 May 2;9:836.

Zheng J1,2, Huang Q1, Huang R1, ***Deng F1***, Yue X3, Yin J1, Zhao W1, Chen Y1, Wen L1, Zhou J1, Huang R1, Riemekasten G2,4, Liu Z5, Petersen F3, Yu X1,3. B Cells Are Indispensable for a Novel Mouse Model of Primary Sjögren's Syndrome. Front Immunol. 2017 Oct 24;8:1384

Chen Y1,2, Zheng J1, Huang Q1, ***Deng F1***, Huang R1, Zhao W1, Yin J1, Song L3, Chen J3, Gao X4, Liu Z5, Petersen F6, Yu X1,6. Autoantibodies against the Second Extracellular Loop of M3R Do neither Induce nor Indicate Primary Sjögren's Syndrome. PLoS One. 2016 Feb 22;11(2):e0149485.

Chen Y1, ***Deng F1***, Zheng J1, Yin J1, Huang R1, Liu W2, Lin Q2, Gao Y2, Gao X3, Yu X1,4, Liu Z5, Chen J2. High circulating level of interleukin-18 in patients with primary Sjögren's syndrome is associated with disease activity. Mod Rheumatol. 2016;26(1):156-8.

Huang R1, Yin J1, Chen Y1, ***Deng F1***, Chen J2, Gao X3, Liu Z1, Yu X4, Zheng J5. The amino acid variation within the binding pocket 7 and 9 of HLA-DRB1 molecules are associated with primary Sjögren's syndrome. J Autoimmun. 2015 Feb;57:53-9.

Zheng J1, Huang R2, Huang Q2, ***Deng F2***, Chen Y2, Yin J2, Chen J2, Wang Y2, Shi G2, Gao X2, Liu Z2, Petersen F2, Yu X3. The GTF2I rs117026326 polymorphism is associated with anti-SSA-positive primary Sjögren's syndrome. Rheumatology (Oxford). 2015 Mar;54(3):562-4.

Zheng J1, Chen Y1, ***Deng F1***, Huang R1, Petersen F2, Ibrahim S3, Yu X4. mtDNA sequence, phylogeny and evolution of laboratory mice. Mitochondrion. 2014 Jul;17:126-31.

8.2. Workshops

Name	**date**
Advanced Topics in Biostatistics	13.10.2017
Time and project management for early stage researchers	19.10.2017
Ethik in der Wissenschaft und gute wissenschaftliche Praxis	22.09.2017
Tierschutz- Tierversuche	12.12.2016
Scientific Writing	13.06.2016
Good scientific Practice	30.07.2017-31.07.2015
Basic statistic course	22.09.2016-23.09.2016

8.3. Scientific conference

Name	date	Contribution
GRK1727 Autumn retreat 2015	26.11.2015-27.11.2015	Presentation
GRK1727 Spring retreat 2016	10.05.2016	Presentation
GRK1727 Autumn retreat 2016	17.11.2016-18.11.2016	Presentation
GRK1727 Autumn retreat 2017	30.11.2017-01.12.2017	Presentation
BBRS retreat 2016	05.07.2016	Presentation
NDI meeting 2015	30.10.2015	Poster
NDI meeting 2016	07.10.2016	Poster
NDI meeting 2017	10.11.2017	organization
IPPF (International Pemphigus & Pemphigoid Foundation)	22.06.2017-23.06.2017	assistant
46th annual meeting of the german society for immunology	27.09.2016-30.09.2016	Poster
The 5th international annual symposium „inflammation at interface“	26.02.2015-28.02.2015	attendance
5th European Congress of Immunology	02.09.2018-05.09.2018	poster

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Declaration

I declare that the dissertation in form and content and except for advices given by my supervisors constitutes my own work. I only used those sources and resources referred to in the thesis, and that I have identified citations as such. This work has been undertaken in compliance with the German Research Foundations (Deutsche Forschungsgemeinschaft, DFG) rules of good academic practice.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.