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Characterization of cellular and humoral immune responses in pemphigus patients and an HLA-transgenic mouse model

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List of Abbreviations

°C	degree Celsius
µg	microgram
µL	microliter
µm	micrometer
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AChR	acetylcholine receptor
ACK	ammonium chloride–potassium hydrogen carbonate
act. P	active pemphigus
AD	atopic dermatitis
AIBD	autoimmune bullous disorder
ALS	amyotrophic lateral sclerosis
alum	aluminum hydroxide
AMP	adenosine monophosphate
APC	allophycocyanin
APC	antigen-presenting cell
AU	autoimmune uveitis
auto-ab	autoantibody
AZA	azathioprine
Bcl6	B cell lymphoma 6
BCR	B cell receptor
BDCA	blood dendritic cell antigen
BL	basal layer
BM	bone marrow
BMDC	bone marrow-derived dendritic cell
BP	bullous pemphigoid
Breg	B regulatory
Ca	calcium
CCL	chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CD	Crohn's disease
CD40L	CD40 ligand
CDP	common dendritic cell progenitor
CFSE	carboxyfluorescein diacetate succinimidyl ester
cGAS	cyclic guanosine monophosphate–adenosine monophosphate synthase
CMP	common myeloid progenitor
CNS	central nervous system

CO_2	carbon dioxide
Col VII	Collagen VII
ConA	concanavalin A
CPDA	citrate-phosphate-dextrose-adenine
CR	complete remission
Csf	colony-stimulating factor
CTLA-4	cytotoxic T lymphocyte antigen 4
CXCR	C-X-C chemokine receptor
D	aspartic acid
DC	dendritic cell
ddH_2O	double-distilled water
DIF	direct immunofluorescence
DMSO	dimethyl sulfoxide
Dsc	Desmocollin
Dsg	Desmoglein
E	glutamic acid
EAE	experimental autoimmune encephalitis
EC	extracellular
ED	Eales' disease
EDTA	(ethylenedinitrilo)tetraacetic acid
e.g.	for example
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
FACS	fluorescence-activated cell sorting
FcγR-III	Fcγ receptor-III
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FOXP3	forkhead box P3
FSC	forward scatter
g	9.81 ms^{-2}
g	gram
G	Gauge
GC	germinal center
GL	granular layer
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	granulocyte and macrophage progenitor
GMP	guanosine monophosphate
HC	healthy control
HLA	human leukocyte antigen
HRP	horse radish peroxidase
HSC	hematopoietic stem cell

huDsg3	human Desmoglein 3
IBD	inflammatory bowel disease
ICOS	inducible T cell costimulator
IDP	inner dense plaque
i.e.	that is
IFN	interferon
Ig	immunoglobulin
IIF	indirect immunofluorescence
IL	interleukin
i.p.	intraperitoneal
iTreg	induced T regulatory
IVIG	intravenous immunoglobulins
JAK	janus kinase
K	lysine
kDa	kilodalton
L	liter
LC	Langerhans cell
LE	lupus erythematosus
LN	lymph node
LPS	lipopolysaccharides
mA	milliampere
mAb	monoclonal antibody
MACS	magnetic-activated cell sorting
MCP-1	monocyte chemoattractant protein-1
mDC	myeloid dendritic cell
MDP	macrophage and dendritic cell precursor
MFI	mean fluorescence intensity
mg	milligram
MG	myasthenia gravis
MHC	major histocompatibility complex
min	minute
mL	milliliter
MMA	mycophenolic acid
MMF	mycophenolate mofetil
MPS	mononuclear phagocyte system
MS	multiple sclerosis
ms^{-2}	meters per $second^2$
MTX	methotrexate
NC1	noncollagenous domain
ND	not determined
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells

ng	nanogram
NK	natural killer
nmol	nanomol
NO	nitric oxide
NOS	nitric oxide synthase
nTreg	natural T regulatory
OD	optical density
ODP	outer dense plaque
p	phosphorylated
P	position
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PD	programed cell death
pDC	plasmacytoid dendritic cell
PD-L	programed cell death ligand
PE	phycoerythrin
PF	pemphigus foliaceus
PMA	phorbol myristate acetate
PR	partial remission
Pred	prednisolone
PRR	pattern recognition receptor
PV	pemphigus vulgaris
Q	glutamine
R	arginine
RA	rheumatoid arthritis
Rag	recombination activating gene
RBC	red blood cell
RE	relative unit
rem. P	remittent pemphigus
RIG	retinoic acid-inducible gene
ROR	RAR-related orphan receptor
ROS	reactive oxygen species
RT	room temperature
S	serine
s.c.	subcutaneous
SC	stratum corneum
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SL	spinous layer
slanDC	6-sulfo N-acetyl-D-lactosamine (LacNAc) dendritic cell
SLE	systemic lupus erythematosus

SS	Sjogren’s syndrome
SSC	side scatter
SSSS	staphylococcal scalded skin syndrome
STAT	signal transducer and activator of transcription
TID	type I diabetes
TCR	T cell receptor
Tfh	T follicular helper
tg	transgenic
TGF	transforming growth factor
Th	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor
Tr1	interleukin-10–secreting T regulatory
Treg	T regulatory
TRL	tacrolimus
TSLP	thymic stromal lymphopoietin
V	valine
V	volt
VitD	vitamin D
W	tryptophan
WB	western blot

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Methods

Summary

The rare, but potentially fatal autoimmune disorder pemphigus is considered as a prototypical antibody-mediated organ-specific disease, in which immunoglobulin (Ig) G autoantibodies (auto-ab) mainly target the desmosomal cadherins Desmoglein 3 (Dsg3) and Dsg1 within the epidermis. This process, referred to as acantholysis, manifests clinically with the formation of flaccid blisters and erosions of the skin and mucous membranes. Still to date, therapeutic intervention is predominantly limited to unspecific immunosuppression causing severe side effects and comorbidities. Although it has long been recognized that auto-ab crucially contribute to the pathology of pemphigus, the exact immunological events leading to the loss of self-tolerance have not yet been fully identified.

Aim of the first part of this doctoral thesis was to investigate antigen-presenting cells (APC) and APC-derived cytokines, as well as their relation to T helper (Th) cell subsets and the auto-ab response in the pathogenesis of pemphigus. Therefore, blood samples were obtained from 34 pemphigus patients, of which twelve clinically well characterized patients with no or minimal intake of immunosuppressive agents were selected for comprehensive analysis. This study group consisted of nine pemphigus vulgaris (PV), as well as three pemphigus foliaceus (PF) patients who were categorized according to either active or remittent state of disease. A cohort of twelve patients suffering from the autoimmune muscle weakness myasthenia gravis (MG) served as a control for a further unrelated auto-ab–mediated organ-specific disorder.

Concerning the APC compartment, a major finding was reduced circulating frequencies of both myeloid and plasmacytoid dendritic cells (mDC and pDC, respectively) in active pemphigus patients. In concordance with this observation, augmented surface expression of the C-C chemokine receptor 2 (CCR2) of mDC suggest an increased migration activity to inflamed peripheral tissues, such as the skin. Similarly, active pemphigus patients displayed an upregulation of CCR2 on blood $CD14^+$ monocytes, as well as elevated numbers of circulating $CD16^+$ monocytes with expansion of the nonclassical subset. However, functional analysis on the cellular level could not confirm an enhanced immunogenicity status of APC in active pemphigus patients, yet we found in these patients increased plasma levels of the proinflammatory tumor necrosis factor (TNF)-α. Interestingly, plasma concentrations of interleukin (IL)-27 known to exert primarily regulatory functions were also elevated in active pemphigus patients, pointing to a rather inflammatory role of IL-27 in disease pathogenesis.

Strikingly, in pemphigus patients, a significant correlation was not only found between serum IgG auto-ab titers and plasma levels of both IL-6 and TNF-α, but also between auto-ab titers and plasma concentrations of IL-27. Furthermore, similarly augmented plasma levels of IL-21 playing a crucial role in B cell activation possibly indicate the mechanism of an IL-27–mediated activation of IL-21–producing Tfh cells.

Finally, as a further major finding, we observed an increase of both Th17 cells and IL-10–secreting T cells in active pemphigus patients.

Aim of the second part of this doctoral thesis was to investigate Dsg3-specific cellular and humoral immune responses in a novel human leukocyte antigen (HLA)-DRB1*04:02–transgenic (tg) mouse model of PV under the genetic restriction by HLA-DRB1*04:02. Rationale for the generation of this model is the high prevalence of distinct HLA class II alleles in PV patients, such as HLA-DRB1*04:02, –DRB1*14:01, and –DQB1*05:03. The occurrence of PV being associated with the presence of specific HLA class II alleles thereby reflects the high relevance of autoreactive $CD4^+$ T cells in disease pathogenesis.

In summary, this experimental study convincingly confirmed that the PV mouse model reproduces the principle mechanisms of HLA-dependent and human Dsg3-specific induction of $CD4^+$ T and B cell responses observed in PV patients. Accordingly, the generation of Dsg3-reactive IgG antibody responses highly depended on prior activation of Dsg3-reactive $CD4^+$ T cells. In turn, APC-mediated activation of T cells critically relied on the recognition of epitopes of the Dsg3 ectodomain, which displayed strong binding affinity to the PV-linked HLA class II allele HLA-DRB1*04:02.

Furthermore, limited cross-reactivity of human Dsg3-specific IgG antibodies with the mouse analogue protein explained lack of the clinical phenotype of PV upon immunization of HLA-DRB1*04:02–tg mice with human Dsg3 protein. Yet, this model accurately reflects that polymorphisms of peptide-binding motifs of specific PV-related HLA-class II alleles tightly regulate $CD4^+$ T cell-mediated induction of Dsg3-reactive IgG antibodies. Therefore, this novel mouse model represents a suitable tool for further investigations of the pathoimmunological mechanisms of PV *in vivo* considering the strong HLA class II association of this disease.

Zusammenfassung

Die prototypische antikörpervermittelte und organspezifische Autoimmunerkrankung Pemphigus zeichnet sich durch Autoantikörper der Immunglobulinklasse G (IgG) aus, welche vornehmlich gegen die desmosomalen Cadherine Desmoglein 3 (Dsg3) und Dsg1 in der Epidermis gerichtet sind. Dieser Prozess, der Akantholyse genannt wird, äußert sich klinisch in der Bildung von fragilen Blasen und Erosionen auf Haut und Schleimhaut. Therapiemöglichkeiten beschränken sich zurzeit überwiegend auf eine unspezifische Immunsuppression, welche mit dem Risiko schwerer Nebenwirkungen und Folgeerkrankungen einhergeht. Obwohl lange bekannt ist, dass IgG-Autoantikörper wesentlich an der Pathologie des Pemphigus beteiligt sind, ist dennoch unklar, welche immunologischen Mechanismen im Einzelnen zum Verlust der Selbsttoleranz führen.

Ziel des ersten Teils dieser Dissertation war es, antigenpräsentierende Zellen (APC) und APC-assoziierte Zytokine sowie deren Beziehung zu T-Helfer (Th)-Zell-Populationen und zur Autoantikörper-Antwort in der Pathogenese des Pemphigus zu untersuchen. Zu diesem Zwecke wurden 34 Pemphigus-Patienten Blutproben entnommen und zwölf klinisch detailliert charakterisierte Patienten für eine umfassendere Analyse ausgewählt, welche keiner oder einer nur minimalen Immunsuppression unterlagen. Diese Kohorte bestand aus neun Pemphigus vulgaris (PV)- und drei Pemphigus foliaceus (PF)-Patienten, die entsprechend eines aktiven oder remittierenden Krankheitsstatus subgruppiert wurden. Als Kontrollgruppe wurden zwölf Patienten der autoimmunen Muskelschwäche Myasthenia gravis (MG) definiert, welche eine unabhängige autoantikörpervermittelte und organspezifische Autoimmunerkrankung darstellt.

Im APC-Kompartiment konnte als wesentliches Ergebnis eine verminderte Anzahl zirkulierender myeloider und plasmazytoider dendritischer Zellen (DC) bei aktiven Pemphigus-Patienten festgestellt werden. Eine gleichzeitig vermehrte Oberflächenexpression des C-C Chemokinrezeptors-2 (CCR2) auf myeloiden DC weist in diesem Zusammenhang auf eine erhöhte Migration dieser Zellen in entzündete periphere Gewebe wie der Haut hin. Analog zeigte sich eine vermehrte Expression des CCR2 auf $CD14^+$ Monozyten sowie eine erhöhte zirkulierende Population an $CD16^+$ Monozyten mit selektiver Expansion der nichtklassischen Subpopulation. Eine funktionelle Analyse auf zellulärer Ebene konnte allerdings keinen erhöhten Immunstatus der APC bei aktiven Pemphigus-Patienten nachweisen, jedoch zeigten sich bei diesen Patienten erhöhte Plasmaspiegel des entzündungsfördernden Tumornekrosefaktors-α (TNF-α). Interessanterweise ergaben sich außerdem bei aktiven Pemphigus-Patienten erhöhte Plasmaspiegel des überwiegend immunregulatorischen Interleukin-27 (IL-27), das hauptsächlich von aktivierten APC produziert wird. Diese Beobachtung deutet auf eine entzündliche Funktion des IL-27 beim Pemphigus hin.

Bemerkenswerterweise ergab sich darüber hinaus bei Pemphigus-Patienten nicht nur eine signifikante Korrelation zwischen Serum-Autoantikörpern und den Plasmaspiegeln von IL-6 und TNF-α, sondern auch eine signifikante Korrelation zwischen IgG-Autoantikörpern und der Plasmakonzentration von IL-27. Die beobachteten, ebenfalls erhöhten Plasmaspiegel des IL-21,

welches eine wesentliche Funktion in der B-Zell-Aktivierung innehat, weisen auf eine mögliche Aktivierung von IL-21–produzierenden follikulären Th-Zellen in Abhängigkeit von IL-27 hin.
Schließlich wurde als weiteres auffälliges Ergebnis der Studie bei aktiven Pemphigus-Patienten eine deutliche Zunahme an inflammatorischen Th-17-Zellen sowie IL-10–sezernierenden Th-Zellen festgestellt.

Ziel im zweiten Abschnitt dieser Dissertation war es, Dsg3-spezifische zelluläre und humorale Immunantworten in einem neuartigen *human leukocyte antigen* (HLA)-DRB1*04:02–transgenen PV-Mausmodell zu untersuchen unter der genetischen Restriktion von HLA-DRB1*04:02. Dieses Modell wird definiert durch die hohe Prävalenz bestimmter HLA-Klasse-II-Allele bei PV-Patienten wie HLA-DRB1*04:02, –DRB1*14:01, und –DQB1*05:03. Der enge Zusammenhang zwischen dem Vorkommen von PV und dem Auftreten spezifischer HLA-Klasse-II-Risikoallele ist ein Indiz für die wesentliche Funktion autoreaktiver $CD4^+$ T-Zellen in der Pathogenese der Erkrankung.
Zusammenfassend geht aus dieser tierexperimentellen Studie schlüssig hervor, dass sich die in PV-Patienten identifizierten Mechanismen der HLA-abhängigen und Dsg3-spezifischen Induktion von $CD4^+$ T– und B-Zell-Antworten in diesem Modell wiederspiegeln. Entsprechend zeigte sich in der Entstehung Dsg3-spezifischer Antikörper eine direkte Abhängigkeit von der Aktivierung Dsg3-reaktiver $CD4^+$ T Zellen. Die APC-vermittelte Aktivierung dieser T-Zellen unterlag wiederum der Erkennung von Epitopen der Dsg3-Ektodomäne, welche eine hohe Bindungsaffinität zu dem PV-assoziierten HLA-DRB1*04:02–Allel aufwiesen.
Darüber hinaus erklärte eine nur geringe Kreuzreaktivität der Antikörper zwischen humanem und murinem Dsg3-Protein das Fehlen eines klinischen Phänotyps bei HLA-DRB1*04:02–transgenen Mäusen trotz wiederholter Immunisierung mit humanem Dsg3-Protein. Jedoch gab dieses PV-Mausmodell konsequent wieder, dass die Peptidbinde-Motive spezifischer PV-assoziierter HLA-Klasse-II-Allele eine wesentliche Rolle in der $CD4^+$-T-Zell-vermittelten Induktion von Dsg3-reaktiven Antikörpern spielen. Dieses PV-Mausmodell stellt daher ein geeignetes Instrument dar, um zukünftig immunpathologische Mechanismen des PV *in vivo* unter Berücksichtigung der starken HLA-Klasse-II-Assoziation dieser Erkrankung zu untersuchen.

1 Introduction

In the state of autoimmunity, aberrant immune responses are directed against endogenous structures of the body, disturbing tissue integrity and culminating in the destruction of the whole organism. Pathological autoantibodies (auto-ab) are a characteristic feature many severe and still to date incurable autoimmune diseases share, such as myasthenia gravis (MG), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) (Martin and Chan). Despite decade-long research into the mechanisms of autoimmune responses, the precise immunological events resulting in the breakdown of self-tolerance are not yet completely understood.

This doctoral thesis deals with the prototypical antibody-mediated autoimmune disorder pemphigus, in which auto-ab are mainly directed against the desmosomal cadherins Desmoglein 3 (Dsg3) and Dsg1, causing loss of keratinocyte adhesion in the suprabasilar layer of the epidermis. Besides autoreactive B cells, the activation of autoreactive Dsg3- and/or Dsg1-specific $CD4^+$ T cells plays a pivotal role in the pathogenesis of pemphigus, which has been demonstrated in numerous studies (Amagai 2008; Hertl, Eming, and Veldman 2006). However, not yet well characterized is the contribution of antigen-presenting cells (APC), including dendritic cells (DC) being the most potent inducers of $CD4^+$ T cell activation, as well as monocytes to disease pathogenesis. Aim of the first part of this study was to investigate APC and APC-derived cytokines, as well as their relation to $CD4^+$ T cell subpopulations and the auto-ab response in the pathogenesis of pemphigus. For this purpose, populations of DC and monocytes were qualitatively and quantitatively analyzed in the peripheral blood of pemphigus vulgaris (PV) and pemphigus foliaceus (PF) patients using flow cytometry. References comprised both healthy controls (HC) and MG patients who served as a control for a further unrelated auto-ab–mediated organ-specific disorder. In addition, using flow cytometry and enzyme-linked immunosorbent assay (ELISA), APC were functionally characterized concerning their capability to produce and secrete cytokines, as well as their capacity to internalize antigen. Moreover, the distribution of $CD4^+$ T cell subpopulations was determined by intracellular detection of their respective signature cytokines using flow cytometry. Finally, serum immunoglobulin (Ig) G auto-ab titers were correlated with the parameters assessed in both the APC and $CD4^+$ T cell compartment to identify novel factors involved in the induction of antibodies in pemphigus.

Aim of the second part of this thesis was to analyze Dsg3-specific $CD4^+$ T and B cell responses under the genetic restriction by human leukocyte antigen (HLA)-DRB1*04:02 in experimental PV. The respective mouse model of PV was established to consider the strong prevalence of distinct HLA class II alleles in PV patients, including HLA-DRB1*04:02. In a cell proliferation assay using flow cytometry, Dsg3-reactive $CD4^+$ T cell responses were tested for regulation by HLA-DRB1*04:02 by stimulation of T cells with APC from HLA-transgenic (tg) mice presenting Dsg3 peptides that bind to HLA-DRβ1*04:02. In the inverse approach using an enzyme-linked immunospot (ELISPOT) assay, mice were immunized with the HLA-DRβ1*04:02-binding Dsg3 peptides to induce a local $CD4^+$ T cell response that was later challenged with Dsg3 protein *ex vivo*. Dependence of Dsg3-reactive IgG antibody production on HLA-DRB1*04:02 was finally

assessed by both ELISA and immunofluorescence microscopy using sera from mice immunized with the HLA-DRβ1*04:02-binding Dsg3 peptides. Mice transgenic for HLA-DRB1*04:01 served as a control in most assays.

The following section now provides information about the biology of APC, T and B lymphocytes, and cytokines, including their role in autoimmunity. The composition of human skin is described, as well as the pathogenesis of pemphigus with presentation of its current experimental mouse models.

1.1 The immune system

1.1.1 Innate immunity

The immune system of vertebrates encompasses two main branches, innate and adaptive immunity. The innate immune system is a universal and ancient form of host defense against infection (Janeway and Medzhitov 2002) and is composed of a humoral arm, which consists of antimicrobial peptides and opsonins, as well as a cellular arm, which mainly involves specialized cells known as phagocytes (Auffray, Sieweke, and Geissmann 2009). Its responses rely on timely recognition of pathogenic or danger signals by a limited number of germline-encoded receptors (Yin et al. 2015; Janeway and Medzhitov 2002). With the help of these receptors, not only pathogenic microbes, but also toxic or allergenic substances that enter through mucosal surfaces are eliminated. Central to the ability of the immune system to mount a response to invading pathogens, toxins, or allergens is its capacity to distinguish self from nonself (Chaplin 2010).
The major decision to respond or not respond to a particular ligand is primarily taken by the genome-encoded receptors of the innate immune response (Janeway and Medzhitov 2002). Multiple of these receptors can be found on the cell surface or within the cytoplasm, from where they transmit pathogenic or danger signals for proper counteractions using various adaptor and effector molecules. Innate immunity comprises four major signaling pathways, including those triggered by toll-like receptors (TLR), retinoic acid-inducible gene (RIG)-I-like receptors, inflammasoms, or cyclic guanosine monophosphate (GMP)–adenosine monophosphate (AMP) synthases (cGAS). Although cellular localization, ligand specificity, and signal relay mechanisms differ between each signal transduction pathway, they collectively entail numerous overlapping consequences, such as nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) activation, interferon (IFN) response, cytokine maturation, and cell death (Yin et al. 2015). Inflammatory responses are initially elicited by foremost macrophages, granulocytes, and mast cells using their innate immune receptors that are also expressed by DC and natural killer (NK) cells. Besides leukocytes, the skin and the epithelia that line internal organs can also be considered as a part of the innate immune system (Janeway and Medzhitov 2002).

1.1.2 Adaptive immunity

The innate immune system broadly expresses recognition molecules on numerous cells that recognize conserved molecular patterns of invading pathogens or toxins. Therefore, the innate

immune response constitutes the initial host response and takes effect rapidly. In contrast, the adaptive immune system provides specific recognition of antigens by a limited number of lymphoid cells that must proliferate upon encounter of the antigen in order to mount an effective immune response. Consequently, in host defense, the adaptive immune response generally develops temporally after the innate response (Chaplin 2010). Specific recognition of proteins, carbohydrates, lipids, nucleic acids, and pathogens is mediated by both Ig and T cell receptors (TCR) of the adaptive immune system (Janeway and Medzhitov 2002). The mechanism of generating these receptors involves great variability and rearrangement of variable, diversity, and joining gene segments (Litman, Rast, and Fugmann 2010). An essential property of the adaptive immune response is to induce long-lived cells that persist in an apparently dormant state, but can exert effector functions again rapidly after another encounter with their specific antigen. Hence, the adaptive immune system is able to establish immune memory, contributing to a more effective host response against specific pathogens or toxins that have been encountered before (Chaplin 2010). Yet, the adaptive immune response can also cause pathological conditions, such as allergy and autoimmunity, as well as rejection of tissue grafts (Janeway and Medzhitov 2002).

1.1.3 Linkage between innate and adaptive immunity

As presented in **1.1.1** and **1.1.2**, the immune system of vertebrates is composed of both innate ("natural") and adaptive ("acquired") immunity. These days, innate control of the adaptive immune response is a well-established paradigm first postulated by Charles Janeway Jr. in 1989 (Janeway 1989). It states that the innate immune system recognizes evolutionary conserved structures of microbial pathogens (pathogen-associated molecular patterns, PAMP), such as bacterial and fungal cell wall components, as well as viral nucleic acids using pattern-recognition receptors (PRR). Detection of PAMP by PRR elicits inflammatory responses and innate host defense mechanisms, whereas sensing of microbes by PRR expressed on APC, especially DC, additionally induces activation of adaptive immune responses (Iwasaki and Medzhitov 2015).

The function of antigen presentation is required for effective responses of lymphoid cells to antigens and is confined to mononuclear phagocytic APC. Receptor-mediated recognition of microbial constituents facilitates the initial uptake and phagocytosis of microbes by APC with both scavenger receptors and receptors of the complement system participating in this process. Upon concurrent maturation of DC, upregulation of major histocompatibility complex (MHC) class I and class II molecules, costimulatory molecules, such as CD40, CD80, and CD86, as well as the production of cytokines enables efficient interaction between DC and naïve $CD4^+$ and $CD8^+$ T cells in an antigen-specific manner (Hoebe, Janssen, and Beutler 2004). This way, recognition of microbes by distinct pathways of the innate immune system is translated into different facets of effector immune responses of the adaptive immune system, principally through specialized populations of DC (Iwasaki and Medzhitov 2015).

1.2 Cellular immunity

1.2.1 Human dendritic cell subsets

Discovered by Steinman and Cohn in 1973 (Steinman and Cohn 1973b), DC form a remarkable cellular network that is recognized for exerting a pivotal function in regulating innate and adaptive immunity (Maldonado and von Andrian 2010). Present throughout the body, DC are sentinels of the immune system that constantly survey tissues to instruct the adaptive immune system in response to peripheral stimuli. The DC system constitutes a unique hematopoietic branch as it originates from a hematopoietic lineage distinct from other leukocytes. Nonlymphoid tissues and the spleen marginal zone are the critical locations, at which DC continuously encounter tissue and blood antigens. Additionally, DC exhibit not only superior abilities to acquire, process, and present antigen to naïve T cells, but also migrate to the T cell zone of lymph nodes (LN) whilst loaded with tissue antigens (Merad et al. 2013).

Characteristically, DC adjust the context of antigen presentation according to inputs that signal the presence of pathogens or tissue damage. These signals cause DC maturation, which entails migration from peripheral tissues into and within secondary lymphoid organs, in which effector T cell responses are efficiently induced and regulated. In contrast, DC maintain immunological tolerance by perpetual presentation of innocuous self and nonself antigens, resulting in the induction of attenuating T regulatory (Treg) cells (Maldonado and von Andrian 2010).

As responses to environmental stimuli cause phenotypic alterations, the definition of discrete DC subsets is based on developmental specificity and functional specialization (Merad et al. 2013). Each DC subset displays distinct cell surface markers and reacts differently to activation by secretion of a unique cytokine profile. Abnormalities in both the differentiation and function of DC are directly associated with several immune disorders (Kim and Diamond 2015). The typical DC phenotype comprises expression of the hematopoietic markers CD45, MHC II, and CD11c, whereas T cell, NK cell, B cell, granulocyte, and erythrocyte lineage markers are absent. In addition, DC demarcate from other leukocytes, including macrophages by the formation of a unique transcriptional entity (Merad et al. 2013).

In general, DC populations split into two major subsets, myeloid DC (mDC) and plasmacytoid DC (pDC). The minor subset of pDC recirculates between the blood stream and lymphoid tissues and in the steady state expresses low levels of MHC class II and costimulatory molecules. Upon detection of foreign nucleic acids by TLR7 and TLR9, pDC produce vast amounts of type I IFN and obtain the ability to present foreign antigens to T cells. DC other than pDC are referred to as mDC that constitute a small subset of mainly tissue hematopoietic cells. Unlike pDC, mDC show enhanced capacities of antigen capture, procession, and presentation to T cells (Merad et al. 2013).

Here presented is an overview of circulating human DC subsets that were investigated in pemphigus patients and control groups within the framework of this doctoral thesis.

In humans, four discrete populations of DC are generally acknowledged to be present in blood, of which all lack expression of the lineage markers CD3, CD19, CD14, CD20, CD56, and glycophorine A, but display surface expression of HLA-DR. Characteristically, human pDC

express low levels of the integrin CD11c, lack expression of CD1a, and are positive for CD123, blood DC antigen (BDCA)-2 (CD303), and BDCA-4 (CD304) (Kim and Diamond 2015). Besides pDC, two mDC subsets expressing the non-overlapping markers BDCA-1 (CD1c) or BDCA-3 (CD141) are found in the human blood circulation (Merad et al. 2013) and account for 5% of monocytic-like cells (Auffray, Sieweke, and Geissmann 2009). DC positive for CD1c represent the most frequent mDC subset (Merad et al. 2013), display additional expression of CD11c, and are negative and low for CD1a and CD11b, respectively. DC positive for CD141 only constitute a minor population and share the surface marker expression profile of the other mDC subset except of expression of CD1c (Kim and Diamond 2015). Furthermore, an inflammatory population termed 6-sulfo LacNAc (slanDC) with expression of Fcγ receptor-III (FcγR-III), HLA-DR, and CD11c, but devoid of lineage markers has also been identified in human blood. Yet, in contrast to mDC positive for CD1c and CD141, slanDC are mainly absent from tissues and are considered to represent a monocyte subset despite their name (Schakel et al. 2002).

Beside the blood circulation, human DC populations are found in nonlymphoid tissues, of which the skin is the affected organ in the pathogenesis of pemphigus. Human skin hosts several distinct DC subsets with Langerhans cells (LC) residing in the epidermis, whilst interstitial DC populate the dermis (Klechevsky 2013).

In the dermis, at least three functionally discrete DC subsets are present, including DC positive for CD1a and negative for CD14, as well as DC expressing the inverse phenotype. Dermal DC with expression of CD1a can be further subdivided according to the presence of CD141. Moreover, a further blood-derived subset with expression of CD141 was identified in human dermis (Merad et al. 2013). Interesting for pemphigus pathogenesis, DC positive for CD14 were shown to impact humoral immune responses, such as the direct activation of B cells by secretion of interleukin (IL)-12 (Klechevsky et al. 2008). Despite being weak stimulators of T cells (Pasparakis, Haase, and Nestle 2014), dermal $CD14^+$ DC also induce $CD4^+$ T cells with a phenotype of T follicular helper (Tfh) cells (Klechevsky et al. 2008) that provide essential costimulation to B cells during the development of antibody-producing plasma cells in germinal centers (GC) (Crotty 2014). Apart from IL-12, dermal $CD14^+$ DC secrete IL-10 and transforming growth factor (TGF)-β that induce the differentiation of B cells into plasma cells and switch of isotype towards plasma IgG and mucosal IgA (Klechevsky et al. 2008).

Identified in 1868 by Paul Langerhans, LC constitute the predominant hematopoietic cells in the human epidermis with their dendritic processes extending to the stratum corneum. Based on the expression of the hematopoietic markers CD45, HLA-DR, the epithelial cell adhesion molecule, and the lectin langerin, human LC are easily detected in the epidermis (Merad et al. 2013). Typically, LC display high levels of CD1a, produce IL-15, and are eminently adept at inducing primary $CD8^+$ T cell responses. Moreover, LC potently induce T helper (Th) 1, Th2, Th17, and Th22 cell responses (Klechevsky et al. 2008). Similarly to dermal DC positive for CD14, LC were also demonstrated to be able to efficiently differentiate naïve $CD4^+$ T cells into IL-21–secreting cells (Shi and Pamer 2011).

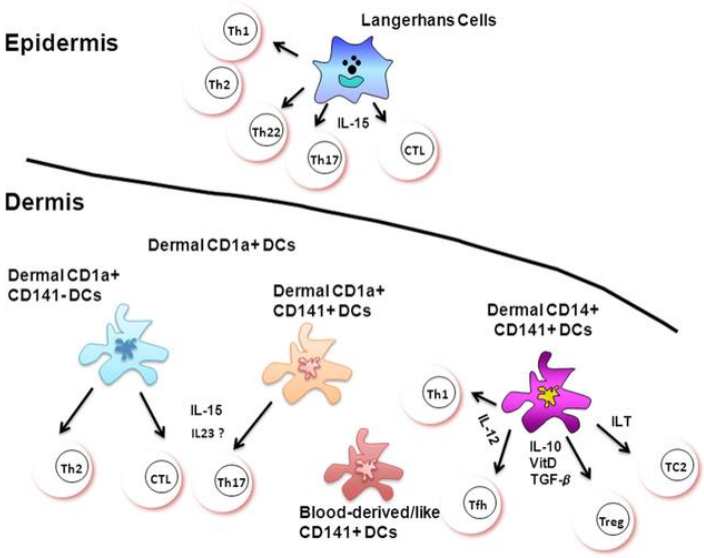

Figure 1.1: Human skin dendritic cell subsets and their interaction with T cells.
Human skin hosts Langerhans cells in the epidermis and at least three interstitial dendritic cell (DC) subsets in the dermis with the respective phenotypes indicated. The dermal CD14$^+$ DC secrete interleukin (IL)-12, vitamin D (VitD), IL-10, and transforming growth factor (TGF)-β and induce the differentiation of T helper (Th) 1, T follicular helper (Tfh), as well as T regulatory (Treg) cells and have limited capacity to prime cytotoxic T lymphocytes (CTL) (Klechevsky et al. Eur J Immunol, 2013).

Concerning lymphoid tissues, two DC subsets positive for either CD1c or CD141 are located in human spleen and tonsils (Kim and Diamond 2015). Beside LN-resident DC, LN cells were also shown to comprise migratory LC, migratory dermal CD1a$^+$ DC, and dermal CD14$^+$ DC, respectively (Merad et al. 2013).

	pDC	BDCA1$^+$ (CD1c)$^+$	BDCA3$^+$ (CD141)$^+$	LC	CD14$^+$	CD1a$^+$
Phenotype:	Lin$^-$ HLA-DR$^+$ CD11c^{low} CD1a$^-$ CD123^{hi} BDCA2$^+$ BDCA4$^+$	Lin$^-$ HLA-DR$^+$ CD11c$^+$ CD1a$^-$ BDCA1$^+$ BDCA3$^{+/-}$ CD11b^{low}	Lin$^-$ HLA-DR$^+$ CD11c$^+$ CD1a$^-$ BDCA1$^-$ BDCA3$^+$ CD11b^{low} CD141$^+$ Necl2$^+$ Xcr1$^+$ Clec9a$^+$ Dec205^{hi}	Lin$^-$ HLA-DR$^+$ CD11c$^+$ CD1a$^+$ CD14$^-$ BDCA1$^+$ Langerin$^+$ EpCAM$^+$ Sirpα$^+$ CD11b$^{+/-}$ E-cadherin$^+$	Lin$^-$ HLA-DR$^+$ CD11c$^+$ CD1a$^-$ CD14$^+$ BDCA1$^+$ Langerin$^-$ EpCAM$^-$ DC-SIGN$^+$ FXIIIa$^-$ CD163$^-$	Lin$^-$ HLA-DR$^+$ CD11c$^+$ CD1a$^+$ CD14$^-$ BDCA1$^+$ Langerin$^-$ EpCAM$^-$ Sirpα$^+$ CD11b^{hi}
PRRs:	TLR1$^+$, TLR2$^-$, TLR3$^-$, TLR4$^-$, TLR6$^+$, TLR7$^+$, TLR8$^-$, TLR9$^+$	ND	TLR1$^+$, TLR2$^+$, TLR3$^+$, TLR4$^-$, TLR6$^+$, TLR7$^-$, TLR8$^+$, TLR9$^-$	TLR1$^+$, TLR2$^+$, TLR3^{lo}, TLR4$^-$, TLR6$^+$, TLR7$^-$, TLR8$^-$, TLR9$^-$	ND	ND
Murine equivalent:	pDC	cDC	CD8$^+$ cDC	LC	ND	Dermal DC
Location:	Blood and lymphoid tissue			Epidermis / Cutaneous tissue	Dermis / Cutaneous tissue	

Figure 1.2: Phenotype of human dendritic cell subsets.
Overview of the phenotype, pathogen recognition receptor (PRR) repertoire, and location of human dendritic cell (DC) subsets, as well as the supposed mouse subset equivalent. LC, Langerhans cell; ND, not determined (Merad et al. Annu. Rev. Immunol., 2013).

In addition to the classification of DC according to localization within the organism and phenotype, DC can also be categorized according to immunological function. As already mentioned, DC represent central regulators of the adaptive immune response, whereby maturation stage and functional capacity of DC critically determine the immunological consequence – tolerance or immunogenicity. Important factors in this process are also form and place of the encountered antigen, as well as the distinct DC subset involved (Kim and Diamond 2015).

At steady state, tissue-resident immature DC constantly acquire antigens from their environment, but are barely immunogenic due to modest expression levels of MHC and costimulatory molecules, as well as lack of inflammatory cytokine production (Maldonado and von Andrian 2010). The capacity of DC to promote peripheral tolerance has been studied in a number of different mouse models. Delivery of antigens to DC *in vivo* using DC-specific antibodies was demonstrated to induce robust T cell tolerance in the steady state (Hawiger et al. 2001; Hawiger et al. 2004). Although DC apparently facilitate the induction (Yamazaki et al. 2003; Sela et al. 2011) and/or the maintenance (Bar-On et al. 2011) of peripheral Treg cells, they are neither indispensable for Treg cell homeostasis (Birnberg et al. 2008) nor do they preferably regulate Treg cells compared to effector T cells (Darrasse-Jeze et al. 2009; Swee et al. 2009; Collins et al. 2012).

In the maintenance of central tolerance, thymic DC can cross-present self-antigens from medullary thymic epithelial cells (Gallegos and Bevan 2004; Hubert et al. 2011) that are primarily responsible for the negative selection of autoreactive T cells in the thymus. In addition, peripheral DC potentially enter the thymus and present peripherally acquired self-antigens to induce clonal deletion of T cells or generation of Treg cells (Bonasio et al. 2006; Proietto et al. 2008).

In contrast to tolerogenic DC, inflammatory DC are transiently induced in the periphery in response to microbial infection or inflammatory stimuli. Inflammatory DC not only comprise monocyte-derived cells, but also those cells derived from DC precursors emerging under different inflammatory conditions (Kim and Diamond 2015). Pathogenic microbes or material associated with cellular stress are sensed by the PRR of immature DC, inducing extensive phenotypical and functional alterations. DC lose their capacity to take up antigen in exchange for upregulation of antigen procession, expression of MHC and costimulatory molecules, as well as production of cytokines. Apart from that, DC leave peripheral tissues and migrate to the draining LN by increased expression of traffic molecules, including C-C chemokine receptor 7 (CCR7) (Ohl et al. 2004). In the LN, now fully mature DC instruct circulating lymphocytes according to the antigenic status of their source tissue (Maldonado and von Andrian 2010).

Tolerogenic and immunogenic DC require tight regulation, as altered or prolonged generation of these DC subsets is believed to result in immunodeficiency or autoimmune diseases (Kim and Diamond 2015).

1.2.2 Human monocyte subsets

Human monocytes are bone marrow (BM)-derived circulating leukocytes that originate from the common macrophage and DC precursor (MDP) (Saha and Geissmann 2011) that also gives rise to common DC progenitors (CDP) (Chow, Brown, and Merad 2011). Monocytes have been

considered circulating precursors for tissue macrophages and DC (Shi and Pamer 2011), yet many DC and macrophage cell types are derived from the MDP independently of monocytes (Saha and Geissmann 2011). Together with macrophages and DC, monocytes form the mononuclear phagocyte system (MPS) (Chow, Brown, and Merad 2011) and exert crucial functions in development and homeostasis of the organism by clearing apoptotic cells and scavenging toxic compounds (Auffray, Sieweke, and Geissmann 2009).

Monocytes represent 10% of leukocytes in human blood (Auffray, Sieweke, and Geissmann 2009) and act as innate effectors of the inflammatory response to microbes, whereby they eliminate pathogens by phagocytosis and the production of reactive oxygen species (ROS), nitric oxide (NO), myeloperoxidase, and inflammatory cytokines (Saha and Geissmann 2011). Expression of numerous trafficking molecules, including CCR2 enables monocytes to exit the blood circulation and enter infected tissues (Shi and Pamer 2011). As accessory cells, monocytes can also bridge inflammation and the innate defense against pathogens to adaptive immune responses, mostly upon differentiating into DC. Despite their limited capacity of antigen presentation (Auffray, Sieweke, and Geissmann 2009), monocytes in some conditions can as well induce and polarize T cell responses in infectious and autoimmune diseases (Saha and Geissmann 2011).

Human circulating monocytes are not a homogenous population, but can be discriminated according to phenotype and function, including size, expression of trafficking and innate immune receptors, as well as their ability to differentiate following stimulation with cytokines and/or microbial molecules (Shi and Pamer 2011). To date, three discrete subsets that exert specific roles in homeostasis and inflammation have been recognized (Auffray, Sieweke, and Geissmann 2009). In general, both human and mouse blood monocytes display surface expression of the colony-stimulating factor 1 (Csf-1) receptor and the chemokine receptor C-X-C chemokine receptor 1 (CXCR1). Monocytes segregate from granulocytes, NK cells, as well as T and B lymphocytes by lack of CD335, CD3, CD19, and CD15 expression. In addition, monocytes sense lipids and various microorganisms using a large range of scavenger receptors and upon stimulation, they produce diverse mediators of inflammation (Auffray, Sieweke, and Geissmann 2009).

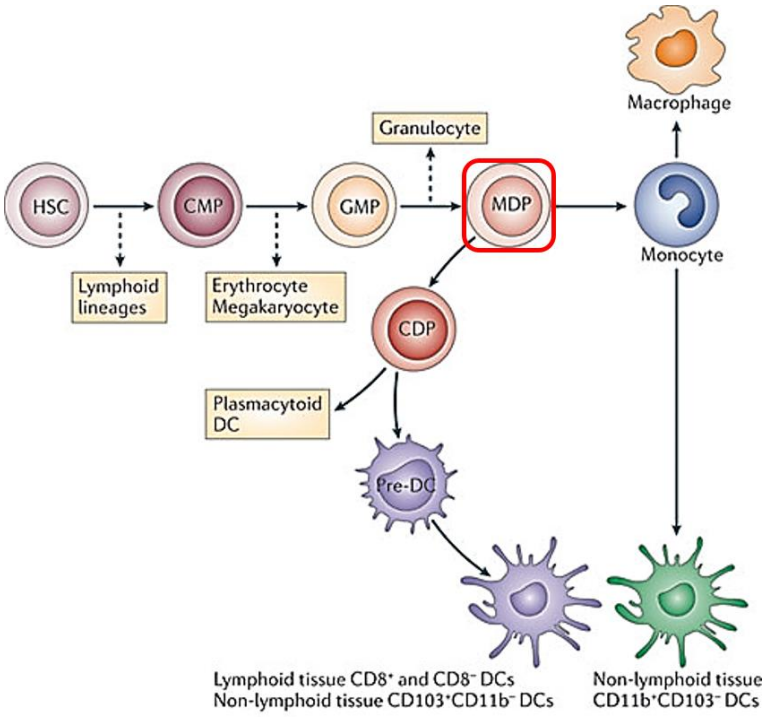

Figure 1.3: Dendritic cells commit to differentiate into a monocyte, macrophage, or dendritic cell at the stage of the macrophage and dendritic cell precursor.
Macrophage and dendritic cell (DC) progenitors (MDP) can give rise to common DC progenitors (CDP) or monocytes. Being committed to the DC lineage, CDP give rise to both $CD8^+$ and $CD8^-$ DC in lymphoid tissues and $CD103^+CD11b^-$ DC in peripheral nonlymphoid tissues. In addition, monocytes can differentiate into some $CD11b^+CD103^-$ DC or macrophages. CMP, common myeloid progenitor; GMP, granulocyte and macrophage progenitor; HSC, hematopoietic stem cell (modified from Chow et al. Nat Rev Immunol, 2011).

In both humans and mice, the monocyte population comprises two main subsets that are primarily located in the circulation, BM, and spleen (Chow, Brown, and Merad 2011). They are segregated on the basis of surface CD14 and CD16 expression (Shi and Pamer 2011). Classical monocytes being devoid of CD16 account for 80% to 90% of circulating monocytes, exhibit high levels of CCR2 and low levels of CX3CR1. Upon stimulation with bacterial lipopolysaccharides (LPS) *in vitro*, they secrete IL-10 rather than tumor necrosis factor (TNF) and IL-1, and carry out high phagocytic activity (Ziegler-Heitbrock 2000).
The minor population of monocytes typically expresses CD16 (Passlick, Flieger, and Ziegler-Heitbrock 1989) and opposed to the major subset displays high expression levels of CX3CR1, but low levels of CCR2. It produces TNF-α in response to LPS stimulation and frequencies were reported to be enhanced in the blood of patients with acute inflammation and infectious diseases (Auffray, Sieweke, and Geissmann 2009). Important for exclusion of pemphigus and MG patients in this study, systemic treatment with glucocorticoids resulted in reduced numbers of circulating $CD16^+$ monocytes (Fingerle-Rowson, Auers, et al. 1998).

Monocytes with CD16 expression can be further subdivided into those displaying diminished and high levels of CD14, referred to as nonclassical and intermediate monocytes, respectively (Ziegler-Heitbrock et al. 2010).

The function of $CD16^+$ monocytes considerably differ (Grage-Griebenow, Flad, and Ernst 2001) with intermediate $CD14^+CD16^+$ monocytes additionally expressing the Fc receptors CD64 and CD32, exhibiting phagocytic activity, and robustly releasing TNF-α and IL-1 upon stimulation with LPS. In contrast, the nonclassical $CD14^{dim}CD16^+$ monocyte subset is devoid of Fc receptors, barely phagocytic, and does not produce TNF-α or IL-1 in response to LPS (Auffray, Sieweke, and Geissmann 2009). Its actual function is not yet well characterized, however increased frequencies were observed in the blood of septic patients (Fingerle-Rowson, Auers, et al. 1998). Apart from that, expression of MHC class II processing and presentation genes was shown to be increased in intermediate monocytes, as well as surface molecules partaking in APC–T cell interaction, particularly CD40 and CD54 (Wong et al. 2012).

1.2.3 Lymphocytes

1.2.3.1 $CD4^+$ T lymphocytes

T lymphocytes are composed of two main classes, cytotoxic T cells that carry CD8 and CD4-bearing Th cells that are involved in activating, rather than killing the cells they recognize. $CD4^+$ T cells support B cells in antibody production, reinforce and maintain $CD8^+$ T cell responses, regulate the function of macrophages, and mediate immune responses against a large array of pathogenic microbes. In addition, they regulate and suppress immune responses to prevent autoimmunity and to adjust the extent and persistence of responses. Importantly, $CD4^+$ T cells are critical mediators of immunological memory and contribute to autoimmunity, asthma, and allergic responses, as well as to tumor immunity. The differentiation of naïve $CD4^+$ T cells into discrete Th cell lineages depends on a distinct cytokine milieu present during APC-mediated activation (Zhu, Yamane, and Paul 2010).

At least four types of Th cells have been identified, Th1, Th2, Th17, and induced Treg (iTreg) cells, each with its own individual phenotype and function (Zhu, Yamane, and Paul 2010). While Th1, Th2, and Th17 cells play a crucial role in eliminating intracellular pathogens, helminths, and extracellular (EC) bacteria or fungi, respectively, both Th1 and Th17 cells are critically involved in many types of autoimmune disorders. Th2 cells are important mediators of allergic responses (Zhu and Paul 2008), whereas iTreg and natural Treg (nTreg) cells ensure self-tolerance and modulate immune responses to infectious pathogens (Belkaid and Tarbell 2009).

Th1 and Th2 cells were the first distinctive populations of differentiated $CD4^+$ T cells identified in mice by Mosmann et al. in 1986 (Mosmann et al. 1986). Typically, Th1 cells secrete IFN-γ as their signature cytokine (Kanno et al. 2012) and represent unique producers of lymphotoxin. In addition, they have been shown to synthesize both IL-2 and TNF-α (Zhu, Yamane, and Paul 2010). Induction of Th1 cell differentiation requires the presence of IL-12 and IFN-γ, and is under control of the master transcription factor T-bet. In Th1 cells, T-bet is induced by signal transducer and activator of transcription 1 (STAT1) and STAT4 receiving signals from IFN and IL-12, respectively (Kanno et al. 2012). Th1 cell development blocks that of Th2 cells, demonstrated by overexpression of T-bet rendering Th2 cells able to produce IFN-γ and lose their capacity to

produce IL-4 (Zhu, Yamane, and Paul 2010). Beside Th2 cells, T-bet also inhibits Th17 cell differentiation (Kanno et al. 2012).

The signature cytokines of Th2 cells are IL-4, IL-5, IL-10, and IL-13, whereas they neither produce IFN-γ nor lymphotoxin. Moreover, Th2 cells secrete TNF-α and some produce IL-9, whilst IL-2 is synthesized in limited amounts. Under the influence of the cytokines IL-4 and IL-2, naïve $CD4^+$ T cells differentiate into Th2 cells upon stimulation by cognate antigen. As T-bet in Th1 cells, GATA3 propagates the differentiation and growth of Th2 cells and impedes Th1 cell development (Zhu, Yamane, and Paul 2010). STAT5 or STAT6 induces GATA3 in Th2 cells, although GATA3 can also be induced by Notch signaling in a STAT6-independent fashion. In addition, STAT3 has been identified to be involved in Th2 cell differentiation (Kanno et al. 2012).

Apart from Th1 and Th2 cells, Th17 cells, a third major effector population of $CD4^+$ T cells derived from naïve $CD4^+$ T cells was initially described in 2003 (Aggarwal et al. 2003; Langrish et al. 2005). They typically produce IL-17A, IL-17F, and IL-22 as signature cytokines, resulting in a massive tissue reaction due to the broad local distribution of IL-17 and IL-22 receptors (Korn et al. 2009). In addition, they represent solid producers of IL-21 being also secreted by other Th cell types (Zhu, Yamane, and Paul 2010). Th17 cell-specific differentiation factors comprise TGF-β combined with IL-6 or IL-21, while IL-23 acts as a growth and stabilization factor (Korn et al. 2009). RAR-related orphan receptor (ROR)γt is the master regulator of Th17 cell development identified in mice (Zhu, Yamane, and Paul 2010) with contribution of the transcription factors STAT3 and RORα. Apart from Th17 cells, TGF-β also induces differentiation of peripheral naïve T cells into Treg cells, placing the Th17 lineage in close relationship to Treg cells (Korn et al. 2009).

Treg cells play a critical role in controlling immune responses, highlighted by the fact that mammals that lack functional Treg cells succumb to fatal autoimmune disease. Based on their origin, Treg cells are composed of two major types, thymus-derived nTreg cells that initially emerge in the fetal circulation (Maldonado and von Andrian 2010) and additional iTreg cells that differentiate from naïve $CD4^+$ T cells in the periphery (Zhu, Yamane, and Paul 2010). Both Treg cell types constitutively express the transcription factor forkhead box P3 (FOXP3) that is essential for Treg cell development and suppressive activity. Moreover, they display surface expression of CD25, the α-chain of the IL-2 receptor, as well as cell surface and intracellular expression of the coinhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA-4) (Sakaguchi et al. 2013). IL-2 is central to the maintenance of Treg cell populations by activation of STAT5 that induces expression of FOXP3. By binding to RORγt, FOXP3 interferes with Th17 cell differentiation (Kanno et al. 2012).

Tfh cells are professional providers of T cell help to B cells and are substantial for GC formation, affinity maturation, and the development of most high-affinity antibodies and memory B cells. The differentiation of Tfh cells is a multistage process that is controlled by the transcription factor B cell lymphoma 6 (Bcl6) (Crotty 2014). Bcl6 is required to induce Tfh cell-related molecules, including CXCR5, programed cell death-1 (PD-1), as well as IL-6 and IL-21 receptors.

Additionally, Bcl6 inhibits production of Th1, Th2, and Th17 cell-related cytokines. CXCR5 permits Tfh cells to locate to B cell follicles, where they interact with B cells (Zhu, Yamane, and Paul 2010). Expression of Bcl6 and IL-21, the signature cytokine of Tfh cells is not a feature unique to Tfh cells, but is also shared by other Th cell subsets (Kanno et al. 2012). Interestingly, Tfh cells contribute to a range of diseases, especially autoimmune disorders (Crotty 2014). In pemphigus, their implication has not yet been elucidated.

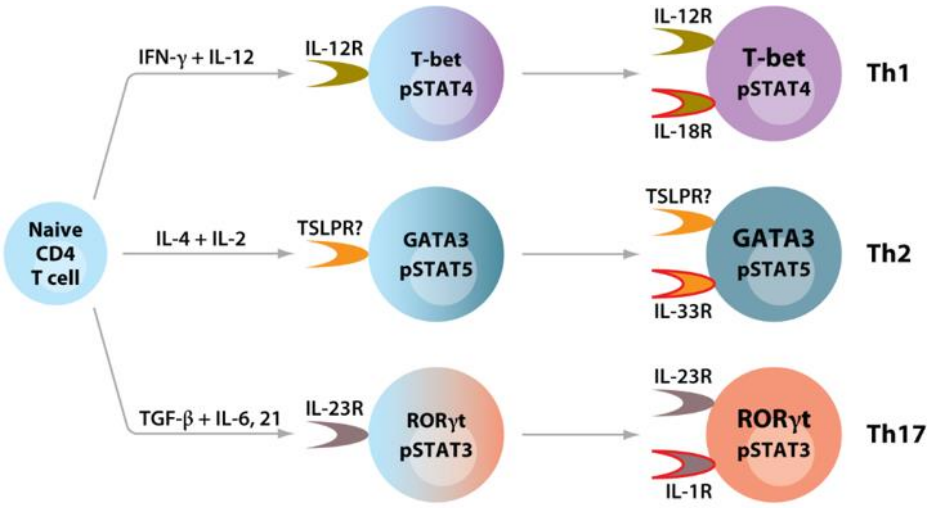

Figure 1.4: Cytokines critically impact differentiation and effector functions of Th1, Th2, and Th17 cells.
Upon antigen-presenting cell (APC)–mediated activation of the T cell receptor, naïve $CD4^+$ T cells differentiate into discrete T helper (Th) cell lineages depending on the presence of specific cytokines. This process involves upregulation of master transcriptional regulators and signal transducer and activator of transcription (STAT) proteins. Each lineage expresses unique cytokine receptors responsive to cytokines secreted by accessory cells. IFN, interferon; IL, interleukin; p, phosphorylated; R, receptor; TGF; transforming growth factor; TSLP, thymic stromal lymphopoietin (Zhu et al. Annu. Rev. Immunol., 2010).

1.2.3.2 B lymphocytes

As pivotal components of humoral immunity, B cells and their antibodies protect the organism against a vast array of pathogens. Misguided B cell development, selection, and function result in autoimmunity, malignancy, immunodeficiencies, and allergy (Pieper, Grimbacher, and Eibel 2013). Plasmablasts and plasma cells represent terminally differentiated B cell populations and are responsible for auto-ab production. Unlike long-lived plasma cells mediating lasting immunity, plasmablasts are short-lived effector cells of the early antibody response (Nutt et al. 2015).
Mature B cells comprise three major subsets, to which follicular B cells, marginal zone B cells, and B1 cells belong (Allman and Pillai 2008; Carsetti, Rosado, and Wardmann 2004). As the dominant subset, follicular B cells are found in lymphoid follicles of the spleen and LN, and are specialized in responding to protein antigens that also trigger $CD4^+$ T cell activation. Marginal zone B cells locate to the marginal sinus of the spleen, where they trap blood-borne and particulate antigens. B1 cells however mainly populate mucosal sites, including the peritoneal and pleural cavities and are thus most susceptible to environmental pathogens (Nutt et al. 2015).

The formation of antibody-secreting cells in response to T cell-dependent antigens involves B cells receiving an antigen receptor-dependent signal and finally differentiating into short-lived plasmablasts (MacLennan et al. 2003). The affinity of the resulting antibodies for antigen are modest and unvarying. Some of the activated B cells re-enter the B cell follicle and with the help of specialized Tfh cells form GC, eventually yielding high-affinity, long-lived plasma cells with robust secretion of antibody (Shlomchik and Weisel 2012; Radbruch et al. 2006). The GC reaction also induces memory B cells that upon re-encounter of antigen rapidly differentiate into antibody-secreting cells (Kometani et al. 2013).

In all mammals, B lymphocytes are derived from hematopoietic precursor cells in the BM, whereby their developmental stages are structured along the functional rearrangement of the Ig gene segments. Immature B cells displaying cell surface expression of IgM exit the BM and migrate to the spleen, where they complete early development upon differentiating into marginal zone or follicular B cells, depending on the specificity of their B cell receptor (BCR) (Pieper, Grimbacher, and Eibel 2013).

B cells are identified by the general expression of CD19 and CD20, components of the BCR complex (Jackson et al. 2008). CD20 represents a target antagonized in the therapy of B cell lymphomas and autoimmune disorders, with its expression extinguished on plasma cells (Riley and Sliwkowski 2000). Plasmablasts, plasma cells, and memory B cells together upregulate surface expression of CD27, whilst expression of CD38 is confined to plasmablasts and plasma cells that exhibit high levels of CD38 (Pieper, Grimbacher, and Eibel 2013).

B cells are critically involved in the initiation and perpetuation of autoimmune disorders by the formation of self-damaging antibodies, release of inflammatory cytokines, participation in antigen presentation, enhancement of T cell activation, and induction of ectopic lymphogenesis. Autoreactive antibodies inflict autoimmune pathology by Fc-mediated activation of the complement system, generation of immune complexes, and Fc receptor-mediated activation of both myeloid and lymphoid cells (Martin and Chan 2006).

1.3 Humoral immunity

1.3.1 Immunoglobulins

Antibodies or Ig molecules represent the secreted form of the BCR and are composed of paired heavy and light polypeptide chains (Murphy et al. 2012). Functionally, they can be divided into variable domains that interact with antigens and constant domains that harness effector functions, including activation of complement or binding to Fc receptors (Schroeder and Cavacini 2010).

Five different classes of antibodies can be distinguished according to their C regions and comprise IgM, IgD, IgG, IgA, and IgE. The class and related effector function are determined by the structure of the heavy chain (Murphy et al. 2012). B lymphocytes that maintain surface expression of IgM and IgD are called unswitched, whereas cells that have undergone Ig class-switching lack IgM and IgD expression and are considered switched memory B cells (Bonilla and Oettgen 2010). In humans, IgG subclasses can be further separated into four subclasses, IgG1, IgG2, IgG3, and IgG4 with IgG1 being most abundant in serum (Murphy et al. 2012).

1.3.2 Cytokines and chemokines

Cytokines and chemokines are central mediators in primarily innate, but also adaptive immunity and are produced by various immune and nonimmune cells of the organism. Commonly, they are secreted in reaction to an activating stimulus and they induce responses by binding to specific receptors (Murphy et al. 2012).

DC release multiple structurally diverse cytokines including IL-1β, IL-6, IL-12, IL-23, TNF-α, and TGF-β to instruct differentiation of T cells into effector cells (Iwasaki and Medzhitov 2015). Because autoreactive $CD4^+$ T cells play a crucial role in the induction and perpetuation of auto-ab production in pemphigus, the particular relevance of cytokines in disease pathogenesis is subject to numerous investigations (Giordano and Sinha 2012).

Here presented are the cytokines IL-6, TNF-α, IL-27, IL-21, and IL-33, as well as the chemokine receptor CCR2 selected for detection in the plasma of pemphigus patients and controls.

The inflammatory cytokine IL-6 is synthesized by many different cell types with stimulated monocytes, fibroblasts, and endothelial cells representing its main sources. The phagocytosis of bacteria-infected apoptotic cells results in the release of both TGF-β and IL-6 by DC, which leads to Th17 cell differentiation. IL-6 activates the janus kinase (JAK) and transcription factors of the STAT family in order to induce the release of distinct cytokines from lymphocytes (Iwasaki and Medzhitov 2015).

The similarly proinflammatory cytokine TNF-α is mainly produced by macrophages, monocytes, neutrophils, T cells, and NK cells upon stimulation with LPS. TNF-α contains local infection by acting on endothelial cells to upregulate adhesion molecules for the extravasation of monocytes and neutrophils, as well as proteins involved in blood coagulation. IL-6, TNF-α, and IL-1β induce production of acute-phase proteins that exhibit broad specificity for PAMP and exclusively require the presence of cytokines for their release (Murphy et al. 2012). Both IL-6 and TNF-α are implicated in the pathogenesis of autoimmune diseases with blockade of IL-6 showing therapeutic benefits in RA (Semerano et al. 2014), while treatment with anti-TNF-α monoclonal antibodies (mAb) ameliorates clinical symptoms in RA, inflammatory bowel disease (IBD), and psoriasis (Brzustewicz and Bryl 2015; Slevin and Egan 2015; Campa, Ryan, and Menter 2015).

Identified in 2002 by Pflanz et al., IL-27 is evolutionary related to IL-12 and its expression is confined to activated APC (Pflanz et al. 2002). IL-27 exerts key modulatory effects on T and B cells with proinflammatory activities comprising the stimulation of $CD8^+$ T cell function and the propagation of Tfh cells (Batten et al. 2010; Gringhuis et al. 2014). In addition, IL-27 is able to directly support B cell production of antibodies (Hunter and Kastelein 2012). IL-27 is involved in multiple disorders of autoimmune origin, including IBD (Schmidt et al. 2005; Cox et al. 2011), RA (Goldberg, Wildbaum, et al. 2004; Cao et al. 2008 ; Shen et al. 2011), experimental autoimmune encephalitis (EAE) (Goldberg, Zohar, et al. 2004; Li et al. 2005), psoriasis (Shibata et al. 2010; Shibata et al. 2013), type I diabetes (TID) (Wang et al. 2008), and Sjogren's syndrome (SS) (Xia et al. 2012). The role of IL-27 in pemphigus pathogenesis has not yet been characterized.

IL-21 is the signature cytokine and produced in high amounts by Tfh cells (Crotty 2014), providing essential stimulation for B cells for the differentiation into class-switched plasma cells (Linterman et al. 2010). Increasing circulating IL-21 levels have been observed in auto-ab–mediated autoimmune diseases including RA and SLE (Liu et al. 2012; Dolff et al. 2011), suggesting that IL-21 is released by potentially autoreactive Tfh cells in the course of an ongoing autoimmune response (Tangye et al. 2013).

IL-33 is a member of the IL-1 family and was identified in 2005 by Schmitz et al. (Schmitz et al. 2005). IL-33 predominantly supports Th2-dependent immune responses and is constitutively expressed by tissue barrier cells, such as epithelial and endothelial cells, as well as macrophages and DC. Upon cellular stress or damage, it functions as an alarmin by signaling the presence of danger to adjacent cells and tissues. Importantly, IL-33 relates to autoimmune disorders, including RA, multiple sclerosis (MS), IBD,TID, and SLE (Pei et al. 2014).

The migration of human blood monocytes underlies distinct trafficking mechanisms with the $CD16^{-}$ $CX3CR1^{low}CCR2^{high}$ subset being preferentially recruited to distressed tissues in a CCR2**–** chemokine (C-C motif) ligand 2 (CCL2)-dependent manner (Auffray, Sieweke, and Geissmann 2009; Alvarez, Vollmann, and von Andrian 2008). Under inflammatory conditions, this route permits monocytes to exit the blood stream and access peripheral nonlymphoid tissues, which may also explain why blocking CCR2 was effective in attenuating disease in models of atherosclerosis, MS, and RA (Viola and Luster 2008; Boring et al. 1998; Izikson et al. 2000; Gong et al. 1997). CCR2 also localizes immature DC or their precursors to inflamed peripheral tissues (Sozzani 2005).

1.4 Autoimmunity

Paroxysomal nocturnal hemoglobinuria caused by anti-erythrocyte antibodies, as well as thyroiditis involving anti-thyroid antibodies represent the first immune disorders identified as autoimmune diseases. To date, more than 80 chronic inflammatory and destructive diseases have been attributed to an autoimmune origin, including RA, Graves' disease, Hashimoto's thyroiditis, and SS with high prevalence in the world's population. These disorders are discriminated from each other by the primary organ targeted, time course of disease manifestation with reference to tissue damage, and major genetic associations. The cause of autoimmunity is idiopathic and multi-factorial with germline genetic variation accounting for less than 20% of disease trait variability. Typically, autoimmunity arises in late childhood to early adulthood, indicating that developmental factors, such as auto-ab formation and epigenetic programming, as well as environmental variables including intestinal microbiome and tobacco use are implicated in disease pathogenesis (Cho and Feldman 2015).

The expression of auto-ab is a feature many autoimmune diseases share, although auto-ab are not essential. It is not yet understood what renders a self-protein autoantigenic, whereby some of them for instance interact with TLR or chemokine receptors (Hradetzky et al. 2013; Harlow et al. 2012). Based on their Ig class, auto-ab display different levels of pathogenicity with for instance

IgM anti-double stranded DNA antibodies being less pathogenic than their IgG counterparts in SLE (Ross, Barland, and Grayzel 1978).

Despite considerably low disease concordance rates in identical twins supporting the role of nongenetic factors in the development of autoimmunity (Bogdanos et al. 2012), the first experimental evidence of the HLA region conferring genetic predisposition to autoimmune diseases was provided in 1970 (Dick 1978). In addition, proinflammatory cytokines, especially TNF have been identified to exert a central function in autoimmunity with TNF blockade showing beneficial therapeutic effect in RA, Crohn's disease (CD) and psoriasis (Cho and Feldman 2015). Strikingly, the TNF pathway as analyzed in disease tissue microarrays or in genome-wide association studies did not show genetic association in diseases, in which anti-TNF treatment has proven very effective. Apart from that, human genetic studies revealed a contribution of the IL-23 pathway to the pathogenesis of autoimmune diseases (Cho 2008) with antagonizing IL-12/23 demonstrating positive results in psoriasis (Griffiths et al. 2010). IL-23 propagates IL-23 receptor-expressing pathogenic cells, including Th17 cells (Zhou, Chong, and Littman 2009). Aside from the MHC, the R620W polymorphism in *PTPN22* was also found to be related to a number of autoimmune disorders, such as RA, TID, SLE, and autoimmune thyroid diseases (Criswell et al. 2005). The tryptophan (W) allele impairs binding of PTPN22 to the c-Src tyrosine kinase altering thresholds for T and BCR signaling (Stanford and Bottini 2014).

1.4.1 Associations of HLA polymorphisms with autoimmune diseases

The prevalence of pemphigus is strongly associated with distinct HLA class II alleles, so here provided is some information about the HLA region representing the most genetically diverse region in the genome.

The HLA region comprises genes with diverse immune functions beside those encoding for the classical antigen-presenting molecules. For the majority of autoimmune disorders, genetic associations with these genes are the strongest with multiple independent HLA alleles conferring predisposition to autoimmune diseases (Cho and Feldman 2015). HLA class II molecules are surface heterodimers expressed by thymic epithelial cells and APC that present antigenic peptides to TCR on cognate $CD4^+$ T cells (Tsai and Santamaria 2013). They consist of three isotypes, including HLA-DR (encoded by HLA-DRA and –DRB1, and –DRB3, 4, and 5 in distinct haplotypes), HLA-DQ (encoded by HLA-DQA1 and –DQB1), and HLA-DP (encoded by HLA-DPA1 and –DPB1) (Miyadera and Tokunaga 2015).

Antigenic peptides are embedded in the peptide-binding groove of the HLA class II molecule, which is composed of two flanking α-helices on top of a β-pleated sheet. In general, these peptides position in the HLA class II binding cleft via their amino acid residues at four positions, designated pockets 1, 4, 6, and 9 (Tsai and Santamaria 2013). The observation that disease-promoting HLA class II alleles often differ from those that do not propagate disease by only single amino acids of the peptide-binding cleft, neighboring key anchoring pockets (Latek et al. 2000; Jones et al. 2006), implies that the repertory of self-peptides presented to T cells substantially determines the HLA-related disease risk (Tsai and Santamaria 2013).

In support of this theory, the HLA-DRβ1*04:01 protein predisposing to RA was shown to provide the physicochemical requirements for high affinity binding of citrullinated peptides, but not noncitrullinated peptides (Hill et al. 2003). Structural analysis revealed that the presentation of citrullinated vimentin and aggrecan peptides by DRβ1*04:01 and –*04:04 proteins involved interaction of citrulline with lysine (K) or arginine (R) at 71β of the P4-binding pocket (Scally et al. 2013). Similarly, the presence of aspartic acid (D70) and glutamic acid (E71) at P4 of the PV-associated HLA-DRβ1*04:02 molecule enabled $CD4^+$ T cell recognition of distinct HLA-DRβ1*04:02–binding Dsg3 peptides as identified in PV patients (Veldman, Gebhard, et al. 2004) and described in detail in **4.2.1**.

1.4.2 Role of DC in autoimmunity

As specialized sentinel cells bridging innate and adaptive immunity, the role of DC in the initiation and perpetuation of autoimmune diseases is currently highly discussed. Based on their bidirectional nature, DC might mediate immune tolerance by the generation and maintenance of Treg cells, as well as the induction of T cell anergy. However, inadequate activation signals or a cell-intrinsic disruption of negative regulation might also allow for the priming and/or effector differentiation of self-reactive T cells owing to the eminently efficient antigen presentation capacity of DC (Ganguly et al. 2013). Accordingly, DC transferred from mice with autoimmune neural inflammation were able to induce disease in naïve recipients (Knight et al. 1983). Observations in both experimental models and humans have provided considerable evidence for the pathogenic role of DC in autoimmune disease (Ganguly et al. 2013), whereby aberrant production of type I IFN of pDC is considered as a common mechanism underlying disease in psoriasis, SLE, and TID (Nestle et al. 2005; Bennett et al. 2003; Allen et al. 2009).

By contrast, DC were shown to rather promote tolerance in the EAE model of MS (Yogev et al. 2012). In various studies, the question was raised whether size of the encompassing DC population impacted the development of autoimmunity. Apparently, DC are not essential for peripheral tolerance (Birnberg et al. 2008), but rather participate in this process as DC ablation did not lead to overt autoimmunity (Ohnmacht et al. 2009). In addition, cytokine-induced augmented DC numbers have been related to the induction of Treg cells and tolerogenic conditions (Darrasse-Jeze et al. 2009; Swee et al. 2009; Collins et al. 2012). Albeit varying DC frequencies alone do not cause autoimmunity, functional alterations were shown to entail inflammation and/or autoimmune phenotypes (Ganguly et al. 2013). Multiple genes have been identified that upon their DC-specific deletion, cause autoimmune and/or inflammatory manifestations (Travis et al. 2007; Melillo et al. 2010; Kim et al. 2011; Hammer et al. 2011; Kool et al. 2011; Kaneko et al. 2012; Abram et al. 2013). However, these genes represent negative regulators of immune activation, for which reason their silencing in cell types other than DC also induced inflammation and/or autoimmunity (Yogev et al. 2012).

1.5 The skin

The skin represents one of the largest organs of the human organism with its surface being continuously exposed to potentially harmful environmental influences. In order to maintain

homeostasis, the skin not only exerts immunological, but also nonimmune functions, which comprise the formation of a physical and biochemical barrier, as well as providing a sensory-receptive area. A versatile collective of both epithelial and immune cells mediate appropriate immune responses against a variety of trauma, toxins, and infectious agents whilst regulatory mechanisms ensure the perpetuation of immune tolerance (Di Meglio, Perera, and Nestle).

Considering its diverse and wide-ranging tasks, the skin displays a layered composition with an outer epidermis covering an inner dermis, segregated by a basement membrane. The epidermis is structured from the lowermost to the uppermost layer in the stratum basale, the stratum spinosum, the stratum granulosum, and the stratum corneum consisting of dead keratinocytes (Di Meglio, Perera, and Nestle). The physical barrier primarily localizes in the stratum corneum, whereas the nucleated epidermis also contributes to barrier function by means of tight, gap, and adherens junctions, as well as desmosomes and elements of the cytoskeleton (Proksch, Brandner, and Jensen 2008).

1.5.1 The desmosome

Resistance of the skin towards mechanical stress is mediated by the epidermal cell contact structures adherens junctions and desmosomes that tightly attach adjacent cells to each other. Thus, desmosomes are abundantly expressed in tissues providing high mechanical stability, such as the stratified squamous epithelia of the skin and mucous membranes, as well as the myocardium (Waschke 2008). Besides ensuring cellular adhesion, desmosomes have been recognized as signaling scaffolds that regulate pathways of proliferation and differentiation (Broussard, Getsios, and Green 2015).

Characteristically, desmosomes form disc-shaped junctions with each of the two neighboring cells displaying an electron-dense plaque. This plaque consists of Armadillo and Plakin family proteins and is organized in an outer dense plaque and a less dense inner plaque that interacts with intermediate filaments bundles. The desmosomal cadherins Dsg and Desmocollins (Dsc) constitute the intercellular adhesive interface and mediate adhesion in a strictly Ca^{2+}-dependent manner. Their cytoplasmic domains are assumed to bind to Plakoglobin, which in turn attaches to Desmoplakin. Desmoplakin is eventually anchored to the intermediate filament cytoskeleton with Plakophilin providing lateral stabilization (Waschke 2008).

In the autoimmune disorder pemphigus, Dsg-specific auto-ab disturb desmosomal integrity causing loss of epidermal keratinocyte adhesion, which presents clinically with flaccid blisters and erosions of the skin and mucous membranes (Hertl and Veldman 2001; Amagai 2002).

1.5.2 Desmosomal cadherins

The cadherin superfamily, of which four Dsg members (Dsg1–4) and three Dsc members (Dsc1–3) are expressed in desmosomes are single-spanned transmembrane glycoproteins and specifically depend on Ca^{2+} to mediate cell-cell adhesion (Waschke 2008). Dsg2 and Dsc2 are primarily present in simple epithelia and are expressed at low levels in the basal layer of stratified epithelia, including the epidermis. Dsg1/3 and Dsc1/3 are distributed in stratified epithelia, whilst Dsg4 is localized in stratified epithelia and hair (Broussard, Getsios, and Green 2015).

Desmosomal cadherins exert important tasks in epithelial morphogenesis and differentiation, and their malfunction entails various diseases of the skin, hair, heart, and digestive tract, as well as cancer (Thomason et al. 2010).

Structurally, desmosomal cadherins are type I integral membrane proteins with the amino-terminal EC portion being structured in five cadherin repeats (EC1–5). Ca^{2+} concentrations increase from the basal layers to the superficial layers of the epidermis providing the Ca^{2+} ions required for conformational stability and binding activity of both desmosomal and classical cadherins (Waschke 2008).

The N-terminal EC1 domain is of critical importance for the binding of Dsg3, the isoform that is predominantly targeted in PV. This is based on the finding that both the mAb AK23 from a PV mouse model, as well as Dsg3-specific antibodies from PV patients are mainly reactive against the N-terminal part of EC1 and exhibit high pathogenicity *in vivo*. Desmosomal cadherins use their EC1 domains for both *cis* and *trans* interactions, which involve molecules on the same cell and opposing cells, respectively. Concerning transinteractions, desmosomal cadherins bind in both homophilic and heterophilic manners. Dsg3 was shown to engage in homophilic transinteraction, whereas no binding was observed between Dsg1 and Dsg3 (Waschke 2008).

Dsg1/Dsc1 and Dsg3/Dsc3 distribute inversely in skin epithelium with Dsg1/Dsc1 being enriched in the suprabasal layers, in which Dsg1 contributes to epidermal differentiation, but also skin disorders, such as PF, bullous impetigo, staphylococcal scalded skin syndrome (SSSS), and striate palmoplantar keratoderma (Amagai and Stanley 2012). In contrast, Dsg3/Dsc3 accumulates in the basal epidermal layer with Dsg1/Dsc1 being expressed to only a limited extent (Broussard, Getsios, and Green 2015). Consequently, anti-Dsg1 auto-ab of PF patients cause blister formation in the most superficial layers of the skin, whilst anti-Dsg3 auto-ab of PV patients induce blisters in the basal layer of the skin (Kitajima 2013; Waschke 2008).

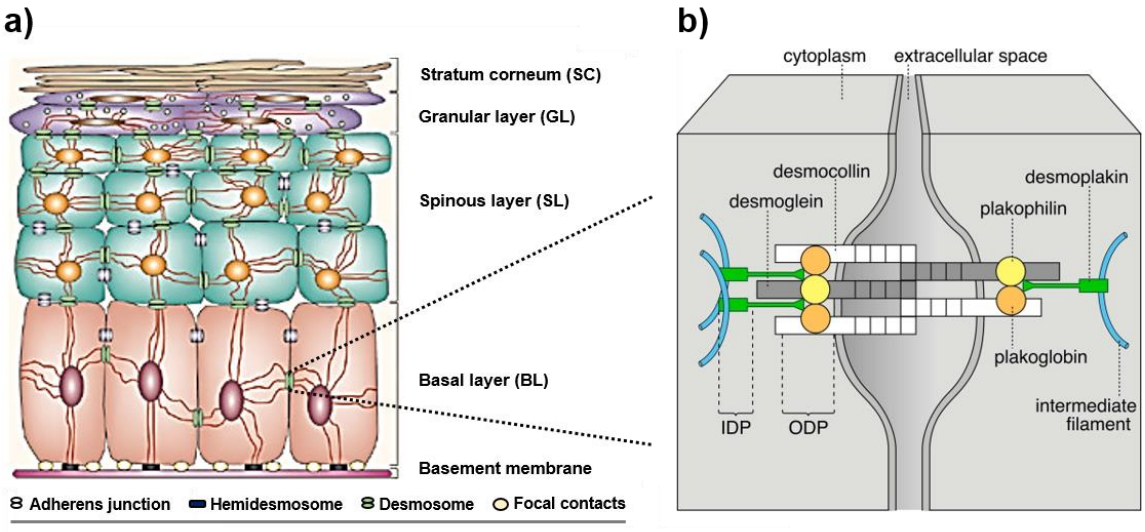

Figure 1.5: Composition of the human epidermis and molecular model of the desmosome.
The human epidermis is a stratified squamous epithelia being composed of several cell layers. The basal layer (BL) is located above the basement membrane and consists of proliferating, transit-amplifying cells. The BL stratifies to give rise to differentiated layers of the spinous layer (SL), granular layer (GL), and the stratum corneum (SC) (**a**). Within the desmosome, the cadherins Desmoglein and Desmocollin interact with partner molecules by homophilic and heterophilic binding to the same (*cis*), as well as adjacent cell (*trans*). Their cytoplasmic domains form the outer dense plaque (ODP), whereas the inner dense plaque (IDP) links adapter molecules to the intermediate filament cytoskeleton (**b**) (modified from Fuchs et al. Nat Rev Genet, 2002 [**a**] and Waschke et al. Histochem Cell Biol, 2008 [**b**]).

1.6 Pemphigus

Autoimmune bullous disorders (AIBD) are a group of rare and organ-specific blistering diseases of the skin and mucous membranes, in which primarily IgG auto-ab target distinct adhesion structures of the epidermis, dermoepidermal basement membrane, and dermis (Hertl, Eming, and Veldman 2006). PV and bullous pemphigoid (BP) are the most frequently reported AIBD with the incidence of pemphigus varying according to geographical area and ethnic affiliation (Alpsoy, Akman-Karakas, and Uzun 2015). High incidence of pemphigus ($16.1/10^6$) particularly among Ashkenazi Jewish in Israel (Pisanti et al. 1974) or in certain regions, such as the endemic form of PF in Brazil (Culton et al. 2008), points to both genetic and environmental factors being implicated in disease pathogenesis. In Europe, the incidence of pemphigus accounts for $0.5–8/10^6$ (Alpsoy, Akman-Karakas, and Uzun 2015).

Pemphigus comprises two major variants, PV with auto-ab specificity against Dsg3 and occasionally Dsg1, and PF only involving Dsg1-reactive auto-ab. More seldom forms include pemphigus herpetiformis, IgA pemphigus, paraneoplastic pemphigus and IgG/IgA pemphigus. Generally, PV is the most frequently reported clinical subtype of pemphigus and represents at least two thirds of patients (Baum et al. 2014).

As a disorder predominantly afflicting the elderly, pemphigus is most often diagnosed between the fifth and sixth decade of life in European countries, with an enhanced prevalence in the female gender (Alpsoy, Akman-Karakas, and Uzun 2015). Compared to the general population, mortality risk of PV patients was found to be approximately threefold increased and if left untreated,

resulted in nearly 100% mortality at the end of five years following diagnosis (Mimouni et al. 2008). In pemphigus, a variety of environmental triggering factors has been proposed with drugs appearing to be the most common cause (Mutasim, Pelc, and Anhalt 1993). Additionally, viral infections, ultraviolet radiation, contact allergens, and diet have been related to the outbreak of pemphigus, although a distinct triggering factor could not yet been identified (Ruocco et al. 2013).

However, associations between specific HLA class II alleles and pemphigus have been reported more than 25 years ago and repeatedly confirmed (Tron et al. 2006). HLA-DRB1*04:02, –DRB1*14:01, and –DQB1*05:03 were the alleles found to be strongly related to the prevalence of pemphigus with HLA-DRB1*04:02 occurring in 92% of Ashkenazi Jewish (Ahmed et al. 1990) and 85% of non-Jewish Iranian (Mobini et al. 1997) patients. In contrast, HLA-DRB1*14:01 was predominantly identified in Japanese (Miyagawa et al. 1997), Italian (Lombardi et al. 1999; Lombardi et al. 1996), and Pakistani (Delgado et al. 1997) PV patients. While the association of PV with HLA-DRB1*04:02 and –DQB1*05:03 is primary, increased frequencies of –DRB1*14:01 and –DQB1*03:02 were shown to be based on a linkage disequilibrium with the respective primary allele (Lee et al. 2006).

Described in the following are clinical presentation, diagnosis, therapeutic treatment options, and current understanding of the pathogenesis of pemphigus. In addition, an established mouse model of pemphigus is presented.

1.6.1 Clinical manifestation

Disease progression of PV is chronic and primarily begins with painful and long-persisting erosions of the mucous membranes, especially that of the oral cavity (Kneisel and Hertl 2011a; Sticherling and Erfurt-Berge 2012). In addition, the throat, esophagus, conjunctivae, nasal, and genital mucosa can also be affected (Bystryn and Rudolph). This termed mucosal dominant type of PV commonly results in the mucocutaneous type upon the emergence of widespread flaccid blisters on normal skin (Sticherling and Erfurt-Berge 2012; Martin et al. 2009).

Skin blisters are easily ruptured and form superficial erosions that first become encrusted and eventually epithelialize again. Blisters preferentially appear on the head's scalp and sites subjected to increased mechanical stress, including the upper trunk and intertriginous areas (Bystryn and Rudolph). The active phase of PV features both direct Nikolsky sign and indirect Nikolsky/Asboe-Hansen/bulla spread sign, of which the former involves slight rubbing of the perilesional skin inducing exfoliation of the outermost layer. In contrast, indirect Nikolsky sign reflects in the lateral shift and enlargement of intact blisters upon application of digital pressure (Baum et al. 2014).

PF is considered as a superficial subtype of pemphigus with lesions usually developing from numerous pruritic, crusted, and demarcated patches in seborrheic areas, including the scalp, as well as the face and trunk. Unlike in PV, blisters are more superficial and result in hyperkeratotic scales if left untreated. In addition, PF does not entail lesions of the oral mucosa and exhibits a more auspicious prognosis than PV (Goon and Tan 2001).

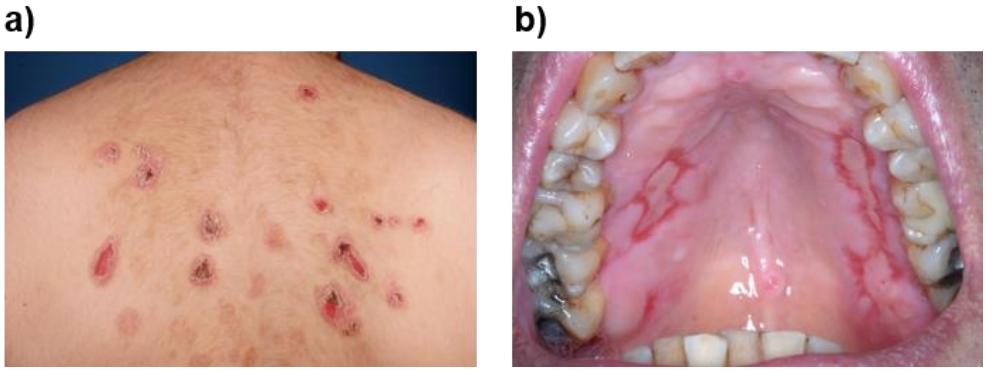

Figure 1.6: Clinical manifestation of the skin and mucosa in pemphigus vulgaris.
Flaccid blisters on the upper back skin are initially exudative, become encrusted, and eventually epithelialize again (**a**). Erosions of the palatinate mucosa (**b**) (Department of Dermatology and Allergology, Philipps University, Marburg).

1.6.2 Diagnostics

Since pemphigus presents clinically with a rather heterogeneous picture, several parameters are taken to secure proper diagnosis, including clinical findings, histology, direct and indirect immunofluorescence (DIF and IIF, respectively) microscopy, and detection of autoantigen-specific serum IgG antibodies (Kneisel and Hertl 2011b).

As described in **1.6.1**, PV patients typically display painful erosions of the mucous membrane, fragile blisters and crusts, as well as positive direct and indirect Nikolsky signs. In contrast, beside fragile blister formation, PF patients exhibit puff pastry-like scales in seborrheic areas. Histological skin biopsies serve as a means of determining the localization of loss of cell adhesion with pemphigus patients generally showing intraepidermal split formation. Specifically, specimens from PV patients display suprabasal acantholysis, while those from PF patients feature superficial subcorneal acantholysis concurrent with a slight inflammatory infiltration of the upper dermis (Kneisel and Hertl 2011b).

Apart from the histological findings, DIF microscopy represents the gold standard of AIBD diagnostics, allowing for the visualization of tissue-bound IgG auto-ab. Using DIF, all pemphigus subtypes characteristically exhibit intercellular net-like deposits in the epidermis with emphasis on the suprabasal and subcorneal epidermis in PV and PF, respectively. By contrast, the technique of IIF microscopy permits detection of circulating auto-ab upon application of patient serum onto mostly monkey esophagus substrate. Compared to DIF microscopy, IIF microscopy yields lower sensitivity. Finally, serological confirmatory tests, such as ELISA, immunoblot, and immunoprecipitation complete the diagnostic procedure of AIBD (Kneisel and Hertl 2011b). In pemphigus, auto-ab primarily target desmosomal structure proteins with mucosal dominant PV showing exclusive reactivity against Dsg3. Following transition into the mucocutaneous type, PV patients additionally display Dsg1-specific auto-ab. Unlike in PV, PF solely features Dsg1-responsive auto-ab (Amagai, Komai, et al. 1999). When monitored in individual pemphigus patients, the titers of serum anti-Dsg3 and anti-Dsg1 IgG auto-ab generally correlate with disease activity (Ishii et al. 1997; Sams and Jordon 1971).

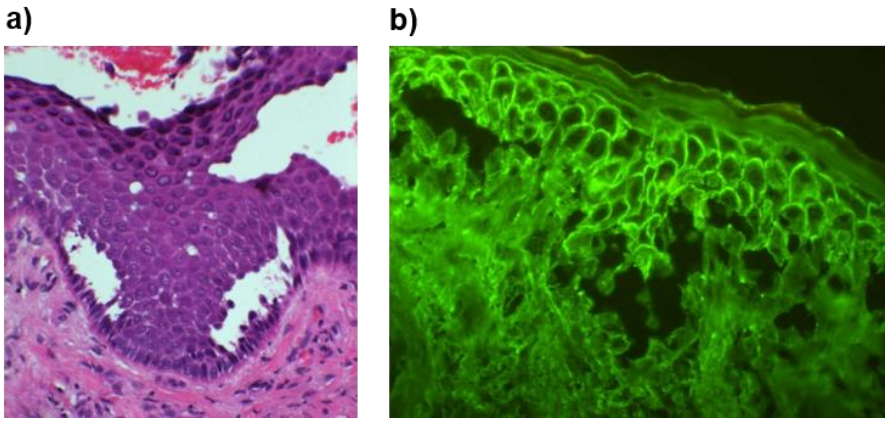

Figure 1.7: Histological picture of acantholysis in pemphigus vulgaris.
Typical loss of keratinocyte adhesion (acantholysis) in the suprabasilar layer of the epidermis of pemphigus vulgaris patients (**a**). Desmoglein-specific IgG antibodies binding to the surface of keratinocytes as detected by direct immunofluorescence microscopy (**b**) (Department of Dermatology and Allergology, Philipps University, Marburg).

1.6.3 Therapy

In the initial phase of pemphigus therapeutic treatment, application of high-dose systemic corticosteroids combined with adjuvant immune suppressants, including azathioprine (AZA), mycophenolate mofetil (MMF), and mycophenolic acid (MMA) is recommended for fast subsidence of clinical symptoms. During the progression of therapy, dosage of systemic corticosteroids is slowly reduced and adjusted to clinical manifestations (Eming 2015). Additional therapy options in particularly severe pemphigus comprise removal of circulating IgG antibodies by extracorporal immunoadsorption, which has proven effective in achieving both rapid and long-term remission, highlighting the pathogenic capacity of pemphigus auto-ab (Eming and Hertl 2006). Administration of high-dose intravenous Ig (IVIG) on the contrary ameliorates disease through various mechanisms, such as the prevention of auto-ab–induced apoptosis of keratinocytes (Michael and Grando 2008).

Depletion of circulating autoreactive B cells, the precursors of auto-ab–secreting plasma cells, represents a targeted treatment strategy with considerable potential of inducing long-lasting clinical remission in pemphigus patients (Eming 2015). Off-label use of the chimeric anti-CD20 mAb Rituximab was shown to cause prompt and complete clearance of B cells in peripheral blood and is indicated for severe and refractory pemphigus (Joly et al. 2007). Apart from systemic immunosuppressive therapy, anti-inflammatory topical medications, such as corticosteroid adhesive creams also contribute to a relief of clinical symptoms (Kneisel and Hertl 2011b).

1.6.4 Pathogenesis

1.6.4.1 Autoantigens

In PV, IgG auto-ab mainly target the cadherin-type desmosomal adhesion proteins Dsg3 and Dsg1. In mucosal dominant PV, IgG reactivity primarily confines to Dsg3, whereas in the mucocutaneous type, auto-ab are directed against both Dsg3 and Dsg1. In contrast, PF only features production of anti-Dsg1 IgG (Baum et al. 2014). Analogous to other typical cadherins,

both Dsg3 and Dsg1 display five tandemly repeated EC domains, termed EC1–EC5 (Amagai, Klaus-Kovtun, and Stanley 1991). Using domain-swapped molecules between Dsg3 and Dsg1, the N-terminal residues 1–161 within EC1 and partly EC2 were identified to contain the major determinant of conformational epitopes of Dsg3 in PV (Futei et al. 2000), and are considered functionally critical for mediating cell-cell adhesion (Amagai et al. 1992). In addition, pathogenic Dsg3-reactive mAb were demonstrated to preferably bind to epitopes in mature Dsg3, whilst nonpathogenic anti-Dsg3 mAb recognized both mature and precursor forms (Sharma et al. 2009). Beside Dsg-responsive IgG, sera from PV patients were found to also exhibit reactivity to further autoantigens, including E-cadherin (Evangelista et al. 2008) and Dsc (Rafei et al. 2011), as well as acetylcholine receptors (AChR) (Grando 2000) and mitochondrial proteins (Marchenko et al. 2010) with not yet clarified function in PV pathology.

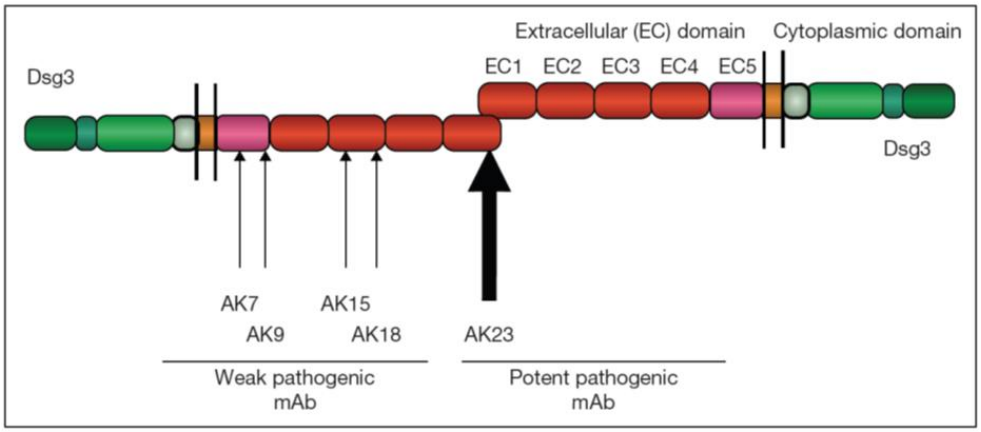

Figure 1.8: Binding sites of potent and weak pathogenic monoclonal antibodies within the Desmoglein 3 molecule.
The desmosomal cadherin Desmoglein 3 (Dsg3) contains a cytoplasmic, transmembrane, and extracellular (EC) domain, the latter of which is structured in five subdomains (EC1–EC5). Pathogenic monoclonal antibodies (mAb) preferably recognize amino-terminal epitopes within the EC1 and EC2 region (Amagai et al. Curr Dir Autoimmun, 2008).

1.6.4.2 B lymphocytes and autoantibodies

In the pathogenesis of pemphigus, substantial data provide evidence of Dsg-specific IgG auto-ab directly inducing loss of epidermal keratinocyte adhesion, a typical hallmark of the disease (Payne et al. 2004). Accordingly, passive transfer of IgG derived from PV patients into neonatal mice was sufficient to trigger pemphigus pathology (Anhalt, Labib, and Voorhes 1982). Similarly, neonates of mothers suffering from active pemphigus transiently displayed blisters due to the transplacental transfer of maternal auto-ab (Avalos-Diaz et al. 2000). By contrast, removal of circulating IgG resulted in rapid subsidence of clinical manifestations in pemphigus patients (Eming and Hertl 2006). Finally, in most pemphigus patients, serum anti-Dsg IgG titers were shown to correlate with disease activity (Amagai, Komai, et al. 1999; Ishii et al. 1997).

The auto-ab response in pemphigus is polyclonal with most auto-ab belonging to the IgG4 subclass in acute onset or active stage of disease, while remittent patients rather exhibit auto-ab

of the IgG1 subtype (Hertl 2000). Recently, a shared gene usage of the IgG heavy chain has been found for pathogenic auto-ab cloned from PV patients (Cho et al. 2014), indicating common humoral immune responses in disease pathogenesis. Interestingly, autoreactive B cells were shown to exert various functions beside the secretion of IgG auto-ab. Based on the observation of PV patients treated with the anti-CD20 mAb Rituximab not only exhibiting depletion of circulating B cells, but also decreased frequencies of autoreactive Th cells, B cells may additionally act as critical APC (Eming et al. 2008).

1.6.4.3 Desmoglein compensation theory

In pemphigus, the mechanism of blister formation is proposed to be based on differing intraepithelial distribution patterns of Dsg1 and Dsg3 between the skin and mucous membranes, and is termed Dsg compensation theory. In mucosal dominant PV, serum IgG auto-ab against Dsg3 cause mucosal lesions, while in mucocutaneous PV, auto-ab targeting Dsg1 and Dsg3 induce blisters and erosions of both the skin and mucous membranes. Contrariwise, sera from PF patients merely contain Dsg1-reactive auto-ab resulting in extensive blistering confined to the skin (Amagai 2002). In mucous membranes, Dsg1 and Dsg3 are similarly distributed throughout the squamous mucosal epithelia with the expression intensity of Dsg1 falling much below that of Dsg3 (Shirakata et al. 1998). By contrast, in the skin, Dsg1 is mainly expressed in the superficial layers, whereas Dsg3 accumulates in the basal and parabasal layers of the epidermis (Amagai et al. 1996).

Anti-Dsg1 IgG exclusively induce blisters in the superficial epidermis of the skin, in which coexpression of Dsg3 is absent. Deeper layers remain unharmed because the presence of Dsg3 compensates for the impaired function of Dsg1. Similarly, anti-Dsg1 auto-ab interfere with Dsg1 of the mucous membranes, yet coexpression of Dsg3 is sufficient to maintain epidermal integrity. Thus, in PF, anti-Dsg1 IgG solely entail superficial blistering of the skin (Amagai 2002).

Inversely, in the skin, in which coexpression of Dsg1 compensates for hampered Dsg3 function, serum displaying merely anti-Dsg3 reactivity does not induce blister formation. However, low expression levels of Dsg1 in mucous membranes are not able to compensate for loss of Dsg3 function. Therefore, anti-Dsg3–reactive sera from PV patients afflicted with the mucosal dominant type rather present with oral lesions, but lack skin manifestations. This compensation principle fails in patients with the mucocutaneous type of PV whose sera exhibit IgG directed against both Dsg3 and Dsg1, leading to extensive blister and erosion formation of the skin and mucous membranes (Amagai 2002).

Certainly, the Dsg compensation theory refers to a simplified model of serum IgG reactivity against Dsg in pemphigus pathology. In favor of this hypothesis is the observation of discrete pemphigus subtypes displaying their own individual Dsg-specific auto-ab profile (Amagai, Tsunoda, et al. 1999). However, multiple additional autoantigens identified in pemphigus gave rise to the proposal of acantholysis relying on the synergistic targeting of several different structures expressed by keratinocytes (Cirillo et al. 2012). The relevance of these auto-ab has not yet been confirmed in the pathogenesis of pemphigus.

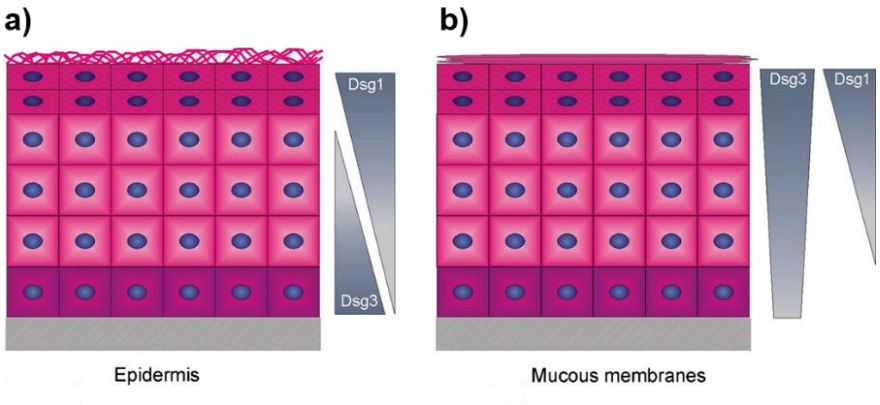

Figure 1.9: The Desmoglein compensation theory.
In pemphigus, blister formation is assumed to be based on the Desmoglein (Dsg) compensation theory. Differing intraepithelial distribution of Dsg isoforms between the skin and mucous membranes determines the localization of auto-ab–induced loss of keratinocyte adhesion. In the skin, Dsg1 accumulates in the superficial layers, while Dsg3 is primarily found in the basal layers of the epidermis. Loss of Dsg1 function cannot, whereas loss of Dsg3 function can be compensated by coexpression of basal Dsg1. Conversely, Dsg1 and Dsg3 are evenly expressed throughout the mucous membranes with reduced expression levels of Dsg1. Hampered Dsg1 function can be compensated by coexpression of Dsg3, whilst disrupted Dsg3 function cannot due to the minimal basal expression intensity of Dsg1 (Sharma et al. J Dermatol Sci, 2007).

1.6.4.4 Effector T lymphocytes

In pemphigus, the generation of circulating IgG auto-ab is critically regulated by autoreactive $CD4^+$ T cells targeting Dsg3 and/or Dsg1 as shown by numerous studies (Amagai 2008; Hertl, Eming, and Veldman 2006). Accordingly, a high association of pemphigus prevalence with the expression of specific HLA class II alleles testifies of APC-mediated activation of autoreactive $CD4^+$ T cells playing a key role in pemphigus pathogenesis (Tong et al. 2006; Wucherpfennig et al. 1995).

Dsg3-reactive T cells have been identified in peripheral blood of PV patients, as well as healthy carriers of PV-related HLA alleles recognizing epitopes of the Dsg3 EC domain that bind to the respective HLA class II molecules (Veldman, Gebhard, et al. 2004). Furthermore, depleting peripheral blood mononuclear cells (PBMC) from PV patients of $CD4^+$ cells *in vitro* resulted in abolished anti-Dsg3 IgG production, which could also be achieved upon addition of an anti–HLA-DR or anti–HLA-DQ mAb to the cultures (Nishifuji et al. 2000). In addition, data obtained from experimental PV demonstrated that induction of a disease-like phenotype relied on loss of Dsg3-specific tolerance of both T and B cells (Tsunoda et al. 2002). In concordance with this observation, application of an anti-CD40 ligand (CD40L or CD154) mAb impeding CD40/CD40L interaction between APC, including B cells and activated T cells, respectively, was able to prevent the generation of anti-Dsg3 IgG (Aoki-Ota et al. 2006).

Concerning a specific pemphigus-associated Th cell polarization, increased Dsg3-specific Th2 cell activity was found in PV patients with active disease compared to autoreactive Th1 cell responses (Rizzo et al. 2005). Correspondingly, the IgG4 subclass of auto-ab being linked with Th2 cell activation predominates in active pemphigus, while IgG1 relies on a Th1 cell-dependent response (Ayatollahi et al. 2004; Romagnani 1992). An active mouse model of PV provided further evidence for autoreactive Th2 cells mediating activation of B cells, resulting in pathogenic anti-Dsg3 IgG production by release of the Th2 cell-related cytokine IL-4 (Takahashi et al. 2008).

Furthermore, frequencies of both circulating and tissue-infiltrating inflammatory Th17 cells were determined in pemphigus patients. Although Th17 cells were detected in skin lesions of PV patients, their occurrence neither correlated with serum autoreactive IgG titers nor with the clinical severity of disease (Arakawa et al. 2009). More recent studies identified significantly enhanced numbers of Th17 cells in the blood of PV patients with yet unclarified relevance in disease pathology (Xu et al. 2013; Asothai et al. 2015).

1.6.4.5 Cytokines

B cell activation and antibody production generally rely on the cognate interaction of B cells with $CD4^+$ T cells and numerous studies have provided evidence for $CD4^+$ T cell recognition of Dsg being central to the initiation and maintenance of pathogenic B cell responses in pemphigus (Hertl 2000; Yokoyama and Amagai 2010). Induction of both cellular and humoral immune responses thereby relies on a large array of different cytokines and chemokines acting as inflammatory mediators.

In concordance with enhanced frequencies of Th2 cells in active pemphigus patients (Rizzo et al. 2005), augmented amounts of circulating Th2 cell-related cytokines could also be detected in PV patients (Satyam et al. 2009; Bhol et al. 2000). However, concerning an expected decline of serum Th1 cell cytokines because of the Th1 cell-inhibitory effects of Th2 cell cytokines, conflicting data exist. Although PV patients were reported to display a Th1 cell profile (Hertl, Amagai, et al. 1998a), another group observed reduced concentrations of circulating Th1 cell signature cytokines (Satyam et al. 2009).

Inflammatory cytokines, including TNF-α and IL-6 have been additionally characterized in pemphigus with IL-6 being also secreted by Th2 cells. Several studies found significantly elevated serum levels of TNF-α in PV patients compared to HC (D/'Auria, Bonifati, and Mussi 1997; Alecu et al. 1999), as well as increasing circulating amounts of IL-6 (Narbutt et al. 2008; Alecu et al. 1999).

1.6.4.6 Immune regulation

The finding of healthy carriers of PV-associated HLA class II alleles exhibiting circulating Dsg3-specific Th1 cells (Veldman et al. 2003b; Hertl, Amagai, et al. 1998a), as well as a considerable proportion of healthy, immediate relatives of PV patients showing Dsg3-reactive auto-ab (Kricheli et al. 2000), hints at the existence of additional regulatory mechanisms taking effect and preventing disease development in those individuals. Accordingly, anti-inflammatory Dsg3-specific IL-10–secreting Treg (Tr1) cells with constitutive expression of FOXP3 were numerically increased in healthy carriers of PV-linked HLA class II alleles compared to PV patients (Veldman, Hohne, et al. 2004). A further study confirmed markedly reduced frequencies of blood Treg cells concurrent with both diminished gene and protein expression of FOXP3 in $CD4^+CD25^+$ T cells in PV patients (Sugiyama et al. 2007). Moreover, frequencies of Treg cells in acute onset PV patients also appeared to fall below those of patients with remittent stage of disease (Xu et al. 2013). In experimental PV, Treg cells induced in a Dsg3-deficient mouse were able to inhibit the

production of anti-Dsg3 IgG upon transfer into mice with active generation of auto-ab, highlighting the therapeutic potential of Treg cells in pemphigus (Yokoyama et al. 2011).

Beside the well-established IL-10–producing Treg cells being subject to numerous studies that explore the pathogenesis of autoimmune disease, B cells with regulatory functions have recently been identified in humans, particularly within the subset of $CD19^{+}CD24^{high}CD38^{high}$ B cells (Blair et al.). Pemphigus patients with active disease displayed enhanced frequencies of blood $CD19^{+}CD24^{high}CD38^{high}$ IL-10–secreting B regulatory (Breg) cells (Zhu et al. 2015), yet concurrent with reduced functional capacities, including the ability to release IL-10 (Cho, Ellebrecht, and Payne). Similarly, patients with other autoimmune disorders also exhibited expanded populations of Breg cells (Iwata et al. 2011) showing deficiencies in IL-10 production, as well as in T cell suppressive function (Blair et al.).

1.6.5 Mouse models of pemphigus vulgaris

Mouse models of PV were created to investigate pathological immunomechanisms of this disease under *in vivo* conditions in a living organism. The currently most established model was developed by Amagai et al. in 2000 reproducing active autoimmune disease by making use of a Dsg3-deficient mouse without acquired tolerance against the absent molecule (Amagai et al. 2000). Immunization with murine Dsg3 protein induces an antigen-specific IgG response, which does not result in PV pathology as the target structure is missing (Amagai et al. 2000). However, adoptive transfer of Dsg3-reactive lymphocytes from these mice into immunodeficient recombination activating gene 2 knockout ($Rag2^{-/-}$) mice competent of Dsg3 expression leads to the generation of IgG auto-ab and manifestation of the pemphigus phenotype (Amagai et al. 2000). A subsequent study identified Dsg3-specific loss of tolerance in both T and B cells as a prerequisite for efficient production of pathogenic IgG auto-ab and induction of PV-like clinical symptoms (Tsunoda et al. 2002). Although this model reliably reproduces ongoing autoimmune responses in PV, it does not take into consideration the triggering events of the disease, such as the strong association of distinct HLA class II alleles in pemphigus patients.

2 Materials

2.1 Ethical approval of experiments

Each patient gave written consent before being included in the study, which was approved by the Ethics Committee of the Medical Faculty of Philipps-University, Marburg (Az.: Studie 20/14). The study was conducted according to the Declaration of Helsinki principles.

All animal experiments were reviewed approved by the local Laboratory Animal Ethics Committees at the Philipps University, Marburg (V 54 – 19 c 20 15 h 01 MR 20/4 – Nr. 15/2008 and V 54 – 19 c 20 15 h 01 MR Nr. 80/2014) and the Karolinska Institutet, Stockholm, respectively. The experiments were done in compliance with local policies and guidelines on the use of laboratory animals.

2.2 Patient material

Fresh peripheral venous blood	Patients of the Department of Dermatology and Allergology, Philipps University, Marburg
Cryopreserved peripheral blood mononuclear cells (PBMC)	

2.3 Mouse material

Fresh peripheral venous blood	HLA-DRB1*04:02–tg mice bred at Experimental Animal Facilities, Philipps University, Marburg (Biomedizinisches Forschungszentrum)
Organs and tissues: spleen, lymph nodes, bone marrow, buccal and palatinal mucosa	

2.4 Immortalized cell lines

Murine hybridoma cell line X6310 transfected with granulocyte-macrophage colony-stimulating factor (GM-CSF) expression construct	Kindly provided by Prof. M. Schnare, Institute of Immunology, Philipps University, Marburg

2.5 Antigens

2.5.1 Recombinant proteins

All recombinant proteins were produced as soluble proteins in a baculovirus expression system and were purified from insect cell culture supernatant by affinity chromatography. Linkage of proteins to an E tag and 6 · histidine tag was introduced for technical reasons.

Recombinant Protein	Description of Recombinant	Amino Acid Sequence	References
Dsg3	EC of human Dsg3 (EC1–5)	1–566	(Hertl, Amagai, et al. 1998b; Veldman, Gebhard, et al. 2004; Veldman et al. 2003a; Eming et al. 2008)
Dsg3	EC of mouse Dsg3 (EC1–5)	1–566	(Anzai et al. 2004)
Col VII	Noncollagenous domain 1 (NC1) of human Collagen VII	17–610	(Muller et al. 2010; Jedlickova et al. 2012)

2.5.2 Peptides

Fifteen- and 17-mer Dsg3 peptides were synthesized by fluorenylmethyloxycarbonyl chloride (FMOC-Cl) chemistry resulting in >95% purity (peptides & elephants, Potsdam) and were solubilized in dimethyl sulfoxide (DMSO) acetic acid buffer (stock concentration, 2 mg/mL).

2.5.2.1 HLA-DRβ1*04:02–binding human Dsg3 peptides:

Dsg3 Peptides							P1			P4		P6					
Dsg3(97–111)			F	G	I	F	**V**	V	D	**K**	N	**T**	G	D	I	N	I
Dsg3(190–204)			L	N	S	K	**I**	A	F	**K**	I	**V**	S	Q	E	P	A
Dsg3(206–220)			T	P	M	F	**L**	L	S	**R**	N	**T**	G	E	V	R	T
Dsg3(251–265)			C	E	C	N	**I**	K	V	**K**	D	**V**	N	D	N	F	P
Dsg3(375–391)	I	N	V	R	E	G	**I**	A	F	**R**	P	**A**	S	K	T	F	T

2.5.2.2 HLA-DRβ1*04:02–non-binding human Dsg3 peptides:

Dsg3 Peptides							P1			P4		P6					
Dsg3(85–101)	Y	R	I	S	G	V	G	I	D	Q	P	P	F	G	I	F	V
Dsg3(145–161)	V	K	I	L	D	I	N	D	N	P	P	V	F	S	Q	Q	I
Dsg3(240–256)	A	D	K	D	G	E	G	L	S	T	Q	C	E	C	N	I	K
Dsg3(295–311)	E	E	Y	T	D	N	W	L	A	V	Y	F	F	T	S	G	N
Dsg3(400–416)	K	L	V	D	Y	I	L	G	T	Y	Q	A	I	D	E	D	T

Marked in bold are the amino acid anchor motifs critical for peptide binding to HLA-DRβ1*04:02 (Wucherpfennig et al. 1995; Tong et al. 2006).

2.5.3 Mitogens

Product	Company
Ionomycin	Merck KGaA, Darmstadt
Phorbol 12-myristate 13-acetate (PMA)	Promega Corporation, Mannheim
Phytohemagglutinin (PHA)	Sigma-Aldrich Chemie GmbH, Schnelldorf

2.6 Growth factors

GM-CSF secreted by X6310 cell line into the supernatant	Department of Dermatology and Allergology, Philipps University, Marburg

2.7 Chemicals and reagents

Product	**Company**
Acetic acid ($C_2H_4O_2$)	Merck KGaA, Darmstadt
Acrylamide (C_3H_5NO)	Carl Roth GmbH & Co. KG, Karlsruhe
Aluminum hydroxide (alum, $Al(OH)_3$)	Sigma-Aldrich Chemie GmbH, Schnelldorf
3-Amino-9-ethylcarbazole (AEC, $C_{14}H_{14}N_2$)	Sigma-Aldrich Chemie GmbH, Schnelldorf
Ammonium chloride (NH_4Cl)	Mallinckrodt Baker, Griesheim
Ammonium persulfate (APS, $(NH_4)_2S_2O_8$)	Sigma-Aldrich Chemie GmbH, Schnelldorf
2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS™, $C_{18}H_{24}N_6O_6S_4$)	Merck KGaA, Darmstadt
Bovine serum albumin – Fraction V (BSA)	Carl Roth GmbH & Co. KG, Karlsruhe
Bromophenol blue solution 2%	Sigma-Aldrich Chemie GmbH, Schnelldorf
5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE)	Thermo Fisher Scientific, Langenselbold
Citric acid ($C_6H_8O_7$)	Grüssing GmbH Analytika, Filsum
Disodium hydrogen phosphate (Na_2HPO_4)	Merck KGaA, Darmstadt
DMSO (C_2H_6OS)	Sigma-Aldrich Chemie GmbH, Schnelldorf
Dulbecco's Phosphate-Buffered Saline (PBS) 1x (without Ca^{2+}, Mg^{2+})	PAN-Biotech GmbH, Aidenbach
ECL Western Blotting Substrate	Thermo Fisher Scientific, Langenselbold
Ethanol 70% (C_2H_6O)	Otto Fischar GmbH, Saarbrücken
(Ethylenedinitrilo)tetraacetic acid (EDTA, $C_{10}H_{16}N_2O_8$)	Sigma-Aldrich Chemie GmbH, Schnelldorf
FACS™ Clean Solution	Becton Dickinson GmbH, Heidelberg
FACSFlow™ Sheath Fluid	Becton Dickinson GmbH, Heidelberg
FACSRinse Solution	Becton Dickinson GmbH, Heidelberg
Fetal calf serum (FCS)	Biochrom AG, Berlin
G 418 disulfate salt solution	Sigma-Aldrich Chemie GmbH, Schnelldorf
Glycerol ($C_3H_8O_3$)	Amersham Pharmacia Biotech, Freiburg
Glycine ($C_2H_5NO_2$)	Carl Roth GmbH & Co. KG, Karlsruhe
GolgiStop™ Protein Transport Inhibitor (contains monensin)	Becton Dickinson GmbH, Heidelberg
Heparin	Sigma-Aldrich Chemie GmbH, Schnelldorf
Horseradish peroxidase (HRP) Streptavidin for ELISPOT	Becton Dickinson GmbH, Heidelberg

Hydrogen peroxide 30% (H_2O_2)	Merck KGaA, Darmstadt
L-Glutamine (200 mM) with Penicillin (10,000 U/mL), Streptomycin (10,000 mg/mL)	PAA Laboratories GmbH, Cölbe
Lipopolysaccharides (LPS) from *Escherichia coli* 055:B5	Sigma-Aldrich Chemie GmbH, Schnelldorf
2-Mercaptoethanol (2-ME, C_2H_6OS) (Gibco® 2-Mercaptoethanol)	Life Technologies GmbH, Darmstadt
Methanol (CH_3OH)	Sigma-Aldrich Chemie GmbH, Schnelldorf
Mounting medium (IF) (VECTASHIELD Antifade Mounting Medium for fluorescence)	LINARIS Biologische Produkte GmbH, Dossenheim
Nonfat dried milk powder	AppliChem GmbH, Darmstadt
Normal Mouse Serum	Thermo Fisher Scientific, Langenselbold
Pancoll human; density 1,077 g/L	PAN-Biotech GmbH, Aidenbach
Paraformaldehyde (PFA, $OH(CH_2O)_nH$)	Merck KGaA, Darmstadt
Potassium hydrogen carbonate ($KHCO_3$)	Merck KGaA, Darmstadt
2-Propanol (C_3H_8O)	Sigma-Aldrich Chemie GmbH, Schnelldorf
Protein ladder (10–250 kDa)	Thermo Fisher Scientific, Langenselbold
Rabbit serum	Thermo Fisher Scientific, Langenselbold
RPMI Media 1640	PAA Laboratories GmbH, Cölbe
Saponin from quillaja bark (20–35%)	Sigma-Aldrich Chemie GmbH, Schnelldorf
Sodium acetate ($C_2H_3NaO_2$)	Merck KGaA, Darmstadt
Sodium azide (NaN_3)	Merck KGaA, Darmstadt
Sodium dodecyl sulfate (SDS, $NaC_{12}H_{25}SO_4$)	Carl Roth GmbH & Co. KG, Karlsruhe
Tetramethylethylenediamine (TEMED, $C_6H_{16}N_2$)	Carl Roth GmbH & Co. KG, Karlsruhe
Tissue-Tek® O.C.T.™ Compound	Sakura Finetek GmbH, Staufen
TiterMax® Gold Adjuvant	Sigma-Aldrich Chemie GmbH, Schnelldorf
Tris(hydroxymethyl)aminomethane (TRIS, $C_4H_{11}NO_3$)	Carl Roth GmbH & Co. KG, Karlsruhe
Trypan Blue solution 0.4%	Sigma-Aldrich Chemie GmbH, Schnelldorf
Tween®-20 (Polysorbate)	Merck KGaA, Darmstadt
X-Vivo™ Medium	Lonza Group Ltd., Köln

2.8 Buffers and culture media

Description	**Production**
ABTS solution	515 mL 0.2 M Na_2HPO_4 + 485 mL 0.1 M citric acid

	Adjust pH to 5.0. + 1 g ABTS Store at -20 °C.
Ammonium chloride–potassium hydrogen carbonate (ACK) lysis buffer	0.15 M NH_4Cl + 1 mM $KHCO_3$ + 0.1 mM EDTA Adjust volume to 1 L ddH_2O, Adjust pH to 7.2–7.4. Store at room temperature (RT).
DC differentiation medium	X-VIVO™ medium + 10% GM-CSF Store at 4 °C.
ddH_2O	Deionization and sterile filtration of tap water by ultrapure water purification system. If necessary sterilization at 121 °C, 20 min. Store at RT.
DIF blocking buffer	PBS 1x + 10% rabbit serum
DIF dilution buffer	PBS 1x + 2% rabbit serum
EDTA–heparin buffer	PBS 1x + 5 U/mL heparin + 1 mM EDTA Store at 4 °C.
ELISPOT assay blocking buffer	PBS 1x + 1% BSA
ELISPOT assay dilution buffer	PBS 1x + 0.5% BSA + 0.02% NaN_3
ELISPOT assay substrate solution	20 mL <u>0.1 M acetate solution</u> 148 mL 0.2 M acetic acid + 352 mL 0.2 M sodium acetate Adjust volume to 1 L ddH_2O, Adjust pH to 5.0. + 666.7 µL AEC stock solution + 10 µL H_2O_2 for activation Sterile filtrate (0.45 µm).
ELISPOT assay washing buffer	PBS 1x + 0.05% Tween®-20
Fixation buffer	PBS 1x

	+ 1% PFA Store at -20 °C.
FACS buffer	PBS 1x + 1% BSA + 0.1% NaN_3 Store at 4 °C.
Freezing medium	FCS, sterile filtered (0.22 µm) + 10% DMSO Store at -20 °C.
Immunoblotting blocking buffer	PBS 1x + 0.05% Tween®-20 + 5% nonfat dried milk powder
Immunoblotting washing buffer	PBS 1x + 0.05% Tween®-20
MACS buffer	PBS 1x + 0.5% BSA + 2 mM EDTA Sterile filtrate (0.22 µm) Store at 4 °C.
Permeabilization buffer	PBS 1x + 1% BSA + 0.1% NaN_3 + 0.1 % saponin Store at 4 °C.
$RPMI^{++}$	RPMI 1640 + 2 mM l-glutamine + 100 U/mL penicillin + 100 µg/mL streptomycin Store at 4 °C.
Running buffer 10x	286 g 190 mM glycine + 60.6 g 25 mM TRIS + 20 g SDS 0.1% Add 2 L ddH_2O. Store at RT.
Sample buffer 5x	1 g SDS + 5 mL glycerol 99% + 4 mL 1 M TRIS, pH= 6.8 + 1 mL 2-mercaptoethanol + 0.5 mL bromophenol blue 2% Store at 4 °C.

SDS-polyacrylamide gel electrophoresis (PAGE) separating gel (*n*= 4) 12.5%	8.7 mL acrylamide 30% + 3.75 mL 2 M TRIS, pH= 8.8 + 7.9 mL ddH2O + 206.3 µL SDS 10% + 77.5 µL APS 10% + 15.5 µL TEMED
SDS-PAGE stacking gel (*n*= 4) 12.5%	833 µL acrylamide 30% + 620 µL 1 M TRIS, pH= 6.8 + 3.54 mL ddH2O + 50 µL SDS 10% + 25 µL APS 10% + 10 µL TEMED
Transfer buffer	6 g TRIS + 28.8 g glycine + 400 mL methanol Adjust volume to 2 L ddH_2O and store at RT.

2.9 Antibodies

2.9.1 Antibodies for cell culture and cell preparation

Specificity	Clone	Isotype	Company
CD3	UCHT1	Mouse IgG1, κ	Becton Dickinson GmbH, Heidelberg
CD13/CD16	2.4G2	Rat IgG2b, κ	Becton Dickinson GmbH, Heidelberg

2.9.2 Antibodies for ELISA

Specificity	Clone	Isotype	Conjugate	Company
Mouse IgG	Polyclonal	Rabbit IgG	HRP	Dako GmbH, Hamburg

2.9.3 Antibodies for ELISPOT

Product	Company
Mouse IFN-γ ELISPOT Pair	Becton Dickinson GmbH, Heidelberg
Mouse IL-4 ELISPOT Pair	Becton Dickinson GmbH, Heidelberg

2.9.4 Antibodies for flow cytometry

Specificity	Clone	Isotype	Conjugate	Company
CCR2	48607	Mouse IgG2b, κ	Allophycocyanin (APC)	R&D Systems GmbH, Wiesbaden

CD3	UCHT1	Mouse IgG1, κ	Phycoerythrin (PE)	Becton Dickinson GmbH, Heidelberg
CD4	RPA-T4	Mouse IgG1, κ	Fluorescein isothiocyanate (FITC)	Becton Dickinson GmbH, Heidelberg
CD4	RPA-T4	Mouse IgG1, κ	PE	Becton Dickinson GmbH, Heidelberg
CD4	RM4-5	Rat IgG2a, κ	APC	Becton Dickinson GmbH, Heidelberg
CD8	RPA-T8	Mouse IgG1, κ	FITC	Becton Dickinson GmbH, Heidelberg
CD11c	B-ly6	Mouse IgG1, κ	APC	Becton Dickinson GmbH, Heidelberg
CD14	M5E2	Mouse IgG2a, κ	FITC	Becton Dickinson GmbH, Heidelberg
CD14	MEM-18	Mouse IgG1, κ	PE	ImmunoTools, Friesoythe
CD16	3G8	Mouse IgG1, κ	PE	Becton Dickinson GmbH, Heidelberg
CD16	B73.1	Mouse IgG1, κ	APC	Becton Dickinson GmbH, Heidelberg
CD86	2331 (FUN-1)	Mouse IgG1, κ	PE	Becton Dickinson GmbH, Heidelberg
CD303	AC144	Mouse IgG1, κ	APC	Miltenyi Biotec, Bergisch-Gladbach
HLA-DR	G46-6	Mouse IgG2a, κ	PE	Becton Dickinson GmbH, Heidelberg
HLA-DQ	Tu169	Mouse IgG2a, κ	FITC	Becton Dickinson GmbH, Heidelberg
I-A[b]	25-9-17	Mouse (C3H) IgG2a, κ	FITC	Becton Dickinson GmbH, Heidelberg
IFN-γ	25723.11	Mouse IgG2b, κ	APC	Becton Dickinson GmbH, Heidelberg
IL-4	MP4-25D2	Rat IgG1, κ	APC	Becton Dickinson GmbH, Heidelberg
IL-6	MQ2-13A5	Rat IgG1, κ	PE	eBioscience, Frankfurt
IL-10	JES3-19F1	Rat IgG2a, κ	APC	Becton Dickinson GmbH, Heidelberg

IL-17A	N49-653	Mouse IgG1, κ	Alexa Fluor® 647	Becton Dickinson GmbH, Heidelberg
TNF-α	MAb11	Mouse IgG1, κ	PE	Becton Dickinson GmbH, Heidelberg
Isotype control	MOPC-21	Mouse IgG1, κ	Alexa Fluor® 647	Becton Dickinson GmbH, Heidelberg
Isotype control	MOPC-21	Mouse IgG1, κ	APC	Becton Dickinson GmbH, Heidelberg
Isotype control	MOPC-21	Mouse IgG1, κ	FITC	Becton Dickinson GmbH, Heidelberg
Isotype control	MOPC-21	Mouse IgG1, κ	PE	Becton Dickinson GmbH, Heidelberg
Isotype control	G155-178	Mouse IgG2a, κ	FITC	Becton Dickinson GmbH, Heidelberg
Isotype control	G155-178	Mouse IgG2a, κ	PE	Becton Dickinson GmbH, Heidelberg
Isotype control	MPC-11	Mouse IgG2b, κ	APC	Becton Dickinson GmbH, Heidelberg
Isotype control	R3-34	Rat IgG1, κ	APC	Becton Dickinson GmbH, Heidelberg
Isotype control	R35-95	Rat IgG2a, κ	APC	Becton Dickinson GmbH, Heidelberg
Isotype control	PPV-06	Mouse IgG1, κ	PE	ImmunoTools, Friesoythe
Isotype control	IS5-21F5	Mouse IgG1, κ	APC	Miltenyi Biotec, Bergisch-Gladbach
Isotype control	133303	Mouse IgG2b, κ	APC	R&D Systems GmbH, Wiesbaden
Isotype control	eBRG1	Rat IgG1, κ	PE	eBioscience, Frankfurt

2.9.5 Antibodies for immunoblotting

Specificity	**Clone**	**Isotype**	**Conjugate**	**Company**
E tag	Polyclonal	Rabbit IgG	-	Abcam plc, Cambridge, U.K.
Mouse IgG	Polyclonal	Rabbit IgG	HRP	Dako GmbH, Hamburg

2.9.6 Antibodies for immunofluorescence

Specificity	Clone	Isotype	Conjugate	Company
Mouse IgG	Polyclonal	Rabbit IgG	FITC	Thermo Fisher Scientific, Langenselbold

2.10 Commercial test kits for serological and cellular diagnostics

Kit	Company
ACHRAB® - Assay	DLD Diagnostika, Hamburg
Alexa Fluor® 488 Protein Labeling Kit	Thermo Fisher Scientific, Langenselbold
Anti-Desmoglein 1/3-ELISA	Euroimmun, Lübeck
CD4 (L3T4) MicroBeads mouse	Miltenyi Biotec, Bergisch-Gladbach
IL-6 ELISA Ready-SET-Go!®	eBioscience, Frankfurt
IL-27 ELISA Ready-SET-Go!®	eBioscience, Frankfurt
LEGEND MAX™ Human IL-33 ELISA Kit	eBioscience, Frankfurt
Neurology Mosaic 1 (titin)	Euroimmun, Lübeck
NOVA Lite® (IFA) Monkey Esophagus	Werfen GmbH, Kirchheim
TNF-α ELISA Ready-SET-Go!®	eBioscience, Frankfurt

2.11 Consumables

Product	Company
6/24/48-well plates	Nunc GmbH & Co. KG, Wiesbaden
96-well plates, flat-bottom	Nunc GmbH & Co. KG, Wiesbaden
96-well plates, round-bottom	Nunc GmbH & Co. KG, Wiesbaden
96-well polyvinylidene fluoride (PVDF) membrane plates (MAIPSWU10)	Millipore GmbH, Schwalbach
Cannulas, 26/30 G (BD Microlance™)	Becton Dickinson GmbH, Heidelberg
Cell culture flasks, 25/75/125 cm^2	Greiner, Frickenhausen
Cell strainers, 40 µm	Becton Dickinson GmbH, Heidelberg
Centrifugation tubes, 15/50 mL	Greiner, Frickenhausen
Combitips®, 5 mL	Eppendorf, Hamburg
Coverslips for Neubauer counting chamber, 20 · 26 mm	Thermo Fisher Scientific, Langenselbold
Cryogenic tubes, 2 mL	Greiner, Frickenhausen
Dissecting instruments (scissors, forceps)	AESCULAP Surgical instruments, Tuttlingen; Dumont, Montignez, Switzerland
Filter papers (Whatman®)	Sigma-Aldrich Chemie GmbH, Schnelldorf
Flow cytometry tubes, polystyrene, 5 mL	Sarstedt, Nümbrecht
Hose connector, metal (P334.1)	Carl Roth GmbH & Co. KG, Karlsruhe
Luer lock syringes, 3 mL (Z248002)	Sigma-Aldrich Chemie GmbH, Schnelldorf

MACS®, MS columns	Miltenyi Biotec, Bergisch-Gladbach
Micro hematocrit blood tubes, heparinized	Brand GmbH + Co KG, Wertheim
Microscope coverslips	Thermo Fisher Scientific, Langenselbold
Microscope slides, 1 mmm, 26 · 76 mm	Thermo Fisher Scientific, Langenselbold
Monovette® (citrate-phosphate-dextrose-adenine, CPDA)	Sarstedt, Nümbrecht
Mouse ear punch	Carl Roth GmbH & Co. KG, Karlsruhe
Pasteur pipettes, glass	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt
Petri dishes, bacteriological, 100 mm	Becton Dickinson GmbH, Heidelberg
Pipette tips	Kalensee, Gießen
Reaction tubes, 1.5 mL	Eppendorf, Hamburg
Rotilabo® tetrafluorethylene-perfluorpropylene (FEP) hose (Y505.1)	Carl Roth GmbH & Co. KG, Karlsruhe
Serological pipettes, 5/10/25 mL	Greiner, Frickenhausen
Stericup® filter units, 250/500 mL, 0.22 µm	Millipore GmbH, Schwalbach
Steriflip® filter units, 0.22 µm	Millipore GmbH, Schwalbach
Steritop™ filter units, 0.45 µm	Millipore GmbH, Schwalbach
Syringes, 1 mL (Omnifix®-F Duo)	B. Braun Melsungen AG, Melsungen
Transfer membrane, nitrocellulose, 0.45 µm	Millipore GmbH, Schwalbach

2.12 Laboratory equipment

Equipment	**Company**
Absorbance reader Sunrise™-Basic	Tecan GmbH, Gröding, Austria
Analytical balance 770	Gottlieb Kern & Sohn GmbH, Balingen-Frommern
Autoclave FVA3	Fedegari Autoclavi Spa, Albuzzano, Italy
CO_2 incubator BBD 6220	Thermo Fisher Scientific, Langenselbold
CO_2 incubator HERAcell®	Heraeus Kendro Laboratory Products GmbH, Langenselbold
Cryogenic container Qualifreeze	Nunc GmbH & Co. KG, Wiesbaden
Documentation device	VWR International GmbH, Erlangen
Electroblotting apparatus	Bio-Rad Laboratories, Hercules, USA
ELISPOT Scanner Eli.Scan	A.EL.VIS, Hannover
Flow cytometer FACSCalibur™	Becton Dickinson GmbH, Heidelberg
Fluorescence microscope Olympus BH-2	Olympus, Tokyo, Japan
Gel electrophoresis apparatus	Bio-Rad Laboratories, Hercules, USA
Heating block	Eppendorf, Hamburg
Light microscope Axiostar	Carl Zeiss MicroImaging GmbH, Göttingen

Magnetic stirrer IKAMAG® RET	IKA Labortechnik, Staufen
MiniMACS® separator	Miltenyi Biotec, Bergisch-Gladbach
Multichannel pipette Discovery 20-200 µL	ABIMED, Langenfeld
Multipette® plus	Eppendorf, Hamburg
Neubauer counting chamber	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen
Nitrogen tank Chronos 200	Cryotherm GmbH & Co. KG, Kirchen
pH meter CyberScan pH 510	Eutech Instruments Europe bv, Nijkerk, Netherlands
Pipettes Research® 10 µL, 100 µL, 200 µL, 1,000 µL	Eppendorf, Hamburg
Refrigerated centrifuge Megafuge® 1.0R	Heraeus Kendro Laboratory Products GmbH, Langenselbold
Sterile bench HERASafe®	Heraeus Kendro Laboratory Products GmbH, Langenselbold
Ultrapure water purification system Astacus	MembraPure GmbH, Henningsdorf
Vortex mixer IKA® Genius 3	IKA Labortechnik, Staufen
Water bath GFL 1083	Gesellschaft für Labortechnik mbH, Burgwedel

2.13 Software

Program	**Company**
CellQuest Pro™ 5.2	Becton Dickinson GmbH, Heidelberg
Eli.Analyse ELISPOT Analysis Office Software 4.2	A.EL.VIS, Hannover
Endnote X7	Thomson ResearchSoft, USA
FlowJo© 7.6.3	TreeStar Inc., Ashland, USA
GraphPad Prism© 6.02	GraphPad Software Inc., La Jolla, USA
Microsoft© Office Excel©	Microsoft Corporation, Redmond, USA
Microsoft© Office PowerPoint©	Microsoft Corporation, Redmond, USA
Microsoft© Office Word©	Microsoft Corporation, Redmond, USA

3 Methods

3.1 Human study

3.1.1 Patients

In this study, 34 pemphigus patients, 31 MG patients serving as a control for a further unrelated auto-ab–mediated organ-specific disorder, and 32 HC were included. Data is primarily shown from nine PV and three PF patients. The clinical diagnosis of pemphigus and MG was confirmed by standard criteria. The MG patients suffered from very mild to moderate disease, whereas the pemphigus patients could be categorized according to active (act. P) or remittent (rem. P) stage of disease. Remission was defined as the absence of any mucosal blisters and/or skin erosions for at least two months (Murrell et al. 2008). Since systemic immunosuppressive therapy with prednisolone (Pred) significantly impacted DC numbers in a dose-dependent manner (**Figure 4.13**), patients with a daily intake of ≥10 mg of Pred were excluded from this analysis. Patients with pathological leukocyte counts were also excluded from this study. HC were matched according to gender and age, and did not display any signs of skin autoimmune inflammation. Pemphigus and MG patients averaged 54 and 57 years, respectively, whilst the average age of HC was 50 years. Here provided is an overview of the pemphigus and MG patients' clinical and serological characteristics indicated in relative units (RE)/mL:

Table 3.1: Clinical phenotype and auto-ab profile of pemphigus vulgaris and pemphigus foliaceus patients.

		Clinical phenotype[1]			Auto-ab profile (IgG)[3]	
		Skin	Mucosa	Medication per day[2]	Dsg3	Dsg1
Patient	Status					
PV1	Active	Isolated erythematous plaques with crusty erosions on legs and feet	Erosions on lower lip	None	18	9
PF2	Active	Crusty erosions on the face, neck, and trunk	None	None	0	34
PV3	Active	None	Erosions in oral cavity	None	529	2
PV4	PR[4]	None	Erosions in oral cavity	None	0	9
PF5	Active	Extensive dry erosions on the scalp, trunk, and legs	None	MMF 2 g	3	632
PV6	CR[5]	None	None	None	2	4
PV7	Active	Isolated crusty erosion on the hand	None	None	491	0
PV8	Active	None	Erosions on palate, tongue, and gingiva	None	40	0
PV9	Active	None	Erosion in oral cavity	None	176	0
PV10	Active	None	Dry erythematous plaques on genitals	None	1148	0

PF11	Active	Extensive open erosions on the face and neck	None	None	0	534
PV12	CR	None	None	None	0	0

[1]At time of study.
[2]MMF, mycophenolate mofetil.
[3]Determined by ELISA with recombinant Dsg1 or Dsg3 in RE/mL (cut-off value: 20 RE/mL).
[4]PR, partial remission off therapy as defined by Murrell et al. J Am Acad Dermatol, 2008.
[5]CR, complete remission off therapy as defined by Murrell et al. J Am Acad Dermatol, 2008.

Table 3.2: Clinical status and auto-ab profile of myasthenia gravis patients.

			Auto-ab profile (IgG)[2]	
		Medication per day[1]	AChR	Titin
Patient	Status			
MG1	Very mild	IVIG	1.6	0
MG2	Very mild	IVIG, MMF 1g, Pred 5 mg	65	640
MG3	No symptoms	MTX 7.5 mg weekly	3.5	80
MG4	Moderate	AZA 150 mg, Pred 5 mg	4.6	0
MG5	Mild	IVIG	0.1	0
MG6	Mild	IVIG	0.1	320
MG7	Moderate	IVIG, TRL	0.39	0
MG8	Very mild	AZA 100 mg	190	0
MG9	No symptoms	AZA 150 mg	0	0
MG10	Moderate	AZA 150 mg	560	0
MG11	No symptoms	None	58	80
MG12	Very mild	MTX 7.5 mg weekly	110	1280

[1]AZA, azathioprine; IVIG, intravenous Ig; MTX, methotrexate; Pred, prednisolone; TRL, tacrolimus.
[2]Determined by ^{125}I-radio receptor assay for anti-acetylcholine receptor (AChR) IgG in nmol/L (cut-off value: 0.25–0.4 nmol/L) and IIF microscopy for anti-titin IgG (cut-off value: titer of 80).

3.1.2 Serological diagnostics

3.1.2.1 Blood collection and autoimmune serology

Peripheral blood samples (~50 mL) were collected from pemphigus, MG patients, and HC by clinical staff of the Department of Dermatology and Allergology of Philipps-University, Marburg. The presence of IgG auto-ab against Dsg1 and/or Dsg3 in the sera of the pemphigus patients was detected by a commercially available ELISA kit (**2.10**) by staff of the diagnostic laboratory of the Department of Dermatology and Allergology of Philipps-University, Marburg according to the manufacturer's protocol. Circulating IgG anti-AChR antibodies in the sera of the MG patients were detected by staff of the Bioscientia Institute of Medical Diagnostics, Ingelheim using a commercially available ^{125}I-radio receptor assay (**2.10**) according to the manufacturer's manual. Auto-ab against striated muscle (titin) were detected by IIF microscopy using a commercially available testing system (**2.10**) by staff of the Department of Laboratory Medicine and Pathobiochemistry, Molecular Diagnostics, Philipps-University, Marburg according to the manufacturer's protocol. For isolation of both mononuclear cells and blood plasma, peripheral

blood was treated with the anticoagulant citrate-phosphate-dextrose-adenine (CPDA) and was prepared on the following day of withdrawal (**3.1.3.1**).

3.1.2.2 Detection of plasma cytokines by ELISA

The cytokines IL-6, TNF-α, IL-21, IL-27, and IL-33 present in the plasma of pemphigus patients were quantified by ELISA with the help of commercially available ELISA kits (**2.10**) according to the manufacturers' protocols. Cytokine concentrations were derived from a four parameter logistic calibration curve using GraphPad Prism© software (**2.13**).

3.1.3 Cellular diagnostics

3.1.3.1 Isolation of PBMC by density gradient centrifugation

PBMC were isolated from peripheral blood treated with the anticoagulant CPDA using density gradient centrifugation. This purification method is based on a watery solution of the high-molecular-weight sucrose polymer Ficoll with a specific gravity of 1.077 g/mL, as well as density differences between mononuclear cells and other components of the blood sample (Fuss et al. 2009). Upon centrifugation of a preparation of Ficoll and whole blood diluted with phosphate-buffered saline (PBS), the single cell populations distribute in distinct layers, with the density of Ficoll exceeding those of lymphocytes, monocytes, and platelets, but falling below those of granulocytes and red blood cells (RBC). Therefore, mononuclear cells and platelets collect between the upper PBS and the lower Ficoll layer, whereas RBC and granulocytes form sediment at the bottom of the Ficoll layer. Platelets are removed within the mononuclear cell fraction by subsequent centrifugation.

Procedure:

The cellular fraction of the patient's CPDA–blood sample is pelleted by centrifugation at 350 g (1 g= 9.81 ms^{-2}) at room temperature (RT) for 10 min, and the blood plasma is subsequently separated, centrifuged at 1,860 g at 4 °C for 10 min and frozen at -20 °C. The leukocyte/RBC fraction is diluted in the ratio of 1:2 with PBS, carefully layered in the ratio of 1:3 onto the Ficoll solution, and centrifuged at 450 g at RT for 30 min with deactivation of the brakes. Following centrifugation, the PBMC fraction located between the PBS and Ficoll layer of the obtained density gradient is aspirated using a Pasteur pipette and is transferred into a fresh centrifugation tube filled with 50 mL PBS at 4 °C and centrifuged at 550 g at 4 °C for 10 min. Thereafter, the cells are again washed with PBS, centrifuged at 350 g at 4 °C for 10 min and resuspended in $RPMI^{++}$ (**2.8**) to determine the cell number utilizing a Neubauer counting chamber (**3.1.3.2**).

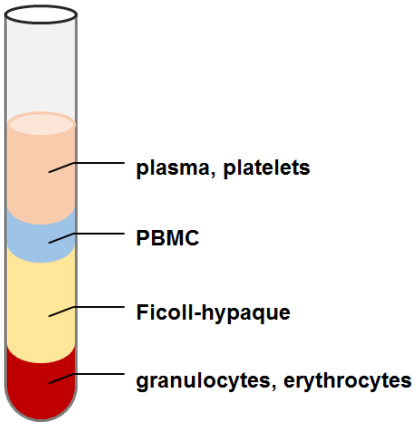

Figure 3.1: Separation of blood components on a Ficoll gradient.
Peripheral blood mononuclear cells (PBMC) collect between the upper phosphate-buffered saline and lower Ficoll layer (modified from Fuss et al. Curr Protoc Immunol, 2009).

3.1.3.2 Cell count determination

The number of viable cells present in a cell suspension was determined under a light microscope with the help of a Neubauer counting chamber and dye exclusion test (Phelan and Lawler 2001; Strober 2001). A sample of the cell suspension was thoroughly diluted in a suitable ratio with 0.4% Trypan Blue solution to obtain a uniform suspension, which was then transferred underneath the coverslip of the counting chamber. Since vital cells possess intact cell membranes that exclude the permeability of distinct dyes, (such as Trypan Blue), only the cytosolic proteins of dead cells are selectively stained. Hence upon optical analysis, live cells appeared clear, whilst dead cells exhibited a blue cytoplasm. Cell counts in each of the four corners of the Neubauer counting chamber were recorded and the cell density was determined by the following calculation: Cells/mL= average count per square · dilution factor · 10^4.

3.1.3.3 Cryopreservation and thawing of cells

Cryopreservation is required for the long-term storage and maintenance of principle cellular functions of mononuclear cells (Yokoyama, Thompson, and Ehrhardt 2012). In order to functionally characterize leukocyte populations of pemphigus patients, PBMC were frozen and thawed according to the following procedure: Upon isolation of the PBMC, cells were resuspended in $RPMI^{++}$ (**2.8**) and centrifuged at 350 g at 4 °C for 10 min. The cell pellet was resuspended at a density of 5–10 · 10^6 cells/mL in freezing medium (**2.8**), which consisted of fetal calf serum (FCS) supplemented with 10% dimethyl sulfoxide (DMSO). The cells were then transferred into cryogenic storage tubes. The addition of the cryoprotective agent DMSO, as well as the selection of appropriate freezing rates prevent cellular damage caused by the formation of intracellular ice crystals and osmotic effects. The optimum freezing rate (1 °C/min temperature reduction until -80 °C) was achieved by storing the cryogenic tubes in specific freezing containers filled with 2-propanol. For long-term preservation, PBMC were stored in liquid nitrogen at -170 °C.

To slowly thaw the cryopreserved PBMC, frozen cells were exposed to a water bath of 37 °C, added dropwise to RPMI^{++}, and subsequently centrifuged at 350 g at 4 °C for 10 min. Thereafter, cells were cultured in their respective growth mediums for further functional analysis.

3.1.3.4 Flow cytometry

The investigational technique of flow cytometry/fluorescence-activated cell sorting (FACS) can perform multiple simultaneous measurements at the single cell level at very rapid rates. It is based on detecting both scattered light and fluorescence from suitably stained constituents in individual cells. Therefore, the stained cells are streamed single file in fluid suspension through the focus of a high-intensity light source (laser beam). Each cell passing through the focus emits a flash of scattered and/or fluorescent light. This light is collected by a system of lenses and filtered before reaching a photodetector that quantitatively converts it into an electronic signal. The diffraction of light (forward scatter, FSC) thereby indicates the size, whereas the refraction of light (side scatter, SSC) indicates the granularity of the cell. A feature of all biological molecules is their capacity to absorb light, which increases the energy state of the molecule above its ground state. The molecule will revert to its ground state by a number of mechanisms, amongst which is the emission of light with a longer wavelength compared to that of the exciting light. A fluorescent dye attached to an antibody absorbs light most efficiently at a distinct wavelength within its specific absorption spectrum. In this study, we applied the fluorochromes fluorescein isothiocyanate (FITC), phycoerythrin (PE), and allophycocyanin (APC) (**2.9.4**) for flow cytometry measurement. The FACSCalibur™ **(2.12**) displays two laser beams operating at specific wavelengths (488 nm, 635 nm) to excite four dyes at the same time.

Analysis of FSC and SSC of peripheral blood leukocytes yields clearly defined populations of lymphocytes, monocytes, granulocytes, and debris clusters. The debris cluster consists of cell fragments, RBC, and platelet aggregates. Because detection of RBC interferes with the detection of lymphocytes, RBC need to be removed from CPDA–blood by adding hypotonic ammonium chloride–potassium hydrogen carbonate (ACK) lysis buffer (**2.8**) causing burst of erythrocytes.

In this thesis, flow cytometry was used to phenotypically and functionally characterize subsets of human circulating DC, monocytes, and lymphocytes. Prior to analyzing antibody binding to distinct surface molecules or intracellular proteins, the respective leukocyte populations were preselected by gating based on FSC and SSC analysis (Watson 1991; Dean and Hoffman 2001).

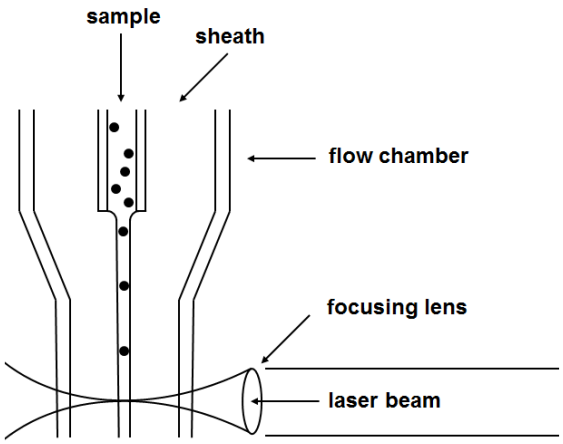

Figure 3.2: Longitudinal cross-sectional view of the flow chamber of a flow cytometer.
The cell sample is contained in the sheath fluid, which guides the cells to the center of the flow chamber. The laser beam is directed onto the cell stream (modified from Dean et al. Current Protocols in Cytometry, 2001).

3.1.3.5 Detection of cell surface markers in whole blood by FACS

In order to phenotypically characterize monocyte and DC populations of pemphigus patients, whole blood was analyzed for cell type-specific and activation/migration cell surface markers. Therefore, peripheral blood–CPDA samples were incubated for 30 min at 4 °C in a dark environment with the respective fluorescently labeled antibodies, as well as matching isotype controls according to the manufacturers' protocols. RBC were lysed upon twofold incubation in ACK lysis buffer (**2.8**) for 5 min at RT. Following double washing with FACS buffer (**2.8**) at 535 g at 4 °C for 5 min, cells were resuspended in 250 µL with the same buffer for measurement. Flow cytometry analysis was performed using FACSCalibur™ (**2.12**) and FlowJo© single cell analysis software (**2.13**).

3.1.3.6 Stimulation of PBMC for detection of intracellular cytokines by FACS

To assess the capacity of monocytes and $CD4^+$ T cells from pemphigus patients to produce distinct cytokines, PBMC were stimulated according to the following procedure: Blood samples were rested overnight at RT before PBMC were isolated using density gradient centrifugation (**3.1.3.1**) and cells were stored at -80 °C until further use. Depending on the assay, after overnight culture in $RPMI^{++}$ (**2.8**) supplemented with 10% FCS, thawed cells were either stimulated with 0.1 µg/mL LPS overnight or 5 ng/mL phorbol myristate acetate (PMA) and 500 ng/mL ionomycin for 5 hours at 37 °C and 5% CO_2. LPS is the major component of the outer membrane of Gram-negative bacteria and it is the best-described ligand of the TLR4 expressed by several immune cells, including DC, monocytes, and macrophages. TLR4 signals from the plasma membrane as part of the LPS-multireceptor complex, which induces the expression of various proinflammatory cytokines, such as TNF-α, IL-1, and IL-6 (Brubaker et al. 2015a). The pharmacological agents PMA and ionomycin together are the most robust and nonspecific stimuli for most T cell cytokines. Their application largely reconstitutes the effects of T cell activation: Whereas ionomycin allows

EC Ca^{2+} to flow into the cell, PMA is an activator of the protein kinase C (Foster et al. 2007). In order to avoid release of the stimulation-induced cytokines into the supernatant, secretory activity of the Golgi apparatus was blocked by adding GolgiStop™ according to the manufacturer's protocol, 5 hours prior to antibody staining.

3.1.3.7 Detection of intracellular proteins of stimulated PBMC by FACS

Activated PBMC were washed with FACS buffer (**2.8**) (535 g, 4 °C, 5 min) and incubated with the respective fluorescently labeled antibodies for staining of cell surface structures, as well as appropriate isotype controls according to the manufacturers' protocols for 20 min at 4 °C in a dark environment. After washing (535 g, 4 °C, 5 min), cells were first fixed with 0.5 mL fixation buffer (**2.8**) for 10 min at RT, washed twice with 2 mL FACS buffer (535 g, 4 °C, 5 min), and then permeabilized with permeabilization buffer (**2.8**) for 10 min at 4 °C in a dark environment. After centrifugation (535 g, 4 °C, 5 min), intracellular cytokines were detected by incubation with the respective fluorescently labeled antibodies, as well as appropriate isotype controls according to the manufacturers' protocols for 30 min at 4 °C in a dark environment. Prior to flow cytometry analysis, cells were washed twice with permeabilization buffer (535 g, 4 °C, 5 min) and then resuspended in 500 µL of the same buffer for flow cytometry analysis.

3.1.3.8 Antigen uptake assay

Antigen uptake belongs to the most important functions of monocytes and DC (Nuñez 2001). In order to check whether this property is altered in monocytes of pemphigus patients their capacity of protein internalization was analyzed by flow cytometry. Therefore $1 \cdot 10^5$ freshly thawed PBMC were resuspended in $RPMI^{++}$ (**2.8**) supplemented with 10% FCS and stored on ice for 10 min. Upon addition of fluorescently labeled recombinant Dsg3 (**2.10**), cells were incubated for various lengths of time (5 min, 10 min, and 30 min) at 37 °C in the incubator or stored on ice in a dark environment as a control. The process of antigen uptake was abolished by adding 2 mL ice-cold FACS buffer (**2.8**). After double washing with FACS buffer (350 g, 4 °C, 10 min) cell surface markers were stained with fluorescently labeled antibodies according to the manufacturers' protocols. Following repeated washing, PBMC were fixed in fixation buffer (**2.8**) and directly analyzed using flow cytometry. The difference in mean fluorescence intensity (MFI) between the sample incubated at 37 °C and its control determines the monocyte uptake capability.

3.1.3.9 Statistics

Statistical evaluation of the data obtained from flow cytometry analysis was performed using GraphPad Prism© software (**2.13**). For comparison of a variable between two groups of a small sample size that are not related to each other, a nonparametric two-tailed test for comparison of unpaired sample sizes (Mann-Whitney-U) was selected. Differences were considered significant for $p < 0.05$ (indicated as *), very significant for $p < 0.01$ (indicated as **), and highly significant for $p < 0.001$ (indicated as ***). Spearman correlation coefficients were displayed for at least medium correlation of $r > 0.6$.

3.2 Mouse study

3.2.1 HLA-DR4–tg mice

HLA-DRA1*01:01-DRB1*04:02/-DQA1*03:01, -DQB1*03:02 (DQ8)–tg DBA/1J mice were generated as described previously (Congia et al. 1998; Cope et al. 1999). The mice express the human CD4 coreceptor and are deficient in I-Aβ (I-Aβ$^{-/-}$) (Killeen, Sawada, and Littman 1993).
Transgenic C57/BL/10 mice coexpressing DRB1*04:01 and human CD4, but devoid of endogenous murine MHC class II and a functional Ncf1 gene were used as control animals by the research group of Johan Bäcklund, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden (Backlund et al. 2002; Hultqvist et al. 2004; Batsalova et al. 2010).

3.2.1.1 Blood collection and phenotyping of HLA-DR4–tg mice

In order to phenotype immune cells of HLA-DRB1*04:02–tg mice, blood samples were extracted from the orbital sinus with a heparinized capillary. Alternatively, blood was drawn from immunized mice to assess Dsg3-specific IgG reactivity in the sera separated by centrifugation at 4,500 g at 4 °C for 10 min. The collected blood was diluted with 100 µL (ethylenedinitrilo)tetraacetic acid (EDTA)–heparin buffer (**2.8**) to prevent blood coagulation. Surface expression of human CD4, HLA-DR, and HLA-DQ, as well as lack of I-Aβ of leukocytes were determined by flow cytometry analysis. For this purpose, unlabeled anti-CD16/CD32 antibody (1:200) was added to 60 µL blood sample to impede unspecific antibody binding by saturation of Fc receptors, and incubated on ice for 10–15 min. Thereafter, fluorescently labeled antibodies were added to the sample in the ratio of either 1:25 (human CD4, HLA-DR, and HLA-DQ) or 1:5,000 (I-Aβ), and incubated on ice for 30 min in a dark environment. Blood cells were washed and RBC removed by twofold addition of ACK lysis buffer (**2.8**) for 5 min at RT in a dark environment (350 g, 4 °C, 5 min). Finally, cells were resuspended with 200 µL FACS buffer (**2.8**) for flow cytometry analysis.

3.2.1.2 Immunization of HLA-DR4–tg mice

To induce Dsg3-reactive T cell responses, 8–12 week old HLA-DRB1*04:02–tg mice were immunized by intraperitoneal (i.p.) injection of 20–40 µg recombinant human Dsg3 protein (**2.5.1**) or the Dsg3 peptides (**2.5.2**) in aluminum hydroxide (alum) on days 0, 14, and 28 (**Figure 3.3a**). Blood samples were taken (**3.2.1.1**) to assess the induction of anti-Dsg3 IgG by ELISA (**3.2.2.1**), immunoblotting (**3.2.2.2**), or immunofluorescence microscopy (**3.2.2.3**).
To induce locally restricted Dsg3-specific T cell responses, mice were injected subcutaneously (s.c.) into the hind foot paws with 20–40 µg Dsg3 peptides in the adjuvant TiterMax™ on day 0. Draining LN were harvested on day seven for ELISPOT analysis (**3.2.3.3**) of Th cell-related cytokine secretion upon *in vitro* restimulation with antigen (**Figure 3.3b**).

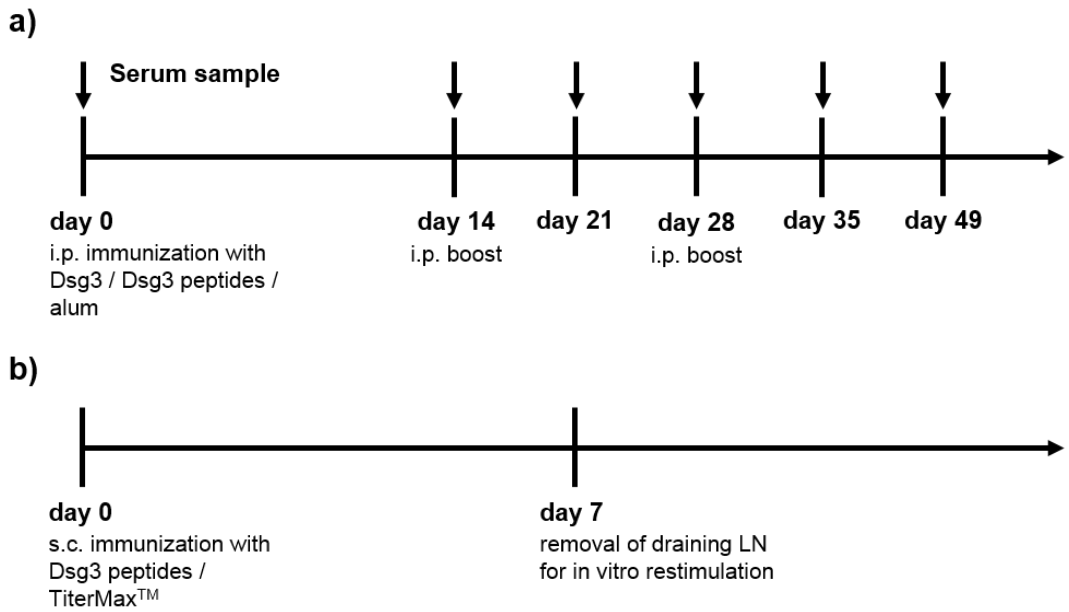

Figure 3.3: Immunization scheme of HLA-DR4–transgenic mice.
For induction of Dsg3-specific $CD4^+$ T cell responses, HLA-DRB1*04:02–tg mice were immunized by intraperitoneal (i.p.) injection of recombinant human Dsg3 protein or the Dsg3 peptides emulsified in aluminum hydroxide (alum) and serum samples were drawn according to the above illustrated scheme (**a**). To induce locally restricted Dsg3-specific T cell responses, mice were injected subcutaneously (s.c.) into the hind foot paws with the Dsg3 peptides in the adjuvant TiterMax™ and draining lymph nodes (LN) were harvested for *in vitro* restimulation according to the above illustrated scheme (**b**).

3.2.2 Serological diagnostics

3.2.2.1 Dsg3 ELISA

With the help of an ELISA the amount of Dsg3-specific IgG antibodies can be assessed in a semi-quantitative manner. The assay relies on binding of antibodies contained in patients' sera or culture supernatant to recombinant Dsg3 coated on a microtiter plate. Following washing off of unbound antibodies, the amount of Dsg3-reactive IgG can be determined by e.g. an horseradish peroxidase (HRP)-coupled detection antibody that catalyzes the reaction of the colorimetric substrate 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Hornbeck 2001).

Procedure:

The presence of circulating IgG antibodies against recombinant Dsg3 (**2.5.1**) in the sera of HLA-DRB1*04:02–tg mice i.p. immunized (**3.2.1.2**) with human Dsg3 or a set of five Dsg3 peptides (**2.5.2.1**) was detected by a commercially available ELISA kit (**2.10**) according to the manufacturer's manual with modification: Mouse sera were diluted in the ratio of 1:20 and an anti-mouse IgG HRP-coupled antibody (1:2,000) was used as secondary antibody.

3.2.2.2 Immunoblotting

IgG cross-reactivity against mouse Dsg3 of sera from HLA-DRB1*04:02–tg mice i.p. immunized (**3.2.1.2**) with human Dsg3 (both **2.5.1**) was analyzed by immunoblotting. This technique is applied to identify specific protein sequences separated by electrophoresis and transferred to a suitable

membrane by polyclonal or mAb. The primary antibody is specific for the protein of interest, whereas the secondary antibody is IgG-reactive and coupled to HRP or alkaline phosphatase enzyme, and upon addition of chromogenic or luminescent substrates, the antigen–antibody complex is visualized (Gallagher et al. 2001).

Procedure:

Protein samples are solubilized with sample buffer (**2.8**) for 5 min at 95 °C and separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) for 30 min at 100 V. Thereafter, the antigens are electrophoretically transferred in a semidry transfer apparatus to a nitrocellulose membrane for 60 min at 150 mA. The gel is thereby positioned on the membrane, which is enclosed by two stacks of four sheets of filter paper soaked with transfer buffer (**2.8**). This way, the proteins bound to the surface of the membrane provide access for the mouse serum IgG antibodies. Remaining binding sites are blocked by immersion of the membrane in blocking buffer (**2.8**) for 2 hours at RT. Mouse sera are diluted in the ratio of 1:200 in the same buffer and incubated for 1 hour at RT. Because proteins are synthesized attached to an E tag, application of an anti-E tag antibody (1:2,000) serves as a positive control. Following threefold washing with washing buffer (**2.8**) for 10 min, the membrane is incubated with the secondary anti-IgG antibody coupled to HRP diluted in the ratio of 1:2,000 in blocking buffer for 1 hour at RT. After repeated threefold washing with washing buffer for 10 min, antigen–antibody complexes are visualized by applying 2 mL of the two ECL chemiluminescent substrates (1:1) on the membrane for 5 min at RT. For record, the membrane is instantly photographed.

3.2.2.3 Immunofluorescence

Proteins can be localized within a histological sample by the technique of immunofluorescence. IIF microscopy relies on proper fixation of cells to retain cellular distribution of antigen and to preserve cellular morphology. Application of permeabilizing reagents, such as saponin, provides access for the primary antibody to the epitope of the protein of interest. The bound primary antibody is labeled by incubation with a fluorescently tagged secondary antibody targeting the primary antibody host species. Finally, the specimen can be viewed under a fluorescence microscope (Donaldson 2001).

DIF is utilized to detect antibodies instantaneously bound to tissues *in vivo* and thus requires exclusive addition of a fluorescently labeled secondary antibody. In order to assess whether IgG induced in HLA-DRB1*04:02–tg mice by i.p. immunization (**3.2.1.2a**) with human Dsg3 cross-reacts with murine Dsg3 (both **2.5.1**) under *in vivo* conditions, DIF was performed on mouse tissue specimens. For this purpose, buccal and palatinal mucosa of immunized mice were embedded in Tissue-Tek® O.C.T.™ Compound, frozen at -80 °C, and cut into sections of 3–4 µm. After blocking with blocking buffer (**2.8**), samples were stained with a rabbit anti-mouse IgG FITC-labeled antibody diluted in the ratio of 1:250 in dilution buffer (**2.8**) for 30 min at RT in a dark environment. To study whether i.p. immunization of HLA-DRB1*04:02–tg mice with human Dsg3 protein (**2.5.1**) or a set of five Dsg3 peptides (**2.5.2.1**) induces IgG against native human Dsg3, IIF microscopy with mouse serum samples (1:25) was performed on monkey esophagus (**2.10**) according to the

manufacturer's protocol with modification: As secondary antibody, a rabbit anti-mouse IgG FITC-labeled antibody (1:500) was used instead.

3.2.3 Cellular diagnostics

3.2.3.1 Generation of bone marrow-derived DC

In order to study APC-induced activation of antigen-specific Th cells, mDC were generated from hematopoietic precursors in mouse BM using granulocyte-macrophage colony-stimulating factor (GM-CSF) (Lutz et al. 1999). For this purpose, femurs and tibiae of 4–12 week old HLA-DRB1*04:02–tg mice were separated and purified from the surrounding muscle tissue by rubbing them with tissues. Thereafter, joints were removed at both ends of the bone with scissors so that the BM could be thoroughly flushed out using a 10 mL syringe and needle (26 G) filled with $RPMI^{++}$ (**2.8**). Following centrifugation at 300 g at 4 °C for 5 min, RBC were destroyed by adding 4 mL ACK lysis buffer (**2.8**) for 5 min at RT and upon neutralization with 8 mL $RPMI^{++}$, bone particles were removed using a cell strainer (40 µm). After repeated centrifugation (300 g, 4 °C, 5 min), cells were resuspended in $RPMI^{++}$ to determine the cell number with the help of a Neubauer counting chamber (**3.1.3.2**). Cells were then centrifuged and seeded at a density of $3–6 \cdot 10^6$ cells per petri dish in 10 mL DC differentiation medium (**2.8**) for seven days. On day three and six of culture, 10 mL fresh DC differentiation medium was added to the BM cells developing into DC. Upon complete differentiation on day seven, bone marrow-derived DC (BMDC) were matured with 0.1 µg/mL LPS, pulsed with 10 µg/mL antigen (**2.5**), and incubated for 18 hours at 37 °C and 5% CO_2.

Murine GM-CSF (**2.6**) was obtained from the GM-CSF–producing cell line X6310 (**2.4**) that was grown in $RPMI^{++}$ supplemented with 10% FCS and 1 mg/mL G418 solution for two weeks. Following additional two weeks of incubation in $RPMI^{++}$ supplemented with 10% FCS without addition of fresh medium, the supernatant was collected, sterile filtered, and stored at 4 °C.

3.2.3.2 Coculture of antigen-pulsed BMDC with antigen-specific $CD4^+$ T cells

Preparation of splenocytes

APC-mediated activation of Th cells was studied in the HLA-DRB1*04:02–tg mouse model by the following approach: BMDC generated *in vitro* using GM-CSF were matured with LPS and pulsed with antigen (**3.2.3.1**) to be cultured together with antigen-specific $CD4^+$ T cells induced by repetitive i.p. immunization (**3.2.1.2a**) of mice with recombinant human Dsg3 protein (**2.5.1**). For this purpose, the spleen of immunized mice was removed and splenocytes were liberated from the capsule by placing it between two microscope slides that were moved carefully against each other. Splenocytes were rinsed off the slides, resuspended in PBS and capsule constituents were removed using a cell strainer (40 µm). After centrifugation (350 g, 4 °C, 10 min), RBC were destroyed upon incubation in ACK lysis buffer (**2.8**) for 5 min at RT, and cells were again centrifuged (350 g, 4 °C, 10 min). Thereafter, cells were resuspended in $RPMI^{++}$ (**2.8**) and prepared for further purification of $CD4^+$ T cells by magnetic-activated cell sorting (MACS).

Isolation of murine $CD4^+$ T cells using magnetic cell separation

The MACS technique is based on covalent binding of paramagnetic nano particles (micro beads) to antibodies that are for instance cell type-specific so that the targeted cells are held back upon application of a magnetic field. It is an efficient method to purify distinct cell populations under sterile conditions.

In order to isolate $CD4^+$ T cells from the splenocyte suspension, the MACS mouse $CD4^+$ T cell positive selection kit (**2.10**) was utilized according to the manufacturer's protocol. Application of magnetically labeled CD4-specific antibodies resulted in the immobilization of $CD4^+$ cells in the magnetic field of the MACS separator. This way cells devoid of CD4 were collected in the flow through of the column and discarded, whereas the $CD4^+$ cells attached to the column were used for the coculture experiment. Purity of the isolated $CD4^+$ T cells was >95% as confirmed by FACS analysis.

CFSE proliferation assay

Usage of carboxyfluorescein diacetate succinimidyl ester (CFSE) is a widely applied method to measure lymphocyte proliferation *in vitro* by flow cytometry. The amino-reactive succinimidyl side chain of the dye allows CFSE to covalently couple to intracellular proteins. Upon cell division, the fluorescence intensity is halved in the two daughter cells, visualized as individual generation peaks by histogram analysis.

$CD4^+$ T cells isolated by MACS were stained with CFSE according to the protocol described by Quah et al. (Quah and Parish 2012). Thereafter, $2 \cdot 10^5$ CFSE-labeled $CD4^+$ T cells were cultured together with titrated numbers of BMDC ($1–5 \cdot 10^4$) in serum-free X-VIVO™ medium in 96-well plate round-bottom microtiter plates and incubated for six days at 37 °C and 5% CO_2. Cell division capacity was assessed using an anti-CD3 antibody as positive control. At the end of the culture, BMDC-induced proliferation of $CD4^+$ T cells was analyzed by flow cytometry.

3.2.3.3 ELISPOT assay for detection of cytokine-secreting T cells

Compared to conventional ELISA, the ELISPOT assay provides information about a single cytokine-secreting cell, but does not require the output of many cells to achieve a measurable protein concentration in culture supernatant. Therefore, cytokine-specific high-affinity antibodies are at first attached to a solid matrix. To prevent nonspecific interactions, the plates are blocked, and the respective cells are incubated in the antibody-coated wells upon addition of the antigen to stimulate the release of cytokines. Throughout a distinct period of time (2–24 hours), the secreted proteins bind to the antibodies located below the producer cells. After removing the cells by extensive washing, the immobilized proteins are recognized by a secondary biotinylated antibody targeting a further epitope of the cytokine. Addition of first streptavidin-conjugated enzyme and second colorimetric substrate yields dark precipitates ("spots") that reflect the location of the cytokine-releasing cell. Hence, the number of spots correlates with the frequency of cytokine-producing cells and can be quantified visually or electronically (Klinman 2008).

Procedure:

ELISPOT assays were performed with cells isolated from popliteal and inguinal LN of mice s.c. injected (**3.2.1.2b**) with the Dsg3 peptides (**2.5.2**) emulsified in adjuvant into the hind food pads for seven days. In order to detect the frequency of antigen-specific Th1 and Th2 cells, LN cells were assayed for release of their signature cytokines IFN-γ and IL-4, respectively. Firstly, the capture antibody (100 µL/well) is diluted in the ratio of 1:200 with sterile PBS and incubated overnight at 4 °C. After discarding the capture antibody, the membranes are washed and then blocked with blocking buffer (**2.8**) for 2 hours at RT. Prior to adding the freshly isolated LN cells (500,000 cells in 100 µL/well), the blocking solution is discarded and the stimulation medium (100 µL/well) consisting of double concentrated (20 µg/mL) antigen solution added. Usage of 1.5% mouse serum supplemented in RPMI^{++} (**2.8**) thereby prevents xenogenic immune reactions of LN cells to FCS proteins. The medium alone serves as a negative control, whereas cell viability is controlled by application of 1% of the mitogen PHA. Cells are added in triplicates and incubated for 24 hours at 37 °C and 5% CO_2. On the next day, cells are aspirated carefully and plates are washed twice with double-distilled water (ddH_2O) (**2.8**) (200 µL/well, 3–5 min) and three times with washing buffer (**2.8**) (200 µL/well). The biotinylated detection antibody is then diluted in the ratio of 1:250 with dilution buffer (**2.8**) (100 µL/well) and incubated for 2 hours at RT. After discarding the detection antibody solution, plates are washed with washing buffer (200 µL/well, 1–2 min) and the enzyme conjugate streptavidin–HRP is diluted in the ratio of 1:100 in dilution buffer (100 µL/well) and incubated for 1 hour at RT. To remove unbound conjugate, plates are washed four times with washing buffer (200 µL/well, 1–2 min) and twice with PBS (200 µL/well). For development of the spots the substrate solution (**2.8**) is added (100 µL/well) and incubated for 60 min. The enzymatic reaction is abolished by washing with ddH_2O (200 µL) twice and plates are subsequently dried overnight at RT in a dark environment. The number of spots is quantified using automatic analyzing software and the frequency of antigen-specific cells is determined by the median of the respective triplicate samples. Finally, the number of spots of the unstimulated controls is deducted from the spot numbers of the samples stimulated with antigen.

3.2.3.4 Statistics

Statistical evaluation of the data obtained from ELISPOT analysis was performed using GraphPad Prism© software (**2.13**). For comparison of a variable between two groups of a small sample size that are not related to each other, a nonparametric two-tailed test for comparison of unpaired sample sizes (Mann-Whitney-U) was selected. Differences were considered significant for $p < 0.05$.

4 Results

4.1 Human study

4.1.1 Patient characteristics

In the first part of this thesis, cellular and humoral immune responses, including APC, $CD4^+$ T cells, plasma cytokines, and the auto-ab response were characterized in pemphigus patients and controls. The cohort of pemphigus patients participating in this cross-sectional study was comprised of 34 test subjects as described in **3.1.1**, whereas data was mainly derived from a group of twelve selected patients as listed in **Table 3.1**. This clinically well characterized group was composed of nine PV patients, the most common subtype of pemphigus with auto-ab reactive against Dsg3 and occasionally Dsg1, as well as three PF patients with exclusively Dsg1-responsive auto-ab. Since the pathoimmunological mechanisms of these two subtypes are believed to not critically differ from each other, results and conclusions presented in this study relate to the pathogenesis of pemphigus in general. The selected pemphigus patients fulfilled the inclusion criteria of a physiological blood analysis concerning quantities of leukocytes, monocytes, and lymphocytes as established by the Department of Laboratory Medicine and Pathobiochemistry, Molecular Diagnostics, Philipps-University, Marburg. In addition, the daily intake of glucocorticoids for medical treatment of pemphigus patients was determined to not exceed 10 mg since systemic immunosuppressive therapy with Pred significantly influenced the composition of blood leukocyte populations in a dose-dependent manner as clearly illustrated in **Figure 4.13**. Consequently, the number of test subjects chosen for profound investigations needed to be limited. In pemphigus patients, immune responses, such as the polarization of $CD4^+$ T cell subpopulations (Rizzo et al. 2005) or IgG subclasses of auto-ab (Zhu et al. 2012) have been shown to be specific for the individual state of disease. Therefore, pemphigus patients were categorized according to either active or remittent stage of disease as defined by Murrell et al. 2008. Active test subjects consisted of patients with acute exacerbation of disease, as well as chronic appearance of lesions that did not heal spontaneously within one week. Remittent test subjects were classified according to partial remission (PR) off therapy, in which transient new lesions healed spontaneously within one week in the absence of systemic therapy for at least two months, and complete remission (CR) off therapy, in which new or established lesions where absent without systemic therapy for at least two months. The study cohort embraced nine active pemphigus patients, as well as two completely remittent and one partially remittent patient off therapy. The IgG antibody titers against Dsg1 and Dsg3 detected in the sera of pemphigus patients were elevated to a varying extent in patients with active disease, whilst remittent patients did not display auto-ab production beyond background level. As an autoimmune disorder primarily affecting the elderly, pemphigus patients averaged 54 years. Furthermore, the male–female ratio amounted four to eight.

In order to specify the findings obtained in this study for pemphigus, 31 patients with MG that clinically presents with fluctuating muscle weakness were selected as control test subjects for a further unrelated auto-ab–mediated disorder that specifically targets tissues or organs. Analogous

to pemphigus patients, twelve MG patients following the inclusion criteria of physiological circulating leukocyte, monocyte, and lymphocyte counts, as well as daily systemic therapy with glucocorticoids below 10 mg were chosen for comprehensive analysis as presented in **Table 3.2**. In contrast to pemphigus patients who were grouped according to active and remittent stage of disease, MG patients were afflicted with very mild to moderate disease or did not exhibit clinical symptoms at all. The MG control cohort comprised three asymptomatic patients, four patients suffering from mild disease, whereas two and three patients presented with mild to moderate disease, respectively. Serum samples from MG patients displayed IgG reactivity against nicotinic AChR located in the neuromuscular junctions of somatic muscles, as well as titin that is found in striated muscle tissue. Unlike pemphigus patients, IgG auto-ab titers of the cohort of MG patients did not relate to disease activity. MG patients averaged 57 years, which was comparable to pemphigus patients and the number of males and females was evenly balanced.

The third study group was composed of 32 HC that were matched according to gender and age of pemphigus patients and did not display any signs of autoimmune inflammation. Compared to pemphigus patients, average age of the twelve selected HC was slightly reduced and amounted 50 years.

4.1.2 Phenotypic characterization of APC in whole blood of pemphigus patients

In the pathogenesis of pemphigus, the role of APC, including populations of monocytes and DC has not yet been elucidated. As potent inducers of $CD4^+$ T cell activation, DC have been shown to be critically involved in the pathogenesis of several autoimmune disorders, such as SLE, TID, and psoriasis (Ganguly et al. 2013). Similarly, alterations in the distribution of distinct monocyte subsets have been mostly implicated in RA and IBD (Wong et al. 2012). Since the major autoantigens of pemphigus, the desmosomal cadherins Dsg3 and Dsg1 are expressed by keratinocytes in the suprabasilar layer of the epidermis, aberrant APC function is suspected to mainly reflect in the intricate network of the skin DC subsets. For reasons of practical feasibility however, both qualitative and quantitative analysis of APC populations was confined to those found in peripheral blood being yet interconnected with their skin counterparts. Assessment of cell type-specific surface markers, as well as markers for activation and migration of APC was performed using flow cytometry analysis according to the procedure described in **3.1.3.5**.

Human DC comprise two major phenotypically and functionally different classes, the mDC and pDC that may represent the precursors of the DC localized to tissues and secondary lymphoid organs (Collin, McGovern, and Haniffa 2013). To date, four different populations of human DC are generally acknowledged to be present in the blood circulation, to which the $CD1c^+$ ($BDCA\text{-}1^+$) DC (mDC type 1), $CD141^+$ ($BDCA\text{-}3^+$) DC (mDC type 2), pDC, and $CD16^+$ DC belong (Kim and Diamond 2015). Opposed to $CD1c^+$ DC and $CD141^+$ DC, $CD16^+$ DC are lacking in tissues and resemble monocytes (Randolph et al. 2002). In this study, $CD1c^+$ DC being the more frequent mDC subtype and $CD141^+$ DC were identified together as mDC based on the simplified pattern of high expression of the integrin CD11c and absence of CD14 ($CD14^-CD11c^{++}$) (Kim and Diamond 2015) as illustrated in **Figure 4.2a**. With regard to morphological characteristics,

analysis of FSC and SSC determining cell size and granularity, respectively, confirmed mDC being localized between the populations of lymphocytes and monocytes as demonstrated in **Figure 4.2b**.

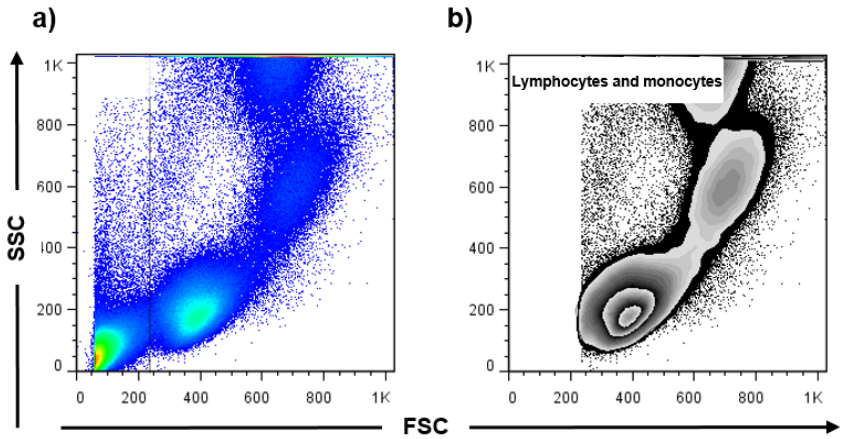

Figure 4.1: Preselected cell populations for phenotypic analysis of antigen-presenting cells.
In order to phenotypically characterize circulating antigen-presenting cell (APC) populations by flow cytometry, whole blood of patients was prepared and stained with fluorescently labeled antibodies. Upon analysis, viable leukocytes were at first segregated from cell debris, platelets, and remaining erythrocytes based on cellular morphology as determined by forward scatter (FSC) and side scatter (SSC) (**a**). For quantitative assessment of APC subsets, granulocytes were excluded from the selected populations of primarily smaller lymphocytes and large monocytes, and 200,000 single events were detected (**b**). Leukocyte, lymphocyte, and monocyte counts were confirmed to be within the specified physiological range as determined by blood analysis.

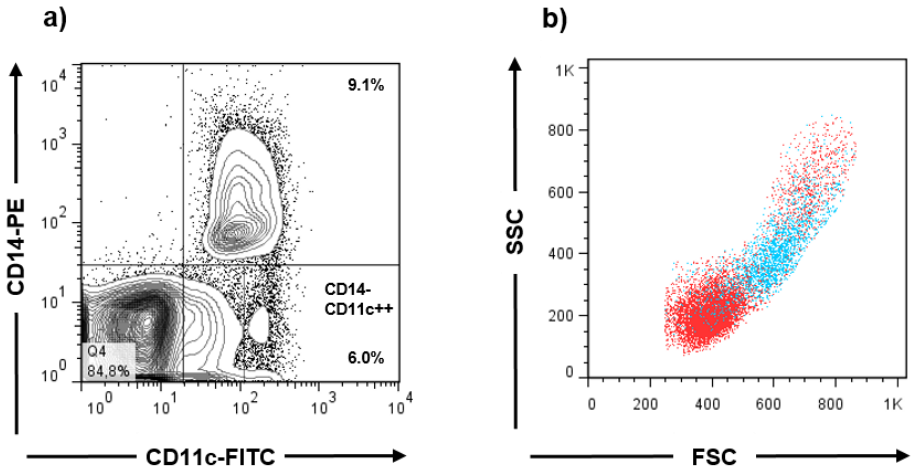

Figure 4.2: Gating strategy of myeloid dendritic cells ($CD14^{-}CD11c^{++}$).
Upon incubation with both an anti-human CD14 antibody and anti-human CD11c antibody labeled with PE and FITC, respectively, the population of myeloid dendritic cells (mDC) clearly segregated as cells negative for CD14 and high in expression of CD11c. Monocytes were separated by expression of both CD14 and CD11c, and cells lacking CD14, but displaying low levels of CD11c represented T and B lymphocytes, as well as NK cells (**a**). As shown by forward scatter (FSC) and side scatter (SSC) analysis of cellular size and granularity, respectively, mDC (blue) typically scattered between lymphocyte and monocyte populations (both red) (**b**). Separation of cell populations was performed using the appropriate isotype controls.

In addition, the subset of pDC was identified by expression of the cell type-specific marker CD303 (BDCA-2), whereas it represents the only circulating DC subset that is typically absent of CD11c (CD11c$^-$CD303$^+$) as presented in **Figure 4.3a**. The recently identified subset of slanDC within the heterogeneous population of blood CD16$^+$CD14$^-$ inflammatory monocytes was shown to be present in the inflammatory infiltrate in psoriasis, RA, and IBD (Schakel et al. 2002; Schakel 2009). As visualized in **Figure 4.4**, the population of slanDC was determined by characteristic expression of CD16 (FcγR-III), HLA-DR, and the costimulatory molecule CD86 (CD14$^-$CD16$^+$HLA-DR$^+$/CD86$^+$). Analogous to all circulating DC subsets, they are negative for Lin markers, including CD14 (Kim and Diamond 2015). In contrast to blood DC populations, monocyte-derived DC positive for CD14 are observed only under inflammatory conditions and mainly locate to cutaneous tissues (Kim and Diamond 2015).

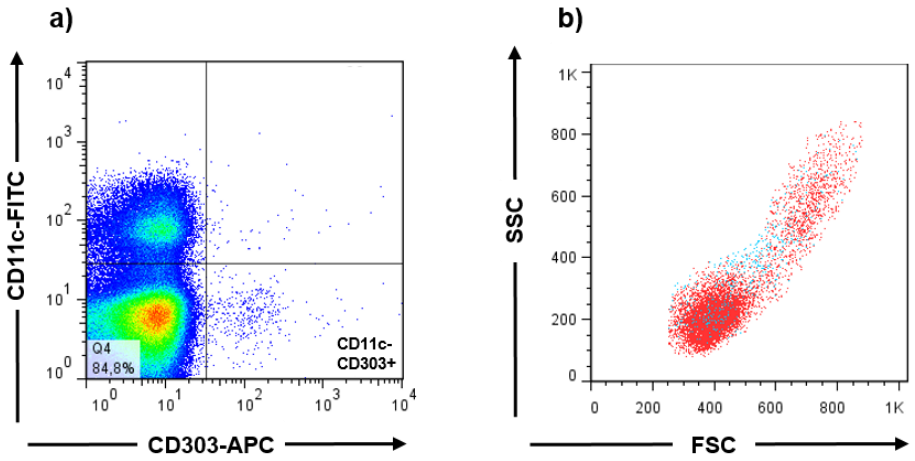

Figure 4.3: Gating strategy of plasmacytoid dendritic cells (CD11c$^-$CD303$^+$).
Addition of both an anti-human CD11c antibody and anti-human CD303 antibody conjugated to FITC and APC, respectively, yielded a clearly distinguished population of plasmacytoid dendritic cells (pDC) devoid of CD11c, but high in expression of CD303. Neutrophils also exhibiting surface expression of CD303 were excluded from analysis as shown in **Figure 4.1**, as well as CD303$^+$ macrophages separating by additional expression of CD11c (**a**). Forward scatter (FSC) and side scatter (SSC) analysis of pDC (blue) confirmed their morphological localization adjacent to subsets of lymphocytes and monocytes (both red) (**b**). Separation of cell populations was performed using the appropriate isotype controls.

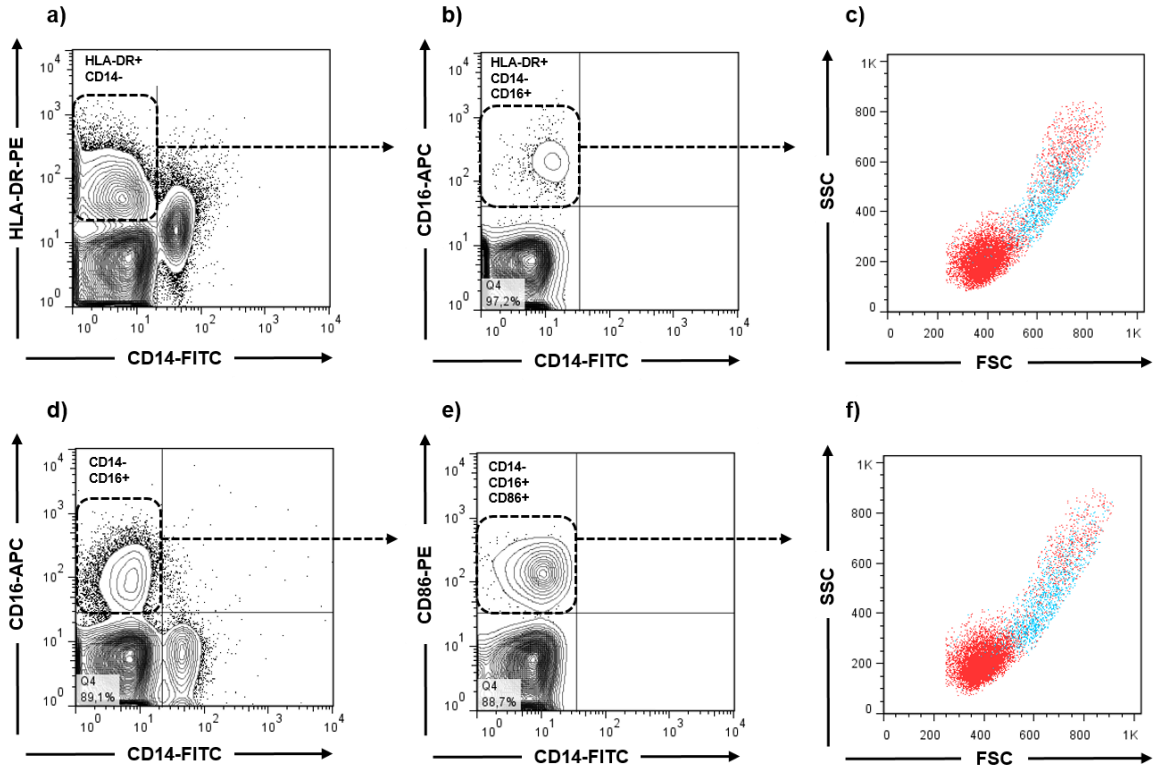

Figure 4.4: Gating strategy of 6-sulfo LacNAc dendritic cells (CD14$^-$CD16$^+$HLA-DR$^+$/CD86$^+$).
Characterization of 6-sulfo LacNAc dendritic cells (slanDC) by independent staining of cells lacking surface expression of CD14 and expressing Fcγ receptor-III (FcγR-III, CD16) with either HLA-DR (**a** and **b**) or CD86. NK cells being also positive for CD16 and devoid of CD14 were segregated by lack of CD86 expression (**d** and **e**). Forward scatter (FSC) and side scatter (SSC) analysis assigned slanDC (blue) morphology between subsets of lymphocytes and monocytes (both red) (**c** and **f**). Separation of cell populations was performed using the appropriate isotype controls.

Besides circulating DC populations, peripheral blood of pemphigus, MG patients, and HC was further analyzed for alterations in the monocyte compartment. Monocytes represent a leukocyte population with augmented cell size and granularity. Besides expression of CD14 being part of the LPS multireceptor complex (Brubaker et al. 2015b), monocytes are positive for the myeloid phenotype marker CD11c, a feature shared with mDC (Auffray, Sieweke, and Geissmann 2009) as presented in **Figure 4.5**. Human blood monocytes are heterogeneous and conventionally comprise two major subsets segregated by differential expression of CD16, the designated classical CD16$^-$ and CD16$^+$ monocytes (Wong et al. 2012) as visualized in **Figure 4.6c**. Classical monocytes account for the majority, i.e. 80% to 90% of circulating monocytes (Auffray, Sieweke, and Geissmann 2009). CD16$^+$ monocytes have been shown to produce TNF-α upon stimulation with LPS and they are found in larger numbers in the blood of patients with acute inflammation and infectious diseases (Auffray, Sieweke, and Geissmann 2009). According to a recent nomenclature, CD16$^+$ monocytes can be further subdivided into two functionally and phenotypically different subsets on the basis of relative expression of CD14 (Wong et al. 2012) as demonstrated in **Figure 4.6c**.

In order to study the migratory behavior of APC, expression of CCR2 was additionally determined on both mDC and CD14$^+$ monocytes based on geometrical mean values of the anti-CCR2

antibody signal intensity as presented in **Figure 4.7**. Classical monocytes and mDC are subject to the axis of CCL2-CCR2 that enables these cells to exit the blood stream and access peripheral nonlymphoid tissues, such as the skin in the state of inflammation (Auffray, Sieweke, and Geissmann 2009).

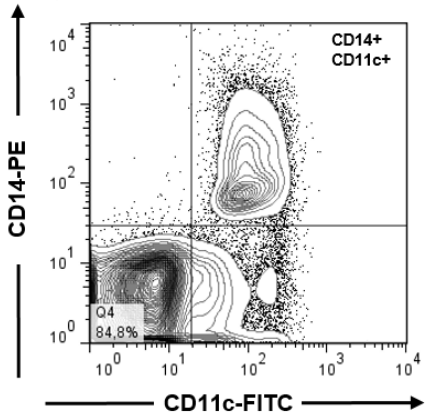

Figure 4.5: Gating strategy of monocytes ($CD14^+CD11c^+$).
Binding of both an anti-human CD14 PE-coupled antibody and anti-human CD11c FITC-coupled antibody segregated circulating monocytes from DC subsets positive for CD11c, but devoid of CD14. Separation of cell subsets was done using the appropriate isotype controls.

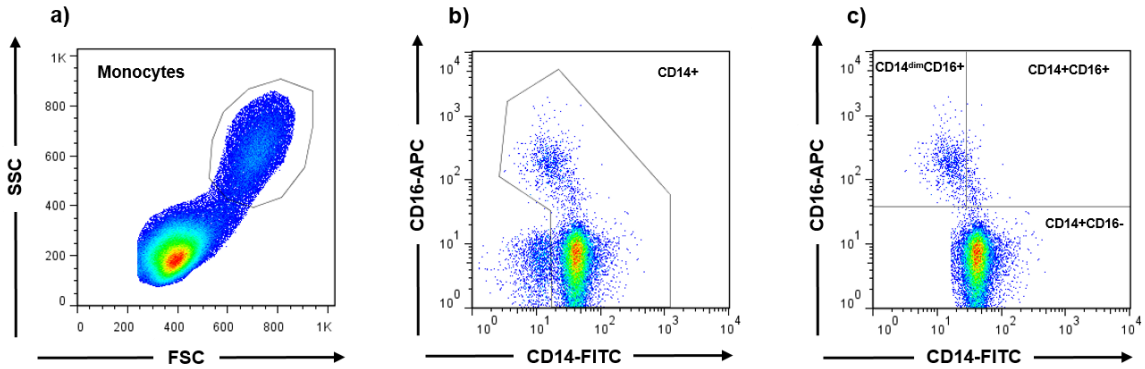

Figure 4.6: Gating strategy of classical ($CD14^+CD16^-$) and $CD14^+CD16^+$ monocytes.
Monocytes were selected based on forward scatter (FSC) and side scatter (SSC) analysis since they represent a morphologically distinct population with increased cell size and granularity compared to lymphocytes (**a**). Upon addition of an anti-human CD14 FITC-labeled antibody and an anti-human CD16 APC-labeled antibody, a subset positive for CD14 was segregated with differential expression of CD16. Cells negative for both CD14 and CD16 may represent myeloid dendritic cells (**b**). Analysis of cells with CD14 expression yielded two populations that comprise monocytes devoid of CD16 and $CD16^+$ monocytes partly displaying high expression of CD16. $CD16^+$ monocytes were further categorized according to diminished and high expression of CD14, referred to as nonclassical ($CD14^{dim}CD16^+$) and intermediate $CD14^+CD16^+$ monocytes (**c**). Separation of cell populations was performed using the appropriate isotype controls.

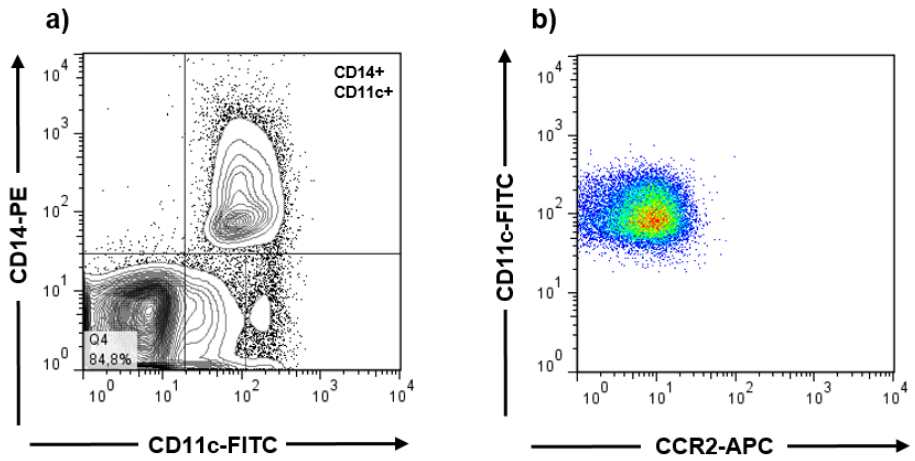

Figure 4.7: CCR2 expression of monocytes.
In order to quantify the expression level of C-C chemokine receptor 2 (CCR2) on circulating monocytes in patients' whole blood using flow cytometry, leukocytes were at first preselected as illustrated in **Figure 4.1**. Incubation with both an anti-human CD14 PE-coupled antibody and anti-human CD11c FITC-coupled antibody identified $CD14^+CD11c^+$ blood monocytes (**a**). Monocytes were additionally stained with an anti-human CCR2 antibody coupled to APC. Cell surface expression of CCR2 was determined by geometrical mean values of the antibody signal intensity (**b**). Gates were set according to the appropriate isotype controls.

Taken together, in order to assess whether an ongoing autoimmune response in active pemphigus shows in alterations within the APC compartment, we both phenotypically and functionally characterized populations of DC and monocytes in peripheral blood of both pemphigus patients and control groups. At first, we determined the frequencies of the main subsets of circulating DC, including mDC and pDC, as well as slanDC. In addition, we assessed the number of peripheral classical and $CD16^+$ monocytes, of which the latter was further subdivided into two subsets. Moreover, we analyzed expression of the activation markers HLA-DR and CD86 of slanDC, as well as the migration marker CCR2 of mDC and $CD14^+$ monocytes.

Compared to HC, the population of circulating mDC was slightly decreased (p= 0.0875) in blood of active pemphigus patients, whereas in MG patients, increasing (p= 0.2113) frequencies of mDC could be observed (**Figure 4.8a**). Similarly, active pemphigus patients displayed slightly reduced (p= 0.1140) and MG patients slightly elevated (p= 0.2987) populations of pDC (**Figure 4.8b**). The subsets of blood slanDC however did not vary (p= 0.9445, $CD14^-CD16^+HLA\text{-}DR^+$ and p= 0.8831, $CD14^-CD16^+CD86^+$) between active pemphigus patients and HC, but were again significantly augmented (p= 0.0083) for $CD14^-CD16^+CD86^+$ cells and slightly augmented (p= 0.2751) for $CD14^-CD16^+HLA\text{-}DR^+$ cells in MG patients (**Figure 4.9**). As mDC frequencies significantly differed (p= 0.0180) between active pemphigus and MG patients, a disease-related alteration in the mDC population of pemphigus patients can be suggested (**Figure 4.8a**). A similar effect of reduced frequencies of both pDC (p= 0.0075) (**Figure 4.8b**) and slanDC (p= 0.3541, $CD14^-CD16^+HLA\text{-}DR^+$ and p= 0.0239, $CD14^-CD16^+CD86^+$) (**Figure 4.9**) was also observed in active pemphigus patients compared to MG controls.

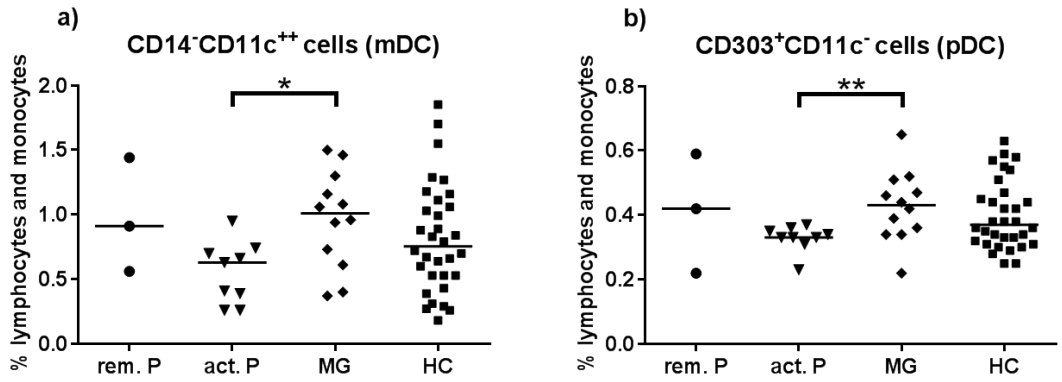

Figure 4.8: Reduced population of mDC and pDC in active pemphigus patients compared to myasthenia gravis controls.

Active pemphigus patients (act. P, *n*= 9) displayed a slightly reduced (*p*= 0.0875) subset of blood myeloid dendritic cells (mDC), whilst myasthenia gravis patients (MG, *n*= 12) exhibited increased frequencies (*p*= 0.2113) of mDC compared to healthy controls (HC, *n*= 32). In addition, mDC significantly differed (*p*= 0.0180) between act. P and MG patients, pointing to a disease-related alteration in the mDC population of pemphigus patients (**a**). Similarly, plasmacytoid DC (pDC) were decreased (*p*= 0.1140) in act. P patients compared to HC, but slightly elevated (*p*= 0.2987) in MG patients. Compared to MG controls, act. P patients displayed a significantly reduced (*p*= 0.0075) population of pDC (**b**). Rem. P, remittent pemphigus patients. Statistical analysis was carried out using Mann-Whitney U test with *p* values indicated as *$p < 0.05$ and **$p < 0.01$.

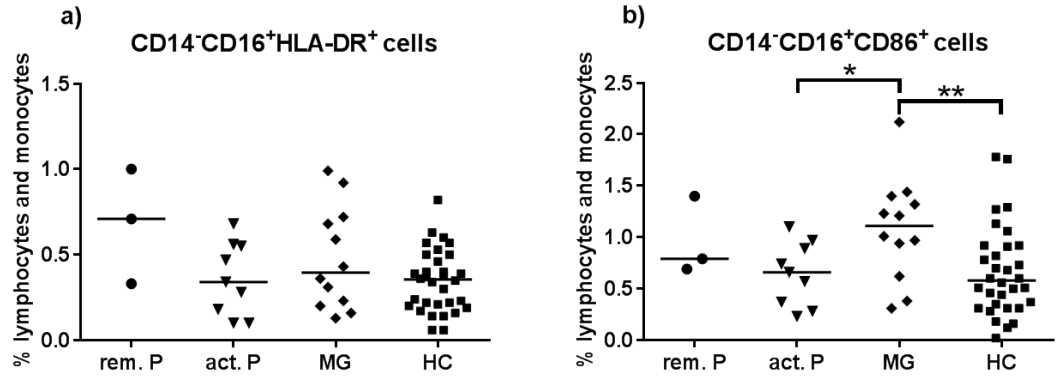

Figure 4.9: Reduced population of CD14$^-$CD16$^+$CD86$^+$ slanDC in active pemphigus patients compared to myasthenia gravis controls.

The subset of circulating 6-sulfo LacNAc dendritic cells (slanDC) was detected by flow cytometry analysis by a combination of two blood samples. Between active pemphigus patients (act. P, *n*= 9) and healthy controls (HC, *n*= 32), the population sizes of CD14$^-$CD16$^+$HLA-DR$^+$ cells (*p*= 0.9445, **a**) and CD14$^-$CD16$^+$CD86$^+$ cells (*p*= 0.8831, **b**) did not differ, yet were significantly elevated (*p*= 0.0083, **b**) for CD14$^-$CD16$^+$CD86$^+$ cells and slightly elevated (*p*= 0.2751, **a**) for CD14$^-$CD16$^+$HLA-DR$^+$ cells in myasthenia gravis patients (MG, *n*= 12). Moreover, act. P patients exhibited reduced frequencies of CD14$^-$CD16$^+$HLA-DR$^+$ cells (*p*= 0.3541, **a**) and CD14$^-$CD16$^+$CD86$^+$ cells (*p*= 0.0239, **b**) compared to MG controls. Rem. P, remittent pemphigus patients. Statistical analysis was carried out using Mann-Whitney U test with *p* values indicated as *$p < 0.05$ and **$p < 0.01$.

In active pemphigus patients, decreased population sizes of circulating mDC could be a sign for enhanced migration from the blood stream possibly into the inflamed skin, for which reason we additionally assessed expression of the migratory receptor CCR2 of mDC. Indeed, compared to HC, we could detect a significantly upregulated (*p*= 0.0239) expression of CCR2 on mDC in acute

pemphigus patients (**Figure 4.11a**). Because of the misguided excessive immune responses in pemphigus, we also expected an upregulation of the activation markers HLA-DR and CD86 of slanDC. However, increased expression of neither HLA-DR nor CD86 as a sign for enhanced DC activation could be detected in active pemphigus patients compared to HC (**Figure 4.12**).

Apart from that, concerning the monocyte compartment, we could observe increased frequencies of $CD16^+$ monocytes in active pemphigus (p= 0.0399), as well as MG patients (p= 0.0087) compared to HC (**Figure 4.10a**). Therefore, these findings could rather reflect an effect unrelated to pemphigus, but characteristic for a general state of inflammation. Furthermore, the recently identified subset of nonclassical monocytes within the population of $CD16^+$ monocytes was elevated in active pemphigus patients with significance close to the threshold (p= 0.0596) (**Figure 4.10b**). Moreover and analogous to mDC, we additionally tested for altered CCR2 expression of $CD14^+$ monocytes to examine their migratory behavior. Similarly, $CD14^+$ monocytes of both active pemphigus (p= 0.0166) and MG patients (p= 0.0083) displayed a significantly upregulated expression of CCR2 compared to HC (**Figure 4.11b**).

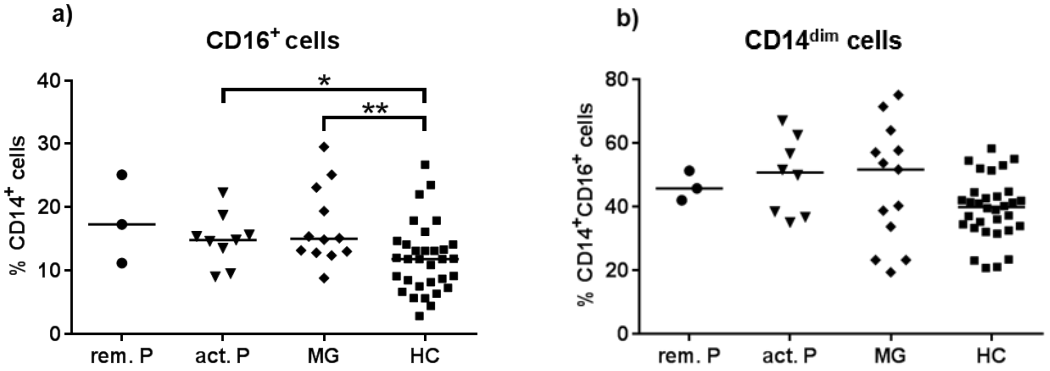

Figure 4.10: Elevated subset of $CD14^+CD16^+$ monocytes in active pemphigus patients compared to healthy controls.

Both active pemphigus (act. P, n= 9; p= 0.0399) and myasthenia gravis patients (MG, n= 12; p= 0.0087) showed significantly increased frequencies of $CD16^+$ monocytes compared to healthy controls (HC, n= 32) (**a**). Concerning the nonclassical subtype within $CD16^+$ monocytes, act. P patients displayed elevated frequencies (p= 0.0596) compared to HC (**b**). Rem. P, remittent pemphigus patients. Statistical analysis was carried out using Mann-Whitney U test with p values indicated as *p< 0.05 and **p< 0.01.

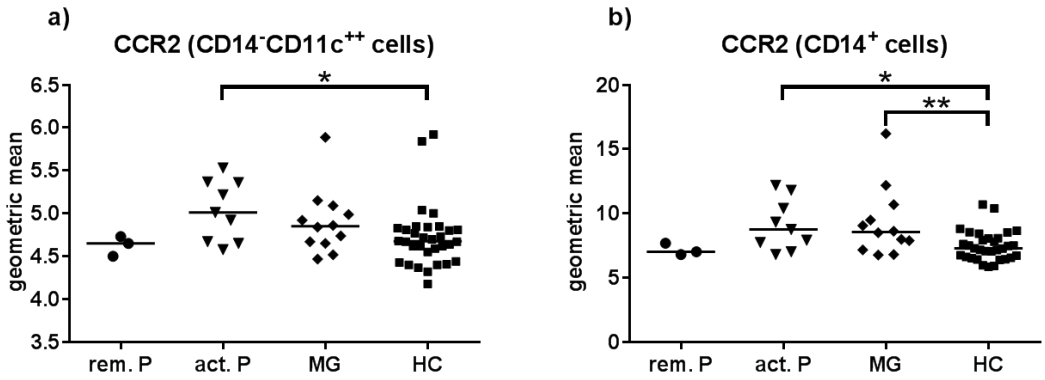

Figure 4.11: Augmented expression of CCR2 on myeloid dendritic cells and CD14$^+$ monocytes in active pemphigus patients compared to healthy controls.
Compared to healthy controls (HC, *n*= 32), C-C chemokine receptor 2 (CCR2) expression of myeloid dendritic cells (mDC) was significantly (*p*= 0.0239) elevated in active pemphigus (act. P, *n*= 9) and slightly elevated (*p*= 0.0526) in myasthenia gravis patients (MG, *n*= 12) (**a**). Moreover, CCR2 expression on CD14$^+$ monocytes was enhanced in both act. P (*p*= 0.0166) and MG patients (*p*= 0.0083) compared to HC (**b**). Rem. P, remittent pemphigus patients. Statistical analysis was carried out using Mann-Whitney U test with *p* values indicated as **p*< 0.05 and ***p*< 0.01.

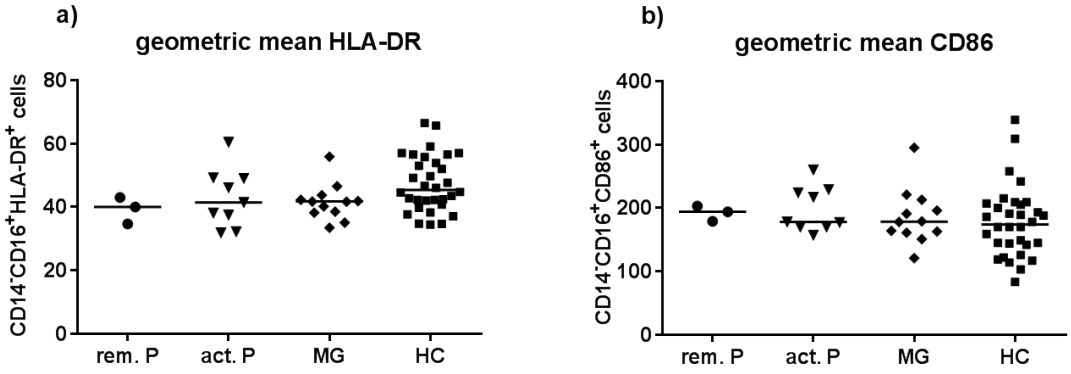

Figure 4.12: No differential expression of both HLA-DR and CD86 of slanDC in active pemphigus patients.
Compared to healthy controls (HC, *n*= 32), active pemphigus patients (act. P, *n*= 9) displayed neither an enhanced expression of HLA-DR (**a**) nor CD86 on circulating 6-sulfo LacNAc dendritic cells (slanDC) (**b**). MG, myasthenia gravis patients; rem. P, remittent pemphigus patients. Statistical analysis was carried out using Mann-Whitney U test.

Furthermore, we assessed the impact of immunosuppressive treatment with Pred on circulating leukocyte populations of both pemphigus and MG patients. As clearly demonstrate in **Figure 4.13**, application of Pred resulted in a drastic reduction of mDC subsets in both pemphigus and MG patients depending on the respective dosage given. Consequently, as described in **4.1.1** test subjects exceeding a daily intake of 10 mg of Pred were excluded from analysis.

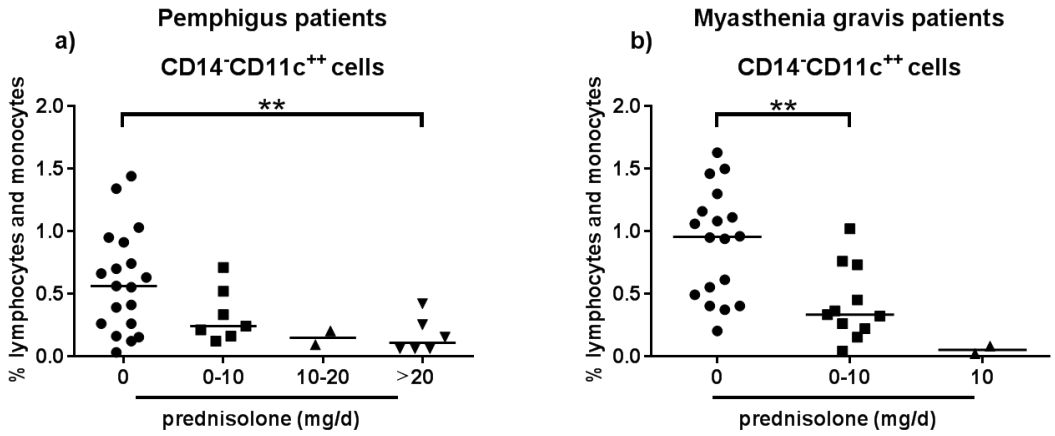

Figure 4.13: Relationship of systemic treatment with prednisolone and myeloid dendritic cell population sizes from both pemphigus and myasthenia gravis patients.
Both pemphigus (*n*= 34, **a**) and myasthenia gravis patients (*n*= 31, **b**) displayed shrinking myeloid dendritic cell ($CD14^{-}CD11c^{++}$) subsets with increasing dosages of prednisolone administered. As a consequence, patients with a daily intake of more than 10 mg of Pred were excluded from analysis. Statistical analysis was carried out using Mann-Whitney U test with *p* values indicated as $^{**}p < 0.01$.

Taken together, active pemphigus patients displayed a reduced subset of mDC and pDC concurrent with an elevated CCR2 expression of mDC, hinting at an enhanced migration of mDC into the inflamed skin. In addition, blood $CD16^{+}$ monocytes and nonclassical $CD14^{dim}CD16^{+}$ monocytes were increased in active pemphigus patients who also displayed augmented CCR2 expression of $CD14^{+}$ monocytes.

4.1.3 Functional characterization of APC from pemphigus patients

4.1.3.1 Cytokine production of monocytes upon stimulation with LPS

Besides their phenotypic analysis, circulating APC were also assayed for functional alterations, including the capability to produce (**3.1.3.6**) and secrete (**3.1.2.2**) distinct cytokines, as well as the capability to take up antigen as described in **3.1.3.8**. For this purpose, PBMC were isolated from peripheral blood of pemphigus patients and control groups by density gradient centrifugation as presented in **3.1.3.1**.

In order to assess the capacity of $CD14^{+}$ monocytes to produce the proinflammatory cytokines IL-6 and TNF-α, monocytes were stimulated overnight with bacterial LPS (**3.1.3.6**), the best-described ligand of TLR4 (Brubaker et al. 2015a). Produced cytokines were detected by flow cytometry analysis of intracellularly stained proteins with fluorescently labeled antibodies as described in **3.1.3.7** and illustrated in **Figure 4.14**. As shown in **Figure 4.15a** and **b**, neither IL-6 nor TNF-α production of $CD14^{+}$ monocytes differed between the groups, but varied over a wide range, particularly concerning TNF-α production in active pemphigus patients. Thus, circulating monocytes of pemphigus patients do not seem to be in a condition of enhanced immunogenicity as assessed by this assay. Yet, interestingly, monocytes of active pemphigus patients produced IL-6 and TNF-α to the same extent (*p*= 0.0002) as visualized in **Figure 4.15c**. Moreover, only monocytes from four active pemphigus patients displayed the highest values of proinflammatory

cytokine production, which could be a sign for enhanced monocyte immunogenicity at least in individual pemphigus patients.

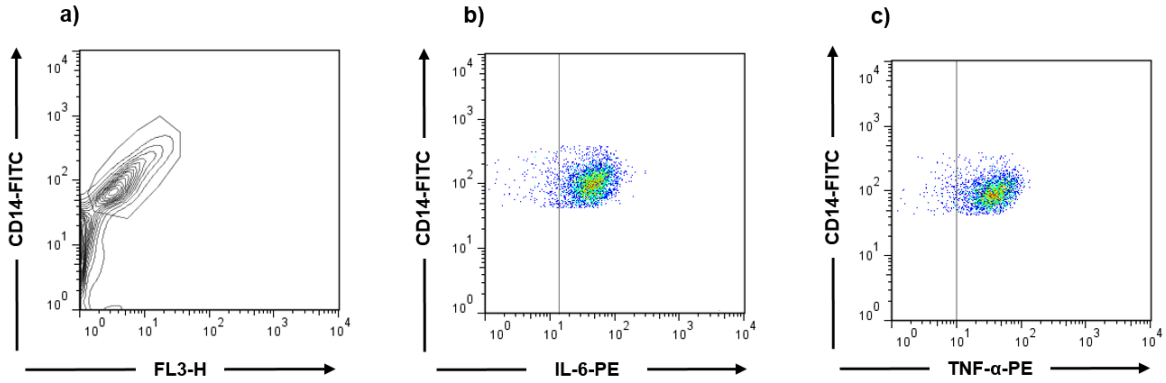

Figure 4.14: Production of IL-6 and TNF-α upon stimulation of monocytes with LPS.
The population of monocytes was selected by opposing binding of an anti-human CD14 FITC-coupled antibody to the autofluorescence signal of monocytes detected in a channel being not in use (**a**). Binding of an anti-human interleukin (IL)-6 PE-coupled antibody and anti-human tumor necrosis factor (TNF)-α PE-coupled antibody, respectively, visualized cytokine synthesis of $CD14^+$ monocytes (**b** and **c**). For quantification of IL-6 (**b**) and TNF-α (**c**) production of monocytes, the geometrical mean values of the respective antibody signal intensities were determined. Separation of cell populations was performed using the appropriate isotype controls. LPS, lipopolysaccharides.

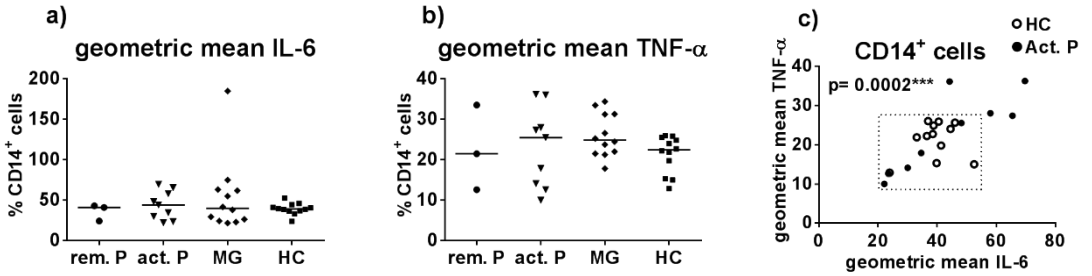

Figure 4.15: No differential production of both IL-6 and TNF-α of monocytes in active pemphigus patients.
Production of interleukin (IL)-6 and tumor necrosis factor (TNF)-α of $CD14^+$ monocytes stimulated with lipopolysaccharides (LPS) was determined by flow cytometry analysis. Neither synthesis of IL-6 (**a**) nor TNF-α (**b**) varied between active pemphigus (act. P, *n*= 9) and myasthenia gravis (MG) patients (*n*= 12). Yet, compared to healthy controls (HC, *n*= 12) only monocytes of act. P patients synthesized IL-6 and TNF-α to the same extent (*p*= 0.0002). Moreover, four act. P patients displayed the highest values of cytokine production, hinting at an enhanced immunogenicity of $CD14^+$ monocytes in at least individual pemphigus patients (**c**). Rem. P, remittent pemphigus patients. Statistical analysis was carried out using Mann-Whitney U test. Spearman correlation coefficients (*r*) are shown as *p* values, indicated as ***$p < 0.001$.

4.1.3.2 Antigen uptake capability of monocyte subsets

The capability to take up and process antigen belongs to the most important functions of APC. In order to test whether this property is altered in pemphigus, PBMC from pemphigus patients and control groups were exposed to fluorescently labeled human Dsg3 protein (**2.5.1**) as described in **3.1.3.8**, and analyzed by flow cytometry as illustrated in **Figure 4.16**. Incubation of monocytes at

37 °C for various lengths of time (5 min, 10 min, and 30 min) enabled efficient antigen take up, whereas control samples stored at 4 °C were used for determining background activity. The difference in MFI between the sample stored at 37 °C and its 4 °C control reflected the uptake capability.

The antigen uptake capacity of neither $CD16^+$ (p= 0.3730, **a**) nor classical (p= 0.2754, **b**) monocytes significantly varied between active pemphigus patients and HC as presented in **Figure 4.18**. In accordance with reports in the literature (Auffray, Sieweke, and Geissmann 2009), internalization capability of classical monocytes exceeded that of $CD16^+$ monocytes and maximum antigen take up was achieved after ca. 30 min of protein incubation as demonstrated in **Figure 4.17**. The time point of 50% antigen take up of classical monocytes was determined to assess the kinetics of antigen internalization as shown in **Figure 4.18c**. Concerning the velocity of antigen take up of classical monocytes, no significant (p= 0.1687) differences were observed between active pemphigus patients and HC (**Figure 4.18c**). Taken together, neither the capability of maximum antigen internalization nor the speed of antigen take up were proven altered in pemphigus.

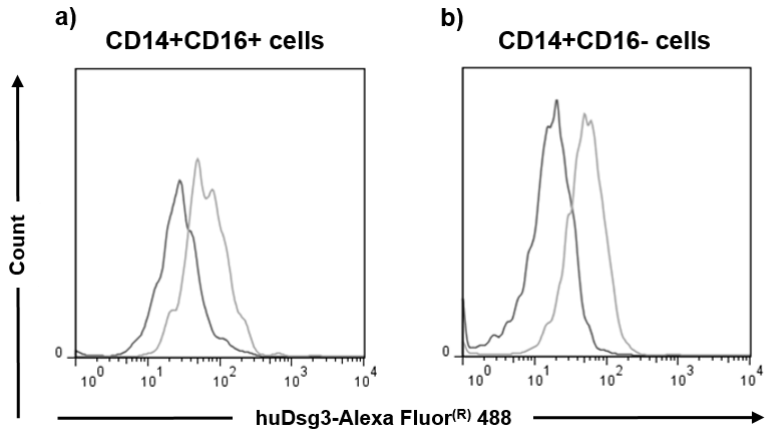

Figure 4.16: Uptake of fluorescently labeled Dsg3 protein of $CD16^+$ and classical monocytes.
Antigen uptake capability of $CD14^+CD16^+$ (**a**) and classical ($CD14^+CD16^-$, **b**) monocytes within the peripheral mononuclear cells (PBMC) from patients' blood was assessed by flow cytometry analysis. Internalization of fluorescently labeled human Desmoglein 3 (huDsg3) was measured at various lengths of time (5 min, 10 min, and 30 min) for PBMC stored at 37 °C and on ice as a control. As illustrated by histogram presentation, the difference in mean fluorescence intensity between the sample stored at 37 °C (bright line) and its control (dark line) determined the monocytes uptake capability. Shown is the antigen uptake of $CD16^+$ monocytes and classical monocytes upon incubation of huDsg3 for 10 min.

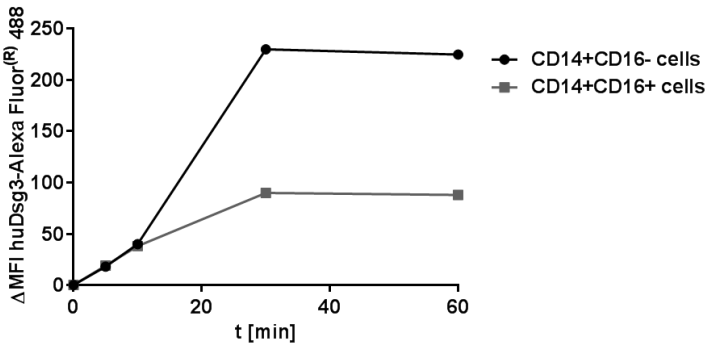

Figure 4.17: Kinetics of antigen uptake of CD16$^+$ and classical monocytes.
Internalization of fluorescently labeled human Desmoglein 3 (huDsg3) protein of CD16$^+$ and classical monocytes was analyzed by flow cytometry. The capability of antigen uptake of CD16$^+$ monocytes (bright line) and classical monocytes (dark line) was assessed after 5 min, 10 min, 30 min, and 60 min of protein incubation. Maximum uptake of antigen was reached following ca. 30 min of protein exposure. Capacity of classical monocytes to internalize protein clearly exceeded that of CD16$^+$ monocytes. ΔMFI huDsg3 describes the difference in mean fluorescence intensity (MFI) between the sample stored at 37 °C and its 4 °C control upon incubation with huDsg3 protein, and determines the monocyte uptake capability.

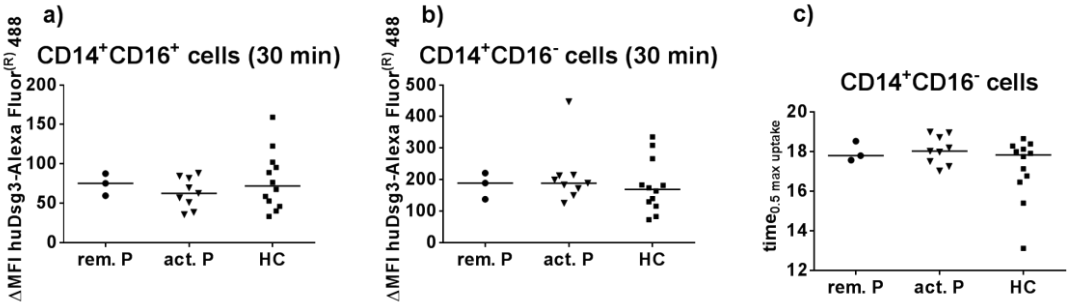

Figure 4.18: Uptake of fluorescently labeled Dsg3 protein of monocytes from active pemphigus patients.
Maximum uptake of fluorescently labeled human Desmoglein 3 (huDsg3) protein of CD16$^+$ and classical monocytes from patients' blood was analyzed by flow cytometry. Following 30 min of protein incubation, neither the antigen uptake capability of CD16$^+$ (**a**) nor classical (**b**) monocytes differed between active pemphigus patients (act. P, *n*= 12) and healthy controls (HC, *n*= 12). Similarly, no differences in the velocity of protein internalization were observed between act. P patients and HC (**c**). Rem. P, remittent pemphigus patients. ΔMFI huDsg3 describes the difference in mean fluorescence intensity (MFI) between the sample stored at 37 °C and its 4 °C control upon incubation with huDsg3 protein, and determines the monocyte uptake capability. $Time_{0.5}$ max uptake reflects the time span until half of the maximum protein internalization capacity was reached. Statistical analysis was carried out using Mann-Whitney U test.

4.1.3.3 Detection of cytokines derived from APC in plasma

Owing to their capacity to mediate T cell function, cytokines are critically involved in the pathogenesis of pemphigus (Giordano and Sinha 2012). Yet, to date, the role of disease-propagating cytokines in pemphigus has not been fully elucidated. Therefore, cytokines related to both APC and T cells, including IL-6, TNF-α, IL-33, and IL-27 were analyzed in the plasma of pemphigus patients and control groups as described in **3.1.2.2**.

As presented in **Figure 4.19a**, the plasma levels of IL-6 tended to be increased in both active pemphigus (*p*= 0.1211) and MG patients (*p*= 0.0543). Similarly, the plasma levels of TNF-α were significantly and visibly elevated in active pemphigus (*p*= 0.0259) and MG patients (*p*= 0.0907), respectively, as shown in **Figure 4.19b**.

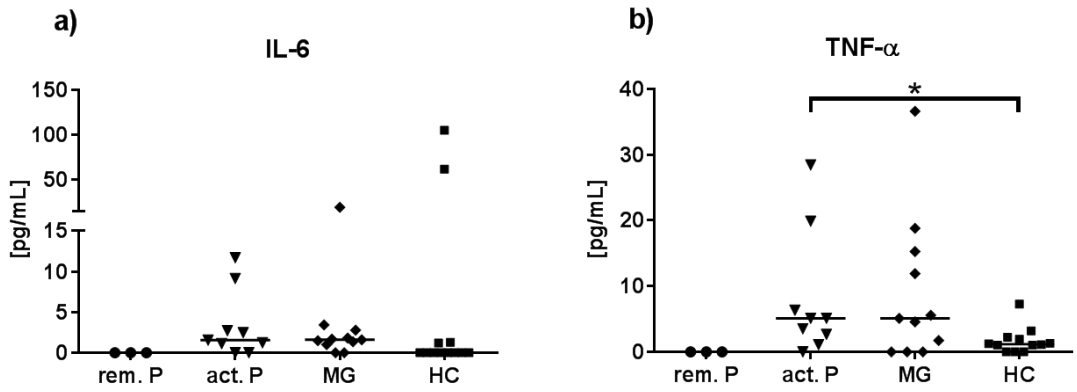

Figure 4.19: Plasma concentrations of IL-6 and TNF-α in pemphigus patients and controls.
Both active pemphigus (act. P, *n*= 9; *p*= 0.1211) and myasthenia gravis (MG) patients (*n*= 12; *p*= 0.0543) displayed elevated plasma concentrations of interleukin (IL)-6 compared to healthy controls (HC, *n*= 12). The plasma of remittent pemphigus (rem. P) patients did not exhibit detectable amounts of IL-6 (**a**). Compared to HC (*n*= 12), both act. P (*n*= 9; *p*= 0.0259) and MG patients (*n*= 12; *p*= 0.0907) exhibited elevated plasma concentrations of tumor necrosis factor (TNF)-α with act. P patients reaching statistical significance. Similar to IL-6, the plasma levels of TNF-α of the three rem. P patients were below the detection limit (**b**). Statistical analysis was carried out using Mann-Whitney U test with *p* values indicated as *$p < 0.05$.

As demonstrated in **Figure 4.20a**, the plasma concentrations of IL-33 were also slightly augmented in both active pemphigus (*p*= 0.1687) and MG patients (*p*= 0.1171). Strikingly, the immunomodulatory IL-27 was significantly increased in active pemphigus (*p*= 0.0136) and MG patients (*p*= 0.0326) compared to HC as presented in **Figure 4.20b**. Of note, the three remittent pemphigus patients displayed very low values for each cytokine measured (**Figures 4.19** and **4.20**).

To summarize, active pemphigus patients exhibited elevated plasma levels of the proinflammatory cytokines IL-6 and TNF-α, as well as IL-27.

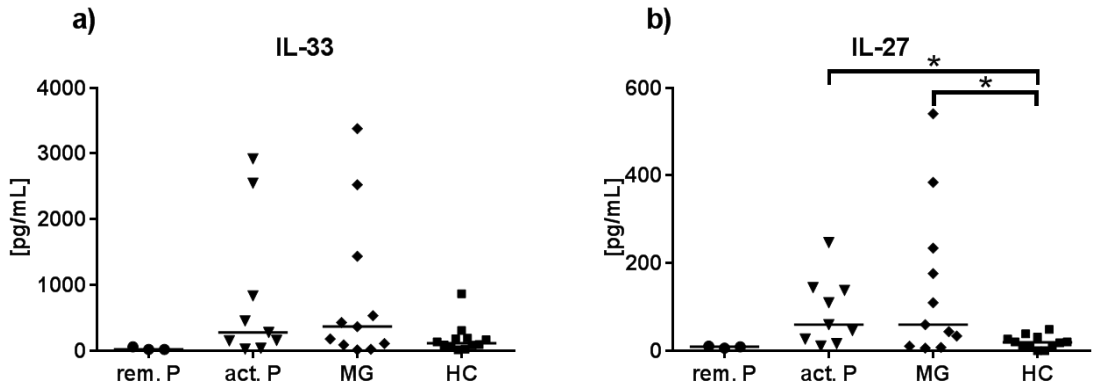

Figure 4.20: Plasma concentrations of IL-33 and IL-27 in pemphigus patients and controls.
The plasma levels of interleukin (IL)-33 tended to be enhanced in both active pemphigus (act. P, *n*= 9; *p*= 0.1687) and myasthenia gravis (MG) patients (*n*= 12; *p*= 0.1171) compared to healthy controls (HC, *n*= 12). The plasma samples of the three remittent pemphigus (rem. P) patients did not exhibit detectable amounts of IL-33 (**a**). Intriguingly, concentrations of IL-27 were significantly elevated in the plasma of both act. P. (*n*= 9; *p*= 0.0136) and MG patients (*n*= 12; *p*= 0.0326) despite the primarily immunomodulatory function of IL-27. In addition, the plasma levels of IL-27 of the three rem P. patients were below the detection limit (**b**). Statistical analysis was carried out using Mann-Whitney U test with *p* values indicated as *$p < 0.05$.

4.1.4 Functional characterization of CD4⁺ T cell subpopulations in pemphigus patients

In the pathogenesis of pemphigus, the generation of auto-ab is critically regulated by autoreactive CD4⁺ T cells specific for Dsg3 and/or Dsg1 as demonstrated by numerous studies (Amagai 2008; Hertl, Eming, and Veldman 2006). Based on the found enhanced production of APC-derived proinflammatory cytokines, including IL-6, TNF-α, and IL-27, we next assessed whether the polarization of CD4⁺ T cells is altered. For this purpose, PBMC were isolated from peripheral blood of pemphigus patients and control groups by density gradient centrifugation as described in **3.1.3.1**. In order to induce synthesis of the Th1, Th2, Th17, and Treg cell signature cytokines IFN-γ, IL-4, IL-17, and IL-10, respectively, PBMC were stimulated with the mitogens PMA and ionomycin in the presence of a secretion inhibitor as presented in **3.1.3.6**. As described in **3.1.3.7** and illustrated in **Figure 4.22**, production of cytokines was detected by flow cytometry analysis of the respective fluorescently labeled antibodies targeting intracellular proteins.
Since surface expression of CD4 is downregulated upon activation (Pelchen-Matthews, Parsons, and Marsh 1993), CD4⁺ T cells were identified by expression of CD3 and lack of CD8 as visualized in **Figure 4.21**.

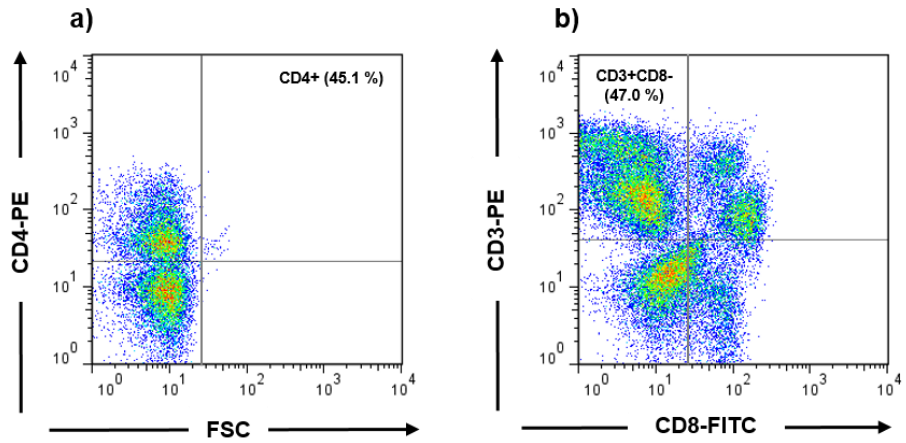

Figure 4.21: Identification of CD4⁺ T cells by flow cytometry.
Staining of $CD4^+$ cells with either an anti-CD4 PE-coupled antibody (45.1% $CD4^+$ T cells, **a**) or the combination of an anti-CD3 PE-coupled and anti-CD8 FITC-coupled antibody (47.0% $CD3^+CD8^-$ cells, **b**) yielded similar population sizes. Separation of cell populations was performed using the appropriate isotype controls. FSC, forward scatter.

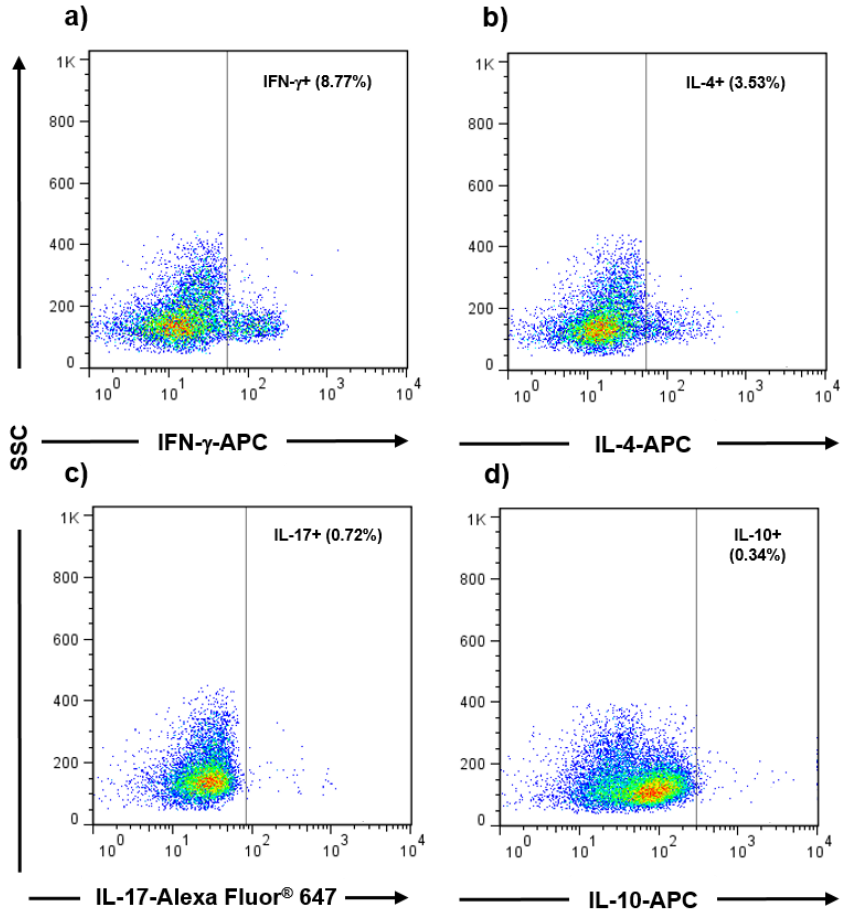

Figure 4.22: Detection of intracellular cytokines of CD4⁺ T cells by flow cytometry.
$CD4^+$ T cells within patients' peripheral blood mononuclear cells were stimulated with phorbol myristate-acetate and ionomycin for 5 hours to induce synthesis of the T helper (Th) 1, Th2, Th17, and T regulatory cell signature cytokines interferon (IFN)-γ (**a**), interleukin (IL)-4 (**b**), IL-17 (**c**), and IL-10 (**d**), respectively. Cytokine production of $CD3^+CD8^-$ cells was detected by flow cytometry analysis of fluorescently labeled antibodies targeting intracellular proteins. Separation of cell populations was performed using the appropriate isotype controls. SSC, side scatter.

In active pemphigus patients, a general shift towards a more pronounced Th1 (p= 0.9047) or Th2 (p= 0.7974) cell response, defined by the frequencies of IFN-γ– and IL-4–producing T cells, respectively, could not be observed compared to HC as presented in **Figure 4.23a** and **b**. Thus, the Th2-biased T cell response in pemphigus seems to be rather confined to single autoreactive Dsg3-specific $CD4^+$ T cells as shown by several studies (Veldman et al. 2003b). Interestingly, IL-17–producing $CD4^+$ T cells were elevated in both active pemphigus (p= 0.0003) and MG patients (p= 0.0200) as demonstrated in **Figure 4.23c**. Moreover, IL-10–producing T cells were significantly increased (p= 0.0278) in active pemphigus patients, whilst in MG patients, their frequency was reduced (p= 0.0283) compared to HC (**Figure 4.23d**). Taken together, active pemphigus patients did not display differing Th1 and Th2 cell responses, but elevated populations of both Th17 cells and IL-10–producing T cells.

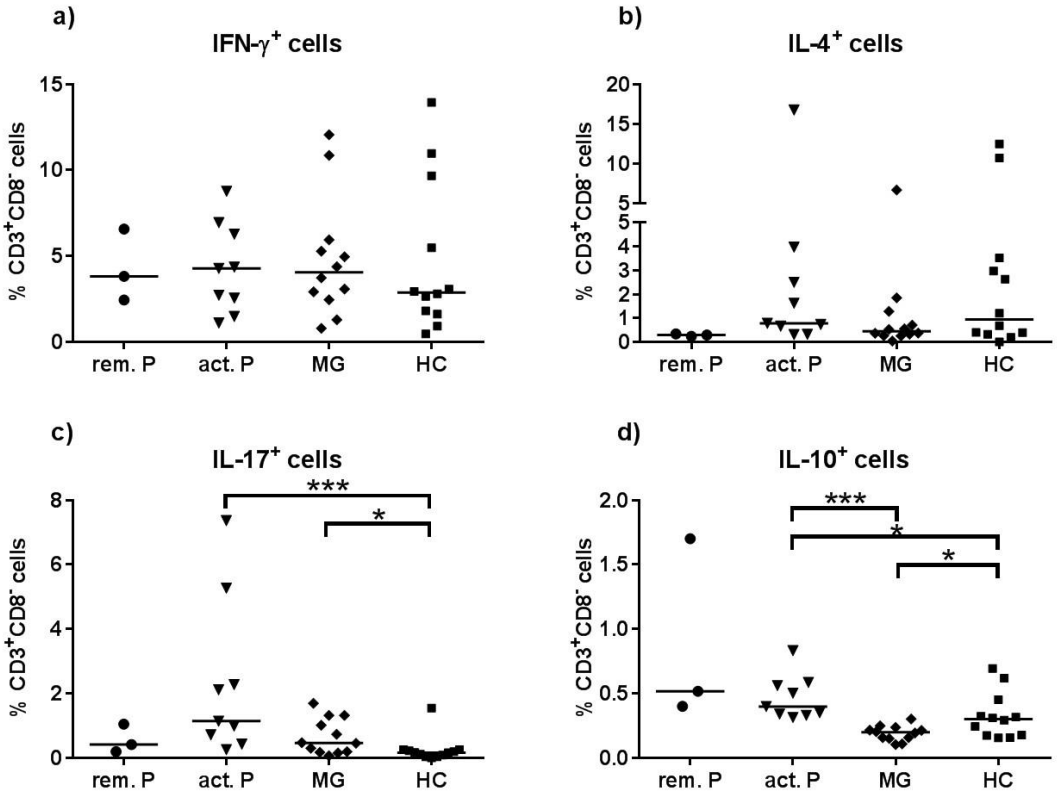

Figure 4.23: $CD4^+$ T cell subpopulations in active pemphigus patients.

Active pemphigus patients (act. P, n= 9) did not display a general imbalance of T helper (Th) 1 (p= 0.9047, **a**) and Th2 cells (p= 0.7974, **b**) compared to healthy controls (HC, n= 12). However, act. P patients showed significantly increased (p= 0.0003) populations of Th17 cells, which was also observed in myasthenia gravis (MG) controls (n= 12; p= 0.0200) (**c**). Unexpectedly, act. P patients similarly exhibited elevated (p= 0.0278) frequencies of interleukin (IL)-10–producing T cells, whereas in MG patients, their frequency was significantly reduced (p= 0.0283) (**d**). Since IL-10 is also secreted by Th2 cells, IL-10–producing cells do not only represent T regulatory cells. IFN-γ, interferon-γ; rem. P, remittent pemphigus patients. Statistical analysis was carried out using Mann-Whitney U test with p values indicated as *p< 0.05 and ***p< 0.001.

4.1.5 Characterization of auto-ab responses in pemphigus patients

Purpose of this study was to characterize immune responses in pemphigus in a phenotypical and functional manner. So far, we have found particularities in the cellular immune response of active pemphigus patients, including qualitative and quantitative alterations in the APC compartment, differing cytokine expression patterns, as well as imbalances between $CD4^+$ T cell subpopulations. Concerning APC behavior, frequencies of both mDC and pDC were slightly reduced in active pemphigus patients (**Figure 4.8**) concurrent with significantly enhanced CCR2 expression of mDC (**Figure 4.11a**). Similarly, active pemphigus patients exhibited significantly elevated CCR2 expression levels of $CD14^+$ monocytes (**Figure 4.11b**), suggesting an increased migration of APC potentially into the inflamed skin. In addition, frequencies of $CD16^+$ monocytes (**Figure 4.10a**) and nonclassical $CD14^{dim}CD16^+$ monocytes (**Figure 4.10b**) were augmented in both active pemphigus patients and MG controls. Moreover, active pemphigus patients displayed slightly and significantly elevated plasma levels of the proinflammatory cytokines IL-6 (**Figure 4.19a**) and TNF-α (**Figure 4.19b**), respectively. Interestingly, significantly increased levels of the immunomodulatory IL-27 reflect its disease-promoting function in pemphigus (**Figure 4.20b**), whereas the plasma levels of the Th2 cell-related IL-33 were only slightly enhanced in active pemphigus patients (**Figure 4.20a**). Considering $CD4^+$ T cell subpopulations, active pemphigus patents displayed both significantly elevated frequencies of Th17 cells (**Figure 4.23c**) and IL-10–producing T cells compared to HC (**Figure 4.23d**).

In pemphigus, the titers of serum anti-Dsg1 and anti-Dsg3 IgG auto-ab generally correlate with disease activity when monitored in individual patients (Ishii et al. 1997). Similarly, in our cohort of PV patients, increased Dsg3-reactive IgG auto-ab titers varied within a wide range and were predominantly exhibited by active, but not remittent PV patients as presented in **Figure 4.24**.

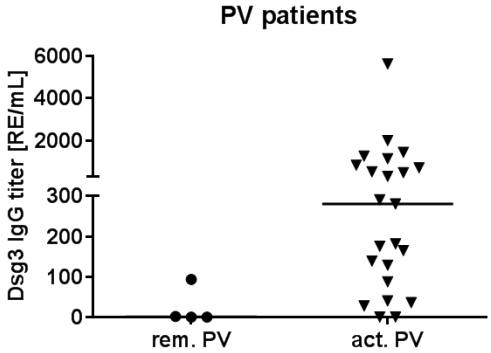

Figure 4.24: Dsg3-specific antibody titer of active and remittent pemphigus vulgaris patients.
Titers of anti-Desmoglein 3 (Dsg3) immunoglobulin (Ig) G antibodies were determined in the sera of pemphigus vulgaris (PV) patients by enzyme-linked immunosorbent assay. In contrast to remittent (rem.) PV patients (*n*= 4), patients with active disease (act. PV, *n*= 23) displayed anti-Dsg3 IgG reactivity that varied within a wide range. The cut-off value of the assay was 20 RE/mL.

In order to investigate the relationship between APC, APC-derived cytokines, $CD4^+$ T cells, and antibody production in pemphigus, Dsg1- and Dsg3-specific IgG auto-ab titers were correlated

with the respective parameters assessed in this study. As a main result and as demonstrated in **Figure 4.25**, the anti-Dsg1 IgG levels from PF patients combined with the anti-Dsg3 IgG levels from PV patients correlated (*r*= 0.6683) with the plasma levels of IL-6. Accordingly, anti-Dsg3 IgG levels from PV patients alone as well correlated (*r*= 0.6967) with the IL-6 plasma concentrations. In contrast, anti-AChR IgG titers from MG controls did not correlate (*r*= -0.1507) with the plasma levels of IL-6.

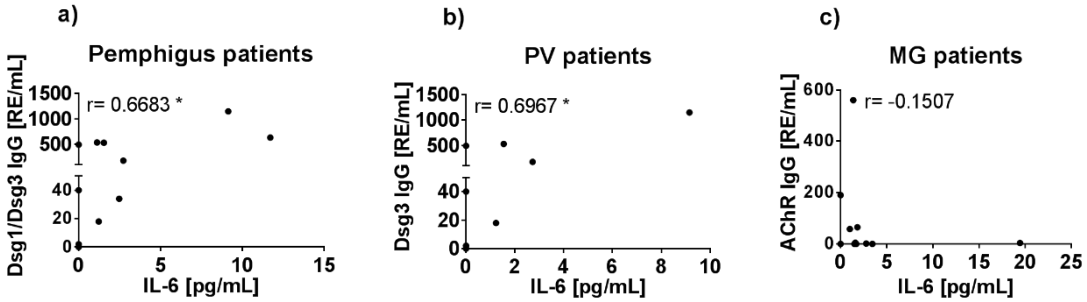

Figure 4.25: Correlation of auto-ab titers with IL-6 plasma levels in pemphigus and myasthenia gravis patients.

Anti-Desmoglein 1 (Dsg1) immunoglobulin (Ig) G levels and anti-Dsg3 IgG levels were determined in the sera from pemphigus foliaceus (PF, *n*= 3) and pemphigus vulgaris (PV) patients (*n*= 9) by enzyme-linked immunosorbent assay. Autoantibody (auto-ab) IgG titers from PF and PV patients together correlated (*r*= 0.6683) with the plasma levels of interleukin (IL)-6 (**a**). Similarly, anti-Dsg3 IgG levels from PV patients alone also correlated (*r*= 0.6967) with the plasma concentrations of IL-6 (**b**). In contrast, a correlation was not observed (*r*= -0.1507) between anti-acetylcholine receptor (AChR) IgG levels and IL-6 plasma levels from myasthenia gravis (MG) controls (*n*= 12) (**c**). Data of pooled active and remittent pemphigus patients is presented. Spearman correlation coefficients (*r*) are shown with *p* values indicated as *$p < 0.5$.

Similarly, auto-ab IgG titers from both PF and PV patients correlated (*r*= 0.6072) with the plasma levels of TNF-α, which was also observed in the cohort of exclusively PV patients (*r*= 0.7902). MG controls however only displayed a correlation (*r*= -0.6299) between anti-AChR IgG levels and TNF-α plasma concentrations in an inverse manner as illustrated in **Figure 4.26**.

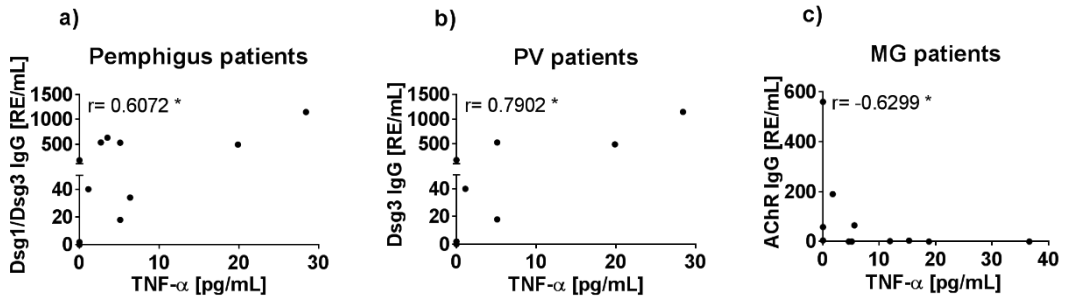

Figure 4.26: Correlation of auto-ab titers with TNF-α plasma levels in pemphigus and myasthenia gravis patients.

In the sera from pemphigus foliaceus (PF, *n*= 3) and pemphigus vulgaris (PV) patients (*n*= 9), anti-Desmoglein 1 (Dsg1) and anti-Dsg3 IgG titers were determined by enzyme-linked immunosorbent assay. Similarly to interleukin-6, autoantibody (auto-ab) immunoglobulin (Ig) G titers from the combination of PF and PV patients correlated (*r*= 0.6072) with the plasma levels of tumor necrosis factor (TNF)-α (**a**). Anti-Dsg3 IgG levels from PV patients alone as well correlated (*r*= 0.7902) with the plasma levels of TNF-α (**b**). However, only a negative correlation was observed (*r*= -0.6299) between anti-acetylcholine receptor (AChR) IgG levels and TNF-α plasma levels from myasthenia gravis (MG) controls (*n*= 12) (**c**). Data of pooled active and remittent pemphigus patients is presented. Spearman correlation coefficients (*r*) are shown with *p* values indicated as *$p< 0.5$.

Interestingly, IL-27 plasma levels not only strongly correlated with the combined anti-Dsg1 and anti-Dsg3 IgG titers from PF and PV patients, respectively (*r*= 0.7965), but also with the anti-Dsg3 IgG levels from PV patients alone (*r*= 0.8909). Yet, in MG controls, we could not observe a correlation between auto-ab IgG titers and IL-27 plasma levels as shown in **Figure 4.27**.

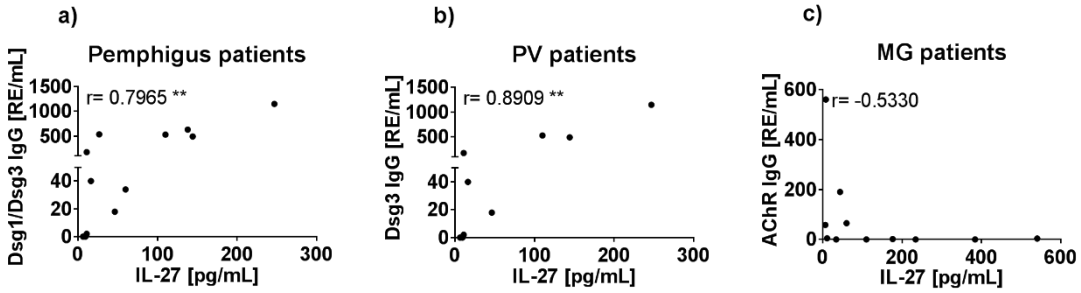

Figure 4.27: Correlation of auto-ab titers with IL-27 plasma levels in pemphigus and myasthenia gravis patients.

Anti-Desmoglein 1 (Dsg1) and anti-Dsg3 immunoglobulin (Ig) G levels were detected in the sera from pemphigus foliaceus (PF, *n*= 3) and pemphigus vulgaris (PV) patients (*n*= 9) by enzyme-linked immunosorbent assay (ELISA). As determined by ELISA, autoantibody (auto-ab) IgG titers from PF and PV patients together strongly correlated (*r*= 0.7965) with the plasma levels of interleukin (IL)-27 (**a**). In accordance with that, anti-Dsg3 IgG levels from PV patients alone again strongly correlated (*r*= 0.8909) with the plasma concentrations of IL-27 (**b**). On the contrary, no correlation was found (*r*= -0.5330) between anti-acetylcholine receptor (AChR) IgG levels and IL-27 plasma levels from myasthenia gravis (MG) controls (*n*= 12) (**c**). Data of pooled active and remittent pemphigus patients is presented. Spearman correlation coefficients (*r*) are shown with *p* values indicated as **$p< 0.01$.

Taken together, in pemphigus, a correlation was found between serum auto-ab IgG titers and the plasma concentrations of proinflammatory cytokines, including IL-6 (**Figure 4.25a** and **b**) and

TNF-α (**Figure 4.26a** and **b**), concurrent with elevated plasma levels of the respective cytokines (**Figure 4.19**).

Again, a disease-promoting function could be confirmed for IL-27 since it was not only elevated (**Figure 4.20b**), but also correlated with auto-ab titers in both pooled pemphigus and PV patients (**Figure 4.27a** and **b**). This observation was further supported by IL-27 plasma levels also strongly correlating (*r*= 0.9287) with TNF-α plasma levels as presented in **Figure 4.28a**. Moreover, IL-27 plasma levels correlated (*r*= 0.7526) with plasma levels of IL-33 in pemphigus patients as shown in **Figure 4.28b**.

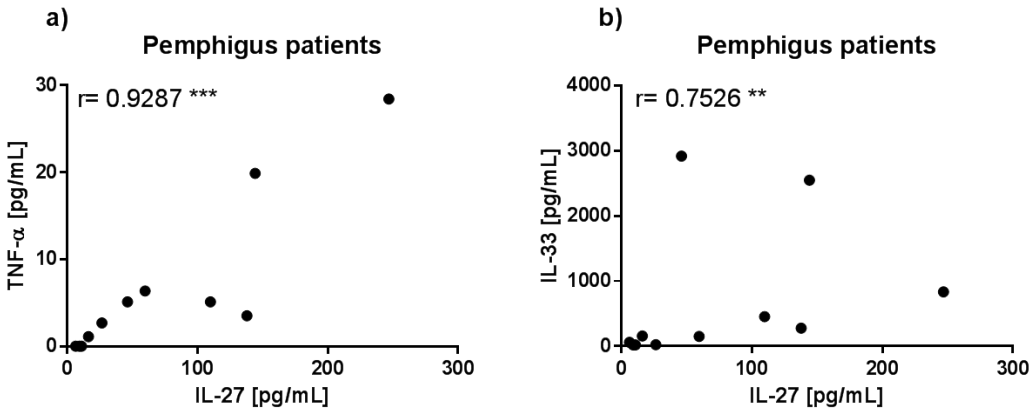

Figure 4.28: Correlation of IL-27 plasma levels with TNF-α and IL-33 in pemphigus patients.
As determined by enzyme-linked immunosorbent assay, interleukin (IL)-27 plasma levels of pemphigus patients (*n*= 12) strongly correlated (*r*= 0.9287) with the plasma levels of tumor necrosis factor (TNF)-α (**a**). Similarly, a strong correlation (*r*= 0.7526) was observed between plasma concentrations of IL-27 (*r*= 0.8909) and the plasma levels of IL-33 (**b**). Spearman correlation coefficients (*r*) are shown with *p* values indicated as $^{**}p < 0.01$ and $^{***}p < 0.001$.

On the basis of the found elevated plasma levels of IL-27 (**Figure 4.20b**) in active pemphigus patients, as well as IL-27 plasma levels correlating with both serum IgG auto-ab titers (**Figure 4.27a** and **b**) and plasma TNF-α levels (**Figure 4.28a**), IL-27 is suggested to exert a disease-propagating function in pemphigus. Although MG controls also exhibited enhanced plasma levels of IL-27 (**Figure 4.20b**), we did not observe a correlation between IL-27 levels and anti-AChR titers (**Figure 4.27c**). Therefore, we assume a disease-specific role of IL-27 in pemphigus. A potential mechanistic link between IL-27 and antibody production was recently provided by Batten et al. who demonstrated that IL-27 signaling to T cells induced the release of IL-21 (Batten et al. 2010). Moreover, IL-27 enhanced both the survival of active $CD4^+$ T cells and the expression of the Tfh cell markers CXCR5, PD-1, and inducible T cell costimulator (ICOS). IL-21–producing Tfh cells are specialized and essential providers of B cell help to both form and maintain GC, and to regulate the maturation of B cells into memory B cells, as well as plasma cells secreting high-affinity antibodies (Tangye et al. 2013). Altered Tfh cell behavior has been reported in several antibody-mediated autoimmune disorders, including SLE and RA, in which the expansion of Tfh cells could be related to disease activity (Simpson et al. 2010; Wang et al. 2013). To investigate

whether the observed increased IL-27 plasma titers possibly affected Tfh cells in pemphigus, we additionally detected circulating IL-21 in plasma samples of the test subjects. Indeed, as presented in **Figure 4.29**, both active pemphigus patients (p= 0.0062) and MG controls (p= 0.0003) exhibited significantly enhanced titers of IL-21 compared to HC. In addition, plasma of the remittent pemphigus patients did not display detectable amounts of IL-21. The cellular source of IL-21 was traced back by our group in a further cohort of pemphigus and MG patients demonstrating elevated populations of $CD4^+CXCR5^+$ Tfh cells in active pemphigus patients, again concurrent with increased plasma levels of IL-21 (Hennerici et al. 2016). Taken together, these findings indicate a novel role of IL-27 and IL-21–producing T cells in the pathogenesis of pemphigus.

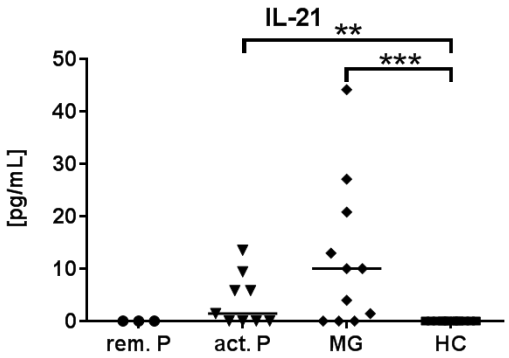

Figure 4.29: Plasma concentrations of IL-21 in pemphigus and myasthenia gravis patients.
Levels of interleukin (IL)-21 were measured in the patients' plasma samples by enzyme-linked immunosorbent assay. Interestingly, concentrations of IL-21 were significantly elevated in the plasma of both active pemphigus (act. P, n= 9; p= 0.0062) and myasthenia gravis (MG) patients (n= 12; p= 0.0003) indicating an expansion of T follicular helper (Tfh) cells in both autoimmune disorders. Moreover, levels of IL-21 of the three remittent pemphigus (rem. P) patients were below the limit of detection. IL-21 is vastly produced by Tfh cells and also by Th 17 cells. HC, healthy control. Statistical analysis was carried out using Mann-Whitney U test with p values indicated as **p< 0.01 and ***p< 0.001.

4.2 Mouse study

4.2.1 HLA-DRB1*04:02–tg mice as a model for PV

The second part of this thesis deals with the HLA-DRB1*04:02–tg mouse model of PV, in which Dsg3-specific pathoimmunological mechanisms, including APC-mediated CD4⁺ T cell activation and antibody production were investigated considering the strong genetic association of this disease. The highly increased prevalence of distinct HLA class II alleles in PV patients, especially HLA-DRB1*04:02 and –DQB1*05:03, was confirmed to provide the physicochemical properties required for efficient positioning of antigenic Dsg3 peptides in the HLA-DR pocket (Tong et al. 2006). This finally results in the activation of autoreactive CD4⁺ T cells by the HLA-DR–peptide complex. Since the β-chain of the HLA-DRβ1*04:02 binding groove displays the negatively charged amino acid residues aspartic acid (D70) and glutamic acid (E71) at the critical relative position four (P4), peptides with a positively charged amino acid at P4 are high affinity-bound and preferably presented to CD4⁺ T cells as illustrated in **Figure 4.30b**. On the basis of this peptide binding algorithm, our group (Veldman, Gebhard, et al. 2004) identified Dsg3-specific CD4⁺ T cell clones from PV patients that recognized a limited set of epitopes within the human Dsg3 EC as shown in **Figure 4.30a**. These designated immunodominant Dsg3 peptides binding to HLA-DRβ1*04:02 typically share a positively charged amino acid residue at P4, either arginine (R) or lysine (K). Besides human Dsg3 protein and the noncollagenous (NC1) domain of Collagen VII (Col VII) as a control (**2.5.1**), a set of five HLA-DRβ1*04:02–binding Dsg3 peptides (**2.5.2.1**) was applied for immunization of the HLA-DRB1*04:02–tg mice. A control set was comprised of five HLA-DRβ1*04:02–non-binding Dsg3 peptides (**2.5.2.2**).

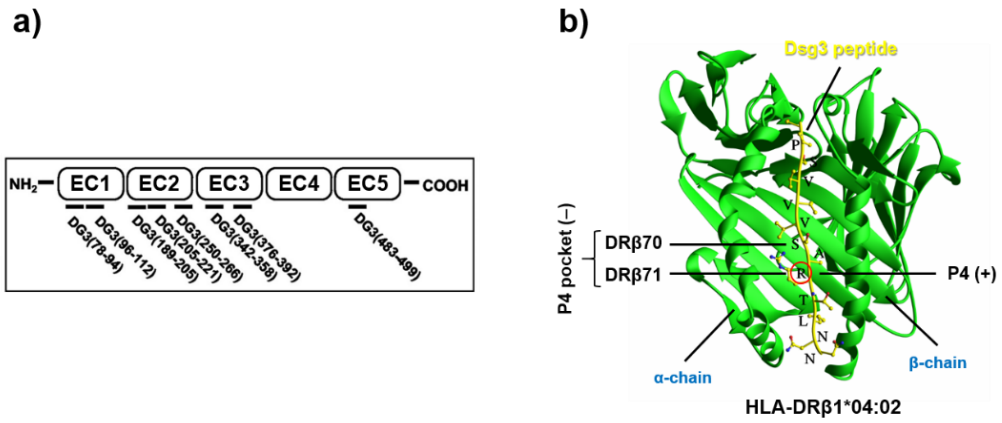

Figure 4.30: Location of Dsg3 peptides within the Dsg3 EC and algorithm for peptide binding to HLA-DRβ1*04:02.

The extracellular portion of Desmoglein 3 (Dsg3) is subdivided into five domains (EC1–5), of which the indicated peptides represent epitopes of Dsg3-specific CD4⁺ T cells (**a**). Genetic susceptibility of pemphigus vulgaris is strongly conferred by expression of distinct HLA class II alleles, especially HLA-DRB1*04:02 and –DQB1*05:03. The molecular basis for this observation is provided by an algorithm for peptide binding to HLA-DRβ1*04:02 proposed by Wucherpfennig et al. (Wucherpfennig et al. 1995) and confirmed by Tong et al. (Tong et al. 2006). The HLA-DRβ1*04:02–binding immunodominant Dsg3 peptides bear a positively charged amino acid residue (R or S) at P4, where it interacts with the negatively charged amino acids D70 and E71 of the HLA-DR binding cleft (**b**) (modified from Veldman et al. J. Immunol., 2004).

In order to study human Dsg3-specific cellular and humoral immune responses of PV *in vivo* under the genetic impact of HLA-DRB1*04:02, DBA/1J mice transgenic for HLA-DRB1*04:02 and –DQB1*03:02 (which is linked to DRB1*04:02 in a disequilibrium) were generated. Expression of the human CD4 coreceptor was introduced to retain efficient APC–$CD4^+$ T cell interaction and mice were devoid of functional murine MHC class II (I-$A\beta^{-/-}$).

At first, the respective phenotype was confirmed for each HLA-DRB1*04:02–tg mouse used in this study by flow cytometry analysis of peripheral venous blood samples as described in **3.2.1.1** and illustrated in **Figure 4.31**. Leukocytes of mice transgenic for HLA-DRB1*04:02 displayed a high intensity of the bound anti-human CD4 PE-coupled antibody unlike those of wild type mice that merely exhibited background fluorescence (**Figure 4.31a**). Similarly, although less well resolved did the anti-human HLA-DR and anti-human HLA-DQ antibodies labeled with PE and FITC, respectively, target the respective cell surface markers expressed by HLA-DRB1*04:02–tg mice only (**Figure 4.31b** and **c**). However, contrary to HLA-DRB1*04:02–tg mice, binding of the anti–I-Aβ FITC-coupled antibody solely occurred on immune cells from wild type mice. Homozygosity of wild type mice for I-Aβ reflects in two single intensity peaks of the bound antibody (**Figure 4.31d**).

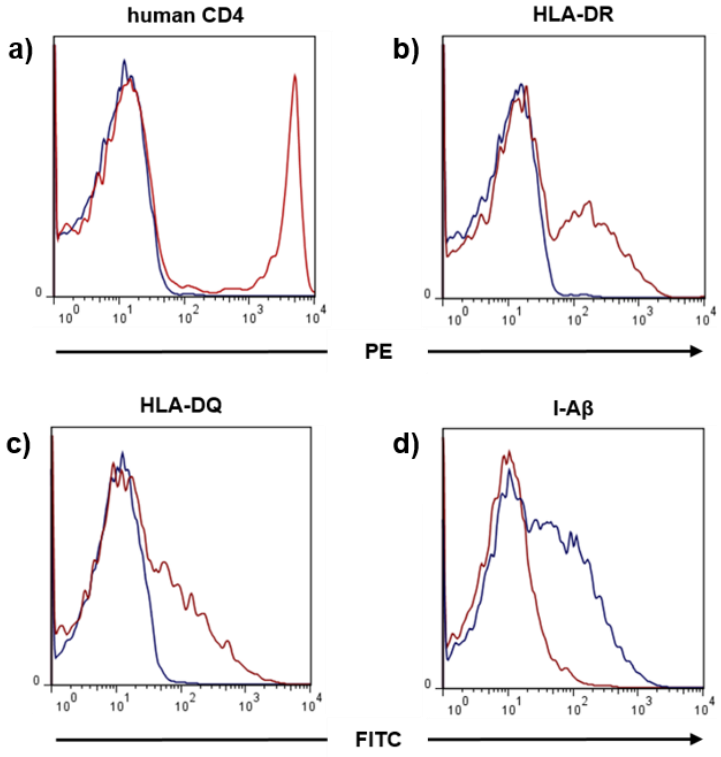

Figure 4.31: Phenotype of HLA-DRB1*04:02–transgenic mice.
Flow cytometry histogram presentation of the transgenes expressed by HLA-DRA1*01:01-DRB1*04:02 /-DQA1*03:01-DQB1*03:02 (DQ8)**–**transgenic (tg) mice on a DBA/1J background: In contrast to wild type mice (blue line) that only exhibited background fluorescence, HLA-DRB1*04:02–tg mice (red line) displayed binding of an anti-human CD4–PE antibody (**a**), anti–HLA-DR–PE antibody (**b**), and anti–HLA-DQ–FITC antibody (**c**). Binding of an anti–I-Aβ–FITC antibody only occurred on leukocytes from wild type mice with two single peaks of fluorescence intensity representing the two different alleles of the gene expressed (**d**). Representative result of each experimental mouse used in this study.

4.2.2 Immunization of HLA-DR4-tg mice with human Dsg3 induces Dsg3-specific IgG

In order to study Dsg3-reactive $CD4^+$ T and B cell responses, as well as determine the level of the Dsg3-specific serum IgG antibody titer, mice transgenic for HLA-DRB1*04:02 were repetitively immunized with recombinant human Dsg3 protein (**2.5.1**) according to the pattern presented in **3.2.1.2a**. For detection of the IgG antibodies by ELISA (**3.2.2.1**), mice sera were diluted in the ratio of 1:20 and optical density (OD) of the colorimetric substrate was measured at 405 nm. As presented in **Figure 4.32**, following two i.p. immunizations with human Dsg3 emulsified in alum on days 0 and 14, the antibody titer strongly rose until a plateau exceeding 2 OD was reached from day 21 on. Maintenance of this titer for at least three weeks was achieved by several booster immunizations. Unlike HLA-DRB1*04:02–tg mice immunized with human Dsg3 protein, control mice injected with PBS and adjuvant alone only displayed background activity.

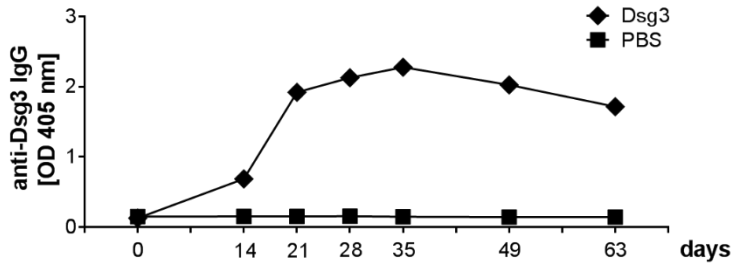

Figure 4.32: Human Dsg3-reactive IgG antibody titer of HLA-DRB1*04:02–transgenic mice.
Immunization of HLA-DRB1*04:02–transgenic (tg) mice with human Dsg3 resulted in a robust Desmoglein 3 (Dsg3)-specific immunoglobulin (Ig) G antibody response as detected by enzyme-linked immunosorbent assay. Two immunizations with human Dsg3 on days 0 and 14 yielded the maximum level of the antigen-specific IgG titer, which was perpetuated by several booster immunizations. HLA-DRB1*04:02–tg mice injected with phosphate-buffered saline (PBS) and adjuvant only served as controls. OD, optical density. Representative result of two individual experiments is shown.

In addition to detecting antigen-specific IgG antibodies by ELISA (**Figure 4.32**), serum samples from HLA-DRB1*04:02–tg mice repetitively immunized with human Dsg3 (**2.5.1**) in alum were also assayed for binding reactivity to native Dsg3 protein as determined by IIF microscopy (**3.2.2.3**). For this purpose, monkey esophagus epithelium was at first incubated with mouse serum samples diluted in the ratio of 1:25, followed by application of the FITC-labeled anti-mouse detection antibody. Apart from recognizing recombinant human Dsg3 (**Figure 4.32**), antibodies abundantly bound to native Dsg3 antigen present on the cell surface of epidermal keratinocytes as visualized in **Figure 4.33a**. In order to test for antigen specificity of the humoral immune response, mice were also immunized with the NC1 domain of Col VII (**2.5.1**) as a control protein since it is produced in the same insect cell expression system, but does not exhibit sequence homology to human Dsg3. Clearly visible, immunization of mice with Col VII did not result in the respective “chicken wire” pattern characteristic of Dsg3-specific IgG tissue deposition, but antibodies bound to Col VII located in the basal membrane zone revealing a typical linear pattern (**Figure 4.33b**).

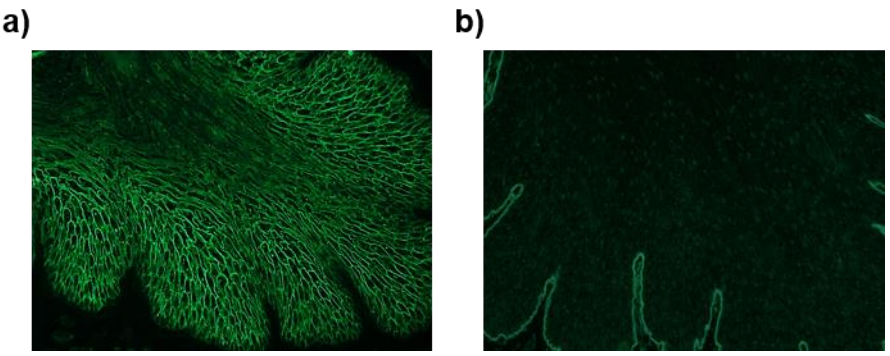

Figure 4.33: Indirect immunofluorescence microscopy of human Dsg3-reactive IgG induced in HLA-DRB1*04:02–transgenic mice.
Immunization of HLA-DRB1*04:02–transgenic mice with human Desmoglein 3 (Dsg3) resulted in the production of Dsg3-specific immunoglobulin (Ig) G antibodies that recognized native Dsg3 protein present on monkey esophagus epithelium as detected by indirect immunofluorescence microscopy (**a**). However, immunization with the noncollagenous domain of Collagen VII (Col VII) as a control protein did not induce IgG antibodies that interacted with Dsg3 antigen, but Col VII located in the basal membrane zone revealing a typical linear pattern (**b**). Representative result of three individual experiments is presented.

4.2.3 T cell recognition of human Dsg3 epitopes is tightly HLA-DRB1*04:02–restricted

4.2.3.1 *In vitro* culture of BMDC with Th cells and CFSE proliferation assay

In PV patients, it was demonstrated that Dsg3-reactive $CD4^+$ T cells are responsive to distinct epitopes of the Dsg3 EC under the restriction by HLA-DRB1*04:02 (Veldman, Gebhard, et al. 2004) based on the peptide binding algorithm presented in **4.2.1**. To assess whether this finding can be reproduced in the HLA-DRB1*04:02–tg mouse model of PV, Dsg3-specific $CD4^+$ T cells were exposed to a set of five HLA-DRβ1*04:02–binding (**2.5.2.1**) and –non-binding (**2.5.2.2**) Dsg3 peptides under *ex vivo* conditions according to the experimental approach described in **3.2.3.2**: As professional APC, mDC were differentiated from BM cells from HLA-DRB1*04:02–tg mice by *in vitro* culture with GM-CSF for seven days as presented in **3.2.3.1**. Since the T cell stimulatory capacity of BMDC rises with cell maturity, BMDC were stimulated with bacterial LPS overnight upon complete differentiation. As confirmed by flow cytometry analysis, maturation of BMDC entailed increased surface expression of HLA-DR required for antigenic peptide presentation and activation of $CD4^+$ T cells (13.8% of $CD11c^+$HLA-DR^{high} cells [**Figure 4.34b**] versus 8.7% of $CD11c^+$HLA-DR^{high} cells in the unstimulated sample [**Figure 4.34a**]). Upon application of LPS, BMDC were also loaded with a set of five Dsg3 peptides that either bind or as a control do not bind to HLA-DRβ1*04:02. Addition of an anti-CD3 mAb was used as a positive control. BMDC pulsed with antigen were subsequently cultured for six days together with Dsg3-responsive $CD4^+$ T cells induced by immunization of HLA-DRB1*04:02–tg mice with human Dsg3 protein (**2.5.1**) according to the pattern described in **3.2.1.2a**. In order to obtain a pure population of $CD4^+$ T cells, splenocytes underwent positive selection by $CD4^+$ MACS, which yielded a purity grade exceeding 95% as confirmed by application of an anti-mouse CD4 APC-coupled antibody using flow cytometry analysis (**Figure 4.35**). To visualize the proliferative response of splenic $CD4^+$ T cells against the Dsg3 peptides presented by activated BMDC using flow cytometry, $CD4^+$ T cells were

stained with the fluorescent dye CFSE that binds to cytosolic proteins. Upon mitosis, distribution and dilution of the dye onto daughter cells was discernible by separated cell generation peaks detected in the FITC channel. For optimum stimulation, Dsg3-responsive $CD4^+$ T cells and antigen-pulsed BMDC were cultured together at different ratios, of which a BMDC–T cell ratio of 1:20 yielded the strongest proliferative response with minimal background cell division activity. A representative result of two respective experiments is presented in **Figure 4.36**.

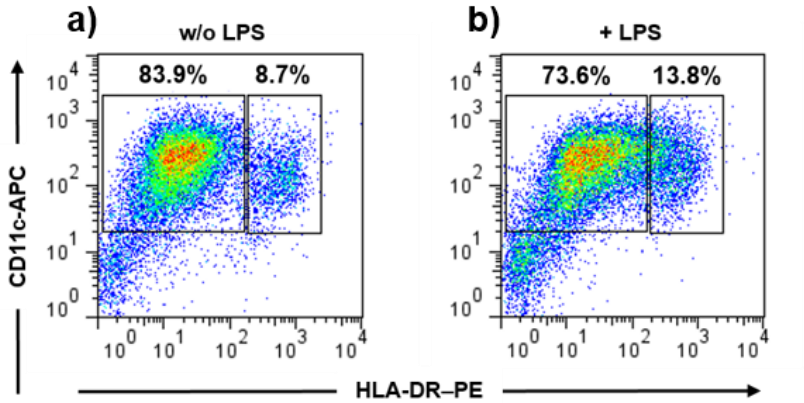

Figure 4.34: Bone marrow-derived dendritic cells from HLA-DRB1*04:02–transgenic mice before and after stimulation with LPS.
Bone marrow-derived DC (BMDC) from HLA-DRB1*04:02–transgenic mice were generated by *in vitro* culture of BM cells with granulocyte macrophage colony-stimulating factor for seven days. Compared to untreated cells (**a**), incubation with lipopolysaccharides (LPS) overnight (**b**) resulted in augmented HLA-DR expression of $CD11c^+$ BMDC as detected by application of an anti-mouse CD11c-antibody and an anti-human HLA-DR antibody conjugated to APC and PE, respectively, using flow cytometry. Representative result of two individual experiments is presented.

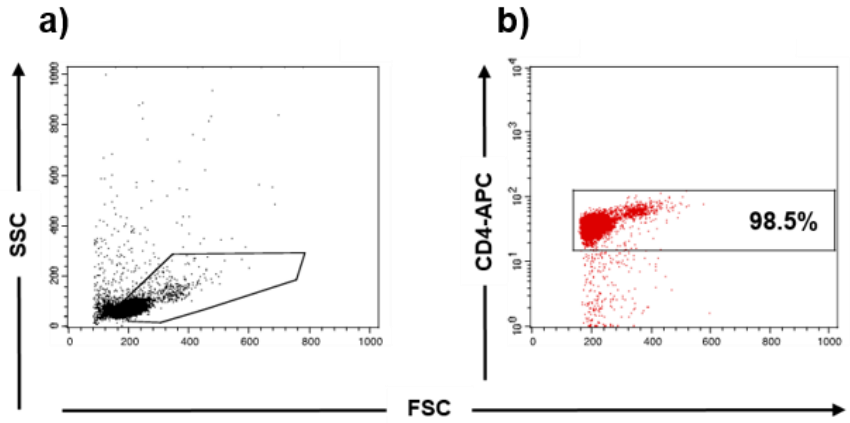

Figure 4.35: Magnetic-activated cell sorting of mouse splenic $CD4^+$ T cells from HLA-DRB1*04:02–transgenic mice.
Positive selection of murine splenic $CD4^+$ T cells from HLA-DRB1*04:02–transgenic mice immunized with human Desmoglein 3 using magnetic-activated cell sorting yielded a population of $CD4^+$ T cells with purity >95% as confirmed by application of an anti-mouse CD4 antibody coupled to APC using flow cytometry analysis (**b**). Based on forward scatter (FSC) and side scatter (SSC), lymphocytes were preselected before quantification of $CD4^+$ T cells (**a**). Representative result of two individual experiments is shown.

As expected and in concordance with the peptide binding algorithm for HLA-DRβ1*04:02 (**4.2.1**), Dsg3-responsive splenic CD4⁺ T cells purified from mice immunized with human Dsg3 protein markedly proliferated (12.0%) upon presentation of a set of five HLA-DRβ1*04:02–binding Dsg3 peptides by BMDC from HLA-DRB1*04:02–tg mice stimulated with LPS (**Figure 4.36a**). *Ex vivo* challenge of splenic CD4⁺ T cells with a set of Dsg3 peptides that do not bind to HLA-DRβ1*04:02 however did not induce a proliferative response beyond background level (5.87%) (**Figure 4.36b**). Culture of splenic CD4⁺ T cells together with BMDC devoid of antigenic cargo served as a negative control (6.64%), whereas addition of an anti-CD3 mAb was used as a positive control (30.7%) (**Figure 4.36c** and **d**). These findings illustrate that in the HLA-DRB1*04:02–tg mouse model of PV, T cell recognition of human Dsg3 epitopes depends on HLA-DRB1*04:02.

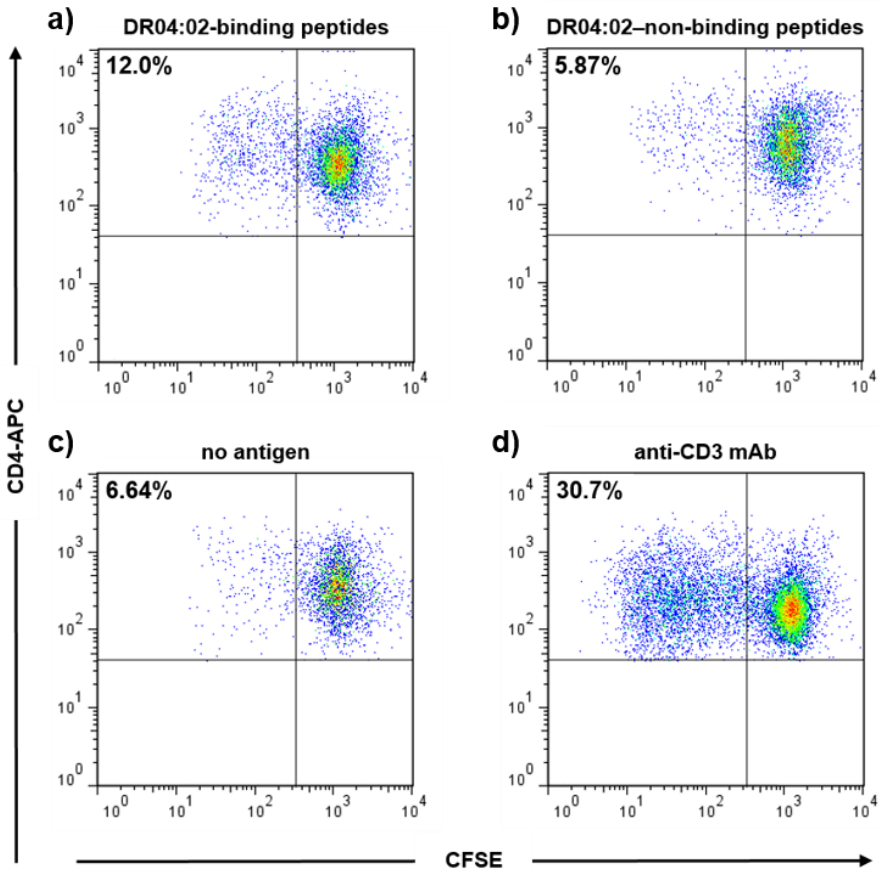

Figure 4.36: Carboxyfluorescein succinimidyl ester proliferation assay of Dsg3-specific T cells and antigen-loaded bone marrow-derived dendritic cells from HLA-DRB1*04:02–transgenic mice.
Reactivity of Desmoglein 3 (Dsg3)-responsive splenic CD4⁺ T cells induced by repetitive immunization of HLA-DRB1*04:02–transgenic mice with human Dsg3 was specified by *ex vivo* challenge of T cells with bone marrow-derived DC (BMDC) from the same mice loaded with a set of either five HLA-DRβ1*04:02–binding or –non-binding Dsg3 peptides. Upon stimulation with BMDC pulsed with a set of five HLA-DRβ1*04:02–binding peptides, Dsg3-specific splenic CD4⁺ T cells mounted a pronounced proliferative response as shown by sequential dilution of the carboxyfluorescein succinimidyl ester (CFSE) dye (**a**). Upon exposure to BMDC loaded with a set of peptides that do not bind to HLA-DRβ1*04:02 however, the proliferative response of T cells was similar to that of exposure to BMDC devoid of antigen (**b**). Challenge with BMDC without addition of antigenic peptides represented the extent of background proliferation (**c**). Application of an anti-CD3 monoclonal antibody to BMDC and splenic CD4⁺ T cells was used as a positive proliferative control (**d**). Representative result of two individual experiments is presented.

4.2.3.2 ELISPOT assay of T cells challenged with Dsg3 epitopes

In the reverse experimental approach, in order to induce a locally restricted $CD4^+$ T cell response in the draining popliteal and inguinal LN following seven days, HLA-DRB1*04:02–tg mice were immunized into the hind foot pads with a set of five HLA-DRβ1*04:02–binding Dsg3 peptides (**2.5.2.1**) emulsified in TiterMax™ according to the pattern described in **3.2.1.2b**. As a control, mice were also immunized with a set of peptides that do not bind to HLA-DRβ1*04:02 (**2.5.2.2**). Upon isolation, LN T cells were challenged *ex vivo* with a panel of antigens consisting of a set of HLA-DRβ1*04:02–binding and –non-binding Dsg3 peptides, as well as human Dsg3 and the NC1 of Col VII protein (**2.5.1**). Stimulation-induced cytokine secretion was quantified following incubation of antigens for 24 hours by ELISPOT assay as described in **3.2.3.3**. As presented in **Figure 4.37**, analysis revealed a significantly increased release of the Th1- and Th2 cell-related signature cytokines IFN-γ and IL-4, respectively, of LN T cells upon both *ex vivo* challenge with human Dsg3 protein (**2.5.1**) or the HLA-DRβ1*04:02–binding Dsg3 peptides. However, *ex vivo* restimulation of LN T cells with the HLA-DRβ1*04:02–non-binding Dsg3 peptides (**2.5.2.2**) or the NC1 of Col VII control protein (**2.5.1**) did not result in a T cell response beyond background level. In contrast, immunization of mice with a set of five Dsg3 peptides that do not bind to HLA-DRβ1*04:02 exclusively resulted in an IFN-γ^+ T cell response to the same set of Dsg3 peptides, whereas importantly, no detectable cytokine release was induced upon *ex vivo* challenge of LN T cells with human Dsg3 protein.

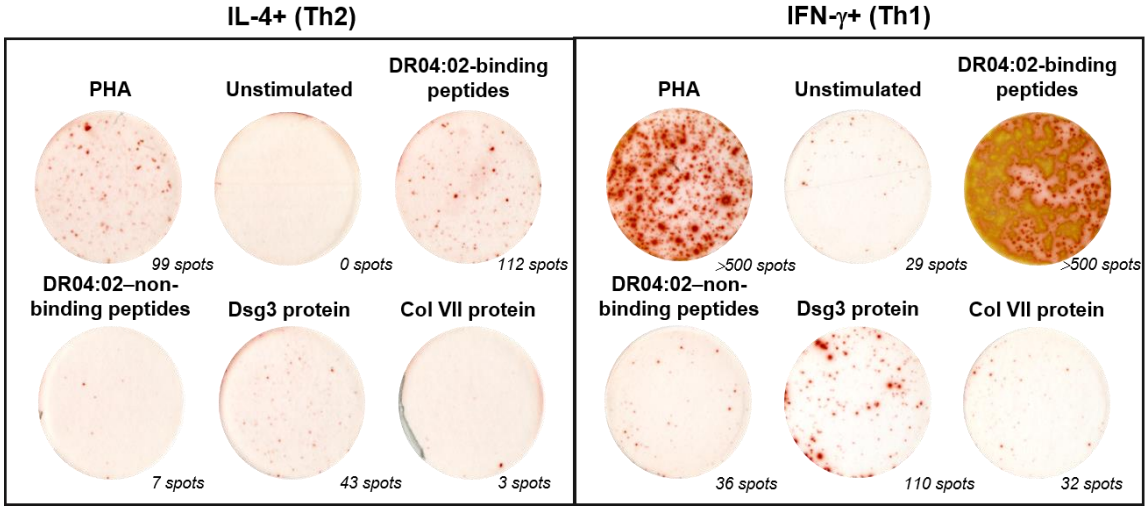

Figure 4.37: Quantitative determination of cytokine-secreting lymph node T cells by ELISPOT assay. Shown are representative enzyme-linked immunospot (ELISPOT) assay membranes seeded with lymph node (LN) cells isolated from HLA-DRB1*04:02–transgenic mice immunized with the HLA-DRβ1*04:02–binding Desmoglein 3 (Dsg3) peptides. The LN T cells were *ex vivo* challenged with phytohemagglutinin (PHA) or left unstimulated as a positive and negative control, respectively. HLA-DRβ1*04:02–binding and –non-binding peptides, as well as the proteins Dsg3 and Collagen VII (Col VII) were the antigens used for *ex vivo* restimulation of LN T cells. Interleukin (IL)-4^+ cells represent T helper (Th) 2 cells, whereas Th1 cells are interferon (IFN)-γ^+. For calculation of the number of cytokine-secreting LN T cells, the number of spots of the unstimulated control was subtracted from the samples stimulated with antigen. The number of spots is indicated per $1 \cdot 10^5$ LN T cells.

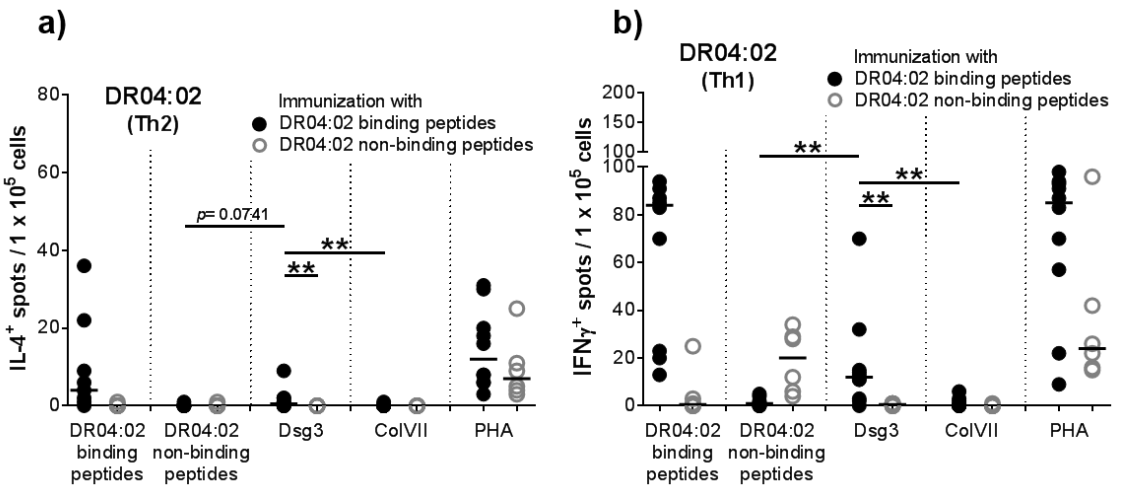

Figure 4.38: Dsg3-specific T cells from HLA-DRB1*04:02–transgenic mice recognized a limited set of Dsg3 peptides.

Draining lymph node T cells from HLA-DRB1*04:02–transgenic mice immunized with a set of five HLA-DRβ1*04:02–binding Desmoglein 3 (Dsg3) peptides displayed both interferon (IFN)-γ^+ (**b**) and interleukin (IL)-4^+ (**a**) T cell responses upon *ex vivo* challenge with human Dsg3 and the HLA-DRβ1*04:02–binding Dsg3 peptides, unlike with the HLA-DRβ1*04:02–non-binding Dsg3 peptides or Collagen VII (Col VII) control protein. Immunization of mice with the Dsg3 peptides that do not bind to HLA-DRβ1*04:02 only resulted in an IFN-γ^+ T cell reaction upon *ex vivo* challenge with the same peptides **b**). Antigens used for restimulation are shown on abscissae. PHA, phytohemagglutinin; Th, T helper cell. Cumulative results of 6–10 mice are presented. Differences were considered significant for $p< 0.05$ (indicated as *) and very significant for $p< 0.01$ (indicated as **) as determined by Mann-Whitney U test.

As a specificity control, this experiment was additionally performed by the collaboration group of J. Bäcklund using mice transgenic for HLA-DRB1*04:01 as shown in **Figure 4.39**. Immunization of these mice with the same set of HLA-DRβ1*04:02–binding Dsg3 peptides did neither yield an IFN-γ^+ (except of one mouse) nor IL-4^+ T cell response upon *ex vivo* restimulation of splenic $CD4^+$ T cells with human Dsg3. Upon both immunization and *ex vivo* challenge with the HLA-DRβ1*04:02–binding Dsg3 peptides, both strong IFN-γ^+ and IL-4^+ T cell responses were induced, which was not observed upon *ex vivo* challenge with the HLA-DRβ1*04:02–non-binding Dsg3 peptides or Col VII protein. Similarly, immunization of HLA-DRB1*04:01–tg mice with a set of five Dsg3 peptide that do not bind to HLA-DRβ1*04:02 did neither lead to an IFN-γ^+ nor IL-4^+ T cell reaction upon *ex vivo* restimulation with human Dsg3. Only again, both immunization and *ex vivo* challenge with the HLA-DRβ1*04:02–non-binding Dsg3 peptides resulted in a pronounced IFN-γ^+ and IL-4^+ T cell response.

Application of the mitogens PHA and concanavalin A (ConA) served as a positive control for LN cell and splenic $CD4^+$ T cell viability, respectively, whereas background stimulation of LN T cells from HLA-DRB1*04:02–tg mice was assessed by injection of mice with PBS and adjuvant only (**Figure 4.40**).

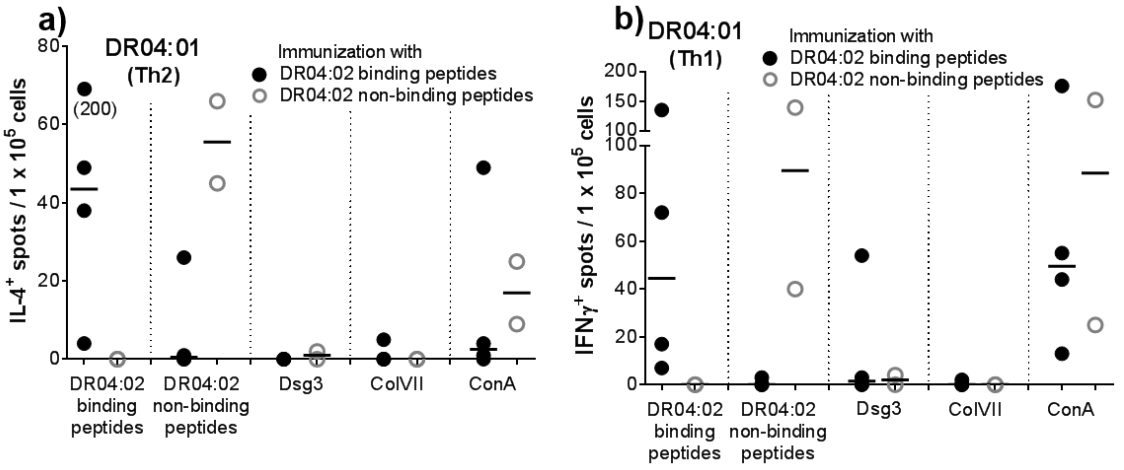

Figure 4.39: Dsg3-specific T cells from HLA-DRB1*04:01–transgenic mice did not recognize the Dsg3 peptides.
Splenic CD4$^+$ T cells from HLA-DRB1*04:01–transgenic mice immunized with either a set of Desmoglein 3 (Dsg3) peptides that bind or do not bind to HLA-DRβ1*04:02 only exhibited interferon (IFN)-γ$^+$ (**b**) and interleukin (IL)-4$^+$ (**a**) T cell reactivity upon *ex vivo* challenge with the respective set of Dsg3 peptides. Importantly, upon *ex vivo* challenge with Dsg3 protein, no T cell responses could be observed except of an IFN-γ$^+$ T cell response of one mouse. Antigens used for restimulation are shown on abscissae. Col VII, Collagen VII; ConA, concanavalin A; Th, T helper cell. Cumulative results of 2–4 mice are presented.

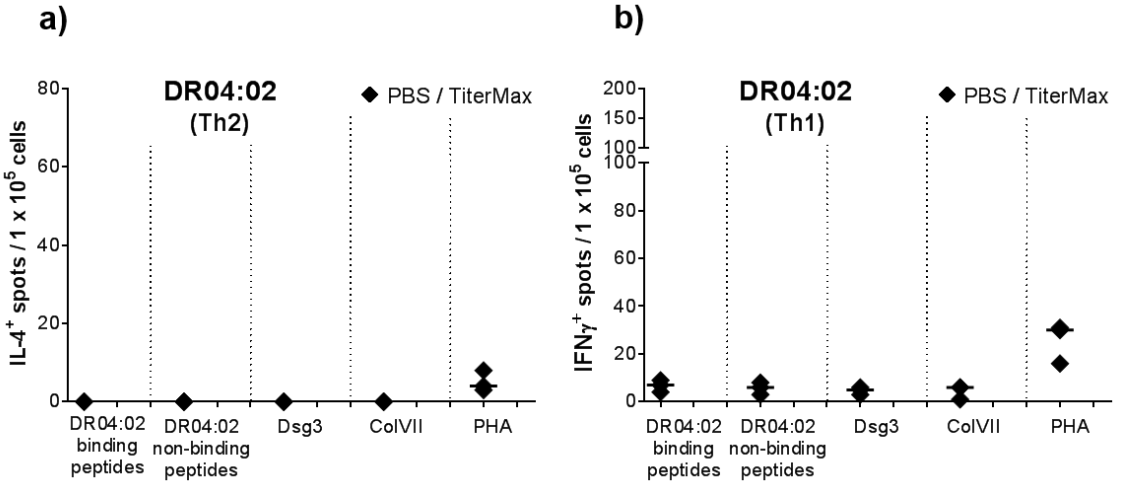

Figure 4.40: Background activity of lymph node T cells from HLA-DRB1*04:02–transgenic mice.
Immunization of HLA-DRB1*04:02–transgenic mice with phosphate-buffered saline (PBS) and adjuvant (TiterMax^{TM}) alone did neither induce background interferon (IFN)-γ$^+$ (**b**) nor interleukin (IL)-4$^+$ (**a**) T cell responses upon *ex vivo* stimulation with the set of protein and peptide antigens used in this study. Application of phytohemagglutinin (PHA) served as a positive control. Antigens used for restimulation are shown on abscissae. Dsg3, Desmoglein 3; Col VII, Collagen VII. Cumulative results of two mice are presented.

To summarize, only *ex vivo* challenge of LN T cells from HLA-DRB1*04:02–tg mice immunized with a set of five HLA-DRβ1*04:02–binding Dsg3 peptides with human Dsg3 protein or the same peptides yielded both an IFN-γ$^+$ and IL-4$^+$ T cell response. In addition, an IFN-γ$^+$ T cell response was induced upon both immunization of these mice and *ex vivo* restimulation with the HLA-DRβ1*04:02–non-binding Dsg3 peptides as a particularity. Contrariwise, splenic CD4$^+$ T cells

from HLA-DRB1*04:01–tg mice generally merely responded to the respective Dsg3 peptides they were immunized with.

These findings confirm the conclusions drawn from the reverse experiment described in **4.2.3.1** of T cell recognition of human Dsg3 epitopes being tightly restricted by HLA-DRB1*04:02.

4.2.4 Immunization of HLA-DR4–tg mice with Dsg3 peptides induces Dsg3-specific IgG

4.2.4.1 ELISA of sera from Dsg3 peptides-immunized HLA-DR4–tg mice

Since the experiments described in **4.2.3.1** and **4.2.3.2** clearly demonstrated that in the HLA-DRB1*04:02–transgenic mouse model of PV, $CD4^+$ T cell recognition of the Dsg3 EC is tightly restricted by HLA-DRB1*04:02, we next addressed the question whether induction of Dsg3-reactive IgG antibodies as well is regulated by HLA-DRB1*04:02. For this purpose, sera from HLA-DRB1*04:02–tg mice immunized according to the scheme presented in **3.2.1.2a** with either a set of HLA-DRβ1*04:02–binding (**2.5.2.1**) or –non-binding (**2.5.2.2**) Dsg3 peptides were assayed for reactivity against recombinant human Dsg3 by ELISA (**3.2.2.1**). Mice sera were diluted in the ratio of 1:25 and OD of the colorimetric substrate was measured at 405 nm.

Interestingly, as shown in **Figure 4.41a**, sera from HLA-DRB1*04:02–tg mice immunized with a set of five HLA-DRβ1*04:02–binding Dsg3 peptides displayed pronounced IgG reactivity against recombinant Dsg3 – approximately half of the antibody titer level achieved by immunization of mice with human Dsg3 protein. In contrast, immunization of these mice with a set of five Dsg3 peptides that do not bind to HLA-DRβ1*04:02 did not result in the production of IgG antibodies recognizing Dsg3 protein.

Intriguingly, opposed to HLA-DRB1*04:02–tg mice, sera from control mice transgenic for HLA-DRB1*04:01 did not exhibit Dsg3-specific IgG antibody responses upon immunization with either the HLA-DRβ1*04:02–binding or –non-binding peptides as shown in **Figure 4.41b**. Analogous to HLA-DRB1*04:02–tg mice (OD of ca. 3.5), immunization of control mice with human Dsg3 protein (**2.5.1**) yielded a strong Dsg3-specific humoral immune response, although to a much lesser extent (OD of ca. 1.1). These findings clearly illustrate that HLA-DRB1*04:02**–**restricted $CD4^+$ T cell recognition of specific Dsg3 epitopes is essential for the induction of Dsg3-reactive IgG antibodies.

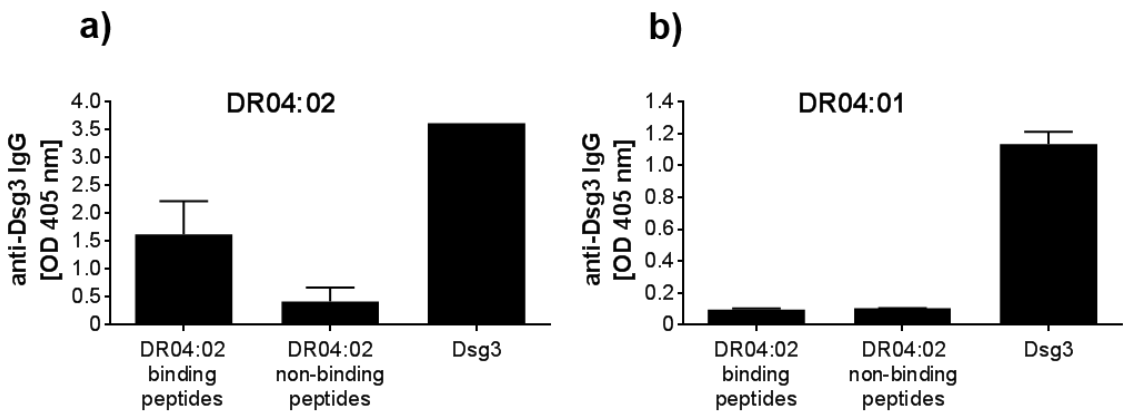

Figure 4.41: Enzyme-linked immunosorbent assay of sera from HLA-DR4–transgenic mice immunized with Dsg3 peptides.

Immunization of HLA-DRB1*04:02–transgenic (tg) mice with a set of HLA-DRβ1*04:02–binding Desmoglein 3 (Dsg3) peptides induced immunoglobulin (Ig) G reactivity against recombinant human Dsg3 as shown by enzyme-linked immunosorbent assay using serum samples diluted in the ratio of 1:25. Contrariwise, immunization of mice with a set of HLA-DRβ1*04:02–non-binding peptides did not yield Dsg3-reactive IgG antibodies. Upon immunization of mice with Dsg3 protein, a robust antibody response was mounted (optical density [OD]≈ 3.5). Cumulative results of three mice per experiment are presented (**a**). In contrast, sera from HLA-DRB1*04:01–tg mice diluted in the same ratio did not display Dsg3-specific IgG antibody induction upon immunization with neither the HLA-DRβ1*04:02–binding nor –non-binding peptides. Immunization with Dsg3 protein resulted in a pronounced, but compared to HLA-DRB1*04:02–tg mice much weaker IgG antibody response (OD≈ 1.1) (**b**). Cumulative results of 2–4 mice are presented.

4.2.4.2 IIF of sera from Dsg3 peptides-immunized HLA-DR4–tg mice

In addition to binding to recombinant Dsg3 protein as determined by ELISA (**3.2.2.1**), serum samples of HLA-DRB1*04:02–tg mice immunized with a set of Dsg3 peptides that either bind (**2.5.2.1**) or do not bind (**2.5.2.2**) to HLA-DRβ1*04:02 according to the pattern shown in **3.2.1.2a** were assayed for IgG reactivity against native Dsg3 protein expressed on monkey esophagus epithelium. Therefore, mice sera were diluted in the ratio of 1:25, incubated on epidermal keratinocytes, and binding of the FITC-tagged secondary antibody was detected by IIF microscopy (**3.2.2.3**).

As presented in **Figure 4.42a** and **b**, immunization of mice with a set of HLA-DRβ1*04:02–binding Dsg3 peptides induced IgG antibodies that strongly interacted with native Dsg3 protein expressed by esophagus epithelium. Immunization of mice with control peptides however did not lead to the generation of anti-epithelial surface IgG, but only unspecific background staining (**Figure 4.42c** and **d**).

Strikingly, immunization of HLA-DRB1*04:01–tg control mice with neither the HLA-DRβ1*04:02–binding (**Figure 4.43a**) nor –non-binding (**Figure 4.43b**) peptides resulted in the “chicken wire” pattern typical of Dsg3-specific IgG tissue deposition.

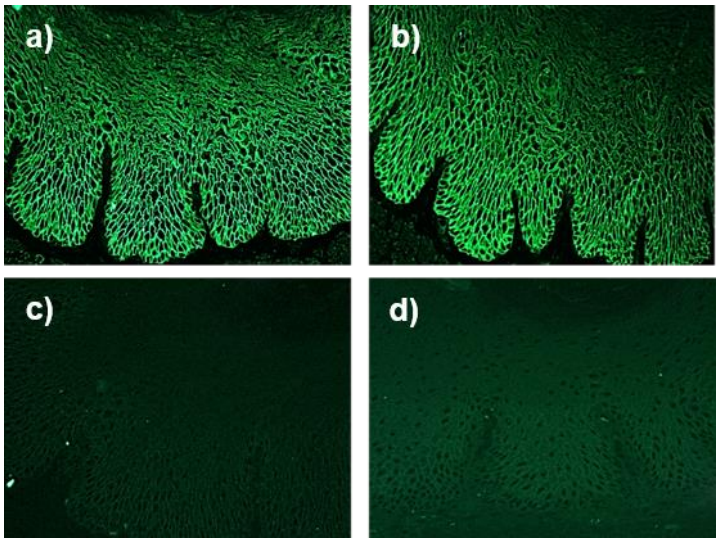

Figure 4.42: Indirect immunofluorescence microscopy of sera from HLA-DRB1*04:02–transgenic mice immunized with Dsg3 peptides.
Sera from HLA-DRB1*04:02–transgenic mice immunized with the Desmoglein 3 (Dsg3) peptides that bind to HLA-DRβ1*04:02 (**a** and **b**) displayed immunoglobulin (Ig) G reactivity against native Dsg3 as shown by indirect immunofluorescence microscopy. However, sera from mice immunized with the HLA-DRβ1*04:02–non-binding peptides did not exhibit Dsg3-specific IgG tissue deposition (**c** and **d**). Mouse sera were diluted in the ratio of 1:25. Representative results of three mice are shown.

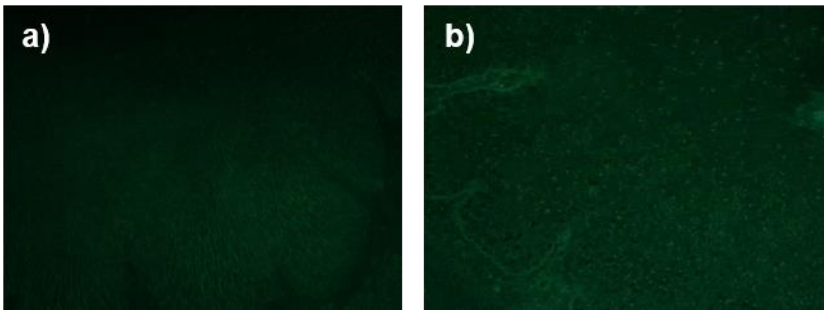

Figure 4.43: Indirect immunofluorescence microscopy of sera from HLA-DRB1*04:01–transgenic mice immunized with Dsg3 peptides.
Immunization of HLA-DRB1*04:01–transgenic mice with neither the HLA-DRβ1*04:02–binding (**a**) nor –non-binding Desmoglein 3 (Dsg3) peptides (**b**) resulted in the production of antibodies recognizing native Dsg3 protein as visualized by indirect immunofluorescence microscopy. Mouse sera were diluted in the ratio of 1:25. Representative result of 2–4 mice are displayed.

As shown in **Figure 4.44**, sera from mice transgenic for either HLA-DRB1*04:02 or –DRB1*04:01 displayed high IgG reactivity against native Dsg3 upon immunization with human Dsg3 protein.

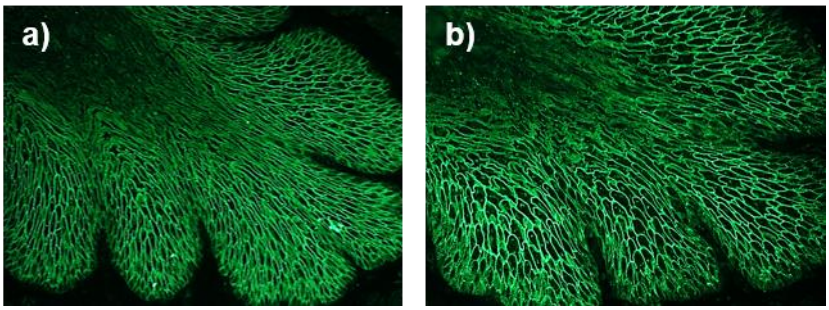

Figure 4.44: Indirect immunofluorescence microscopy of sera from HLA-DR4–transgenic mice immunized with human Dsg3 protein.
Immunization of mice transgenic for HLA-DRB1*04:02 (**a**) or –DRB1*04:01 (**b**) with human Desmoglein 3 (Dsg3) protein led to the generation of antibodies recognizing native Dsg3 protein present on monkey esophagus epithelium as detected by indirect immunofluorescence microscopy. Mouse sera were diluted in the ratio of 1:25. Representative result of 2–4 mice are displayed.

Taken together, the here described findings confirmed those presented in **4.2.4.1** of only sera from HLA-DRB1*04:02–tg mice immunized with the HLA-DRβ1*04:02–binding Dsg3 peptides exhibiting IgG reactivity against both recombinant and native Dsg3 protein (**Figures 4.41a**, and **4.42a** and **b**). In contrast, immunization of HLA-DRB1*04:01–tg with neither the HLA-DRβ1*04:02–binding nor –non-binding peptides induced IgG reactive against recombinant Dsg3 protein (**Figure 4.41b**) or that expressed by monkey esophagus epithelium (**Figure 4.43**). Solely immunization of both HLA-DR–tg mice with human Dsg3 protein led to the production of antibodies interacting with recombinant (**Figure 4.41**) and native Dsg3 (**Figure 4.44**).

4.2.5 IgG cross-reactivity of sera from human Dsg3-immunized HLA-DR4-tg mice

4.2.5.1 WB of sera from human Dsg3-immunized HLA-DR4–tg mice

Repetitive immunization of HLA-DRB1*04:02–tg mice with human Dsg3 protein (**2.5.1**) according to the scheme illustrated in **3.2.1.2a** did not induce blisters and erosions of mucous membranes, the phenotype characteristic of PV. In order to assess whether the homology between the EC of human and mouse Dsg3 is ample for human Dsg3-reactive IgG antibodies to bind to recombinant murine Dsg3 *ex vivo*, serum samples from HLA-DRB1*04:02–tg mice immunized with human Dsg3 were tested for recognition of the mouse Dsg3 EC (**2.5.1**) by western blot (WB) analysis (**3.2.2.2**). As presented in **Figure 4.45**, IgG reactivity against the human Dsg3 EC (72 kDa) was pronounced, whereas it was markedly reduced against the murine Dsg3 EC (75 kDa). Binding of an anti-E Tag antibody served as a positive control and the NC1 of Col VII (**2.5.1**) was used as a noncross-reactive control protein.

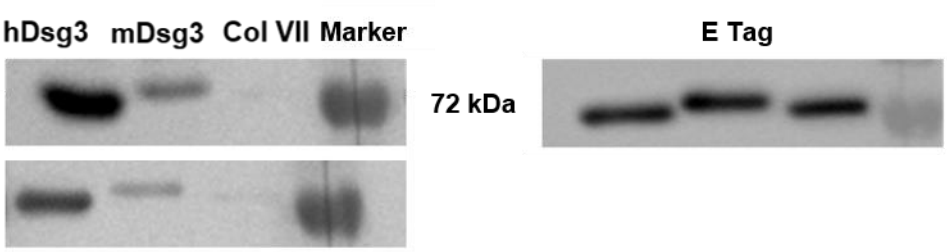

Figure 4.45: Immunoblotting analysis of sera from HLA-DRB1*04:02–transgenic mice immunized with Dsg3 protein.
Sera from HLA-DRB1*04:02–transgenic mice immunized with human Desmoglein 3 (hDsg3) exhibited robust immunoglobulin G reactivity against human Dsg3, but diminished reactivity against murine Dsg3 (mDsg3) as determined by immunoblotting analysis. Recognition of proteins by an anti-E Tag antibody served as a positive control, whereas Collagen VII (Col VII) was used as control protein produced in the same expression system. Representative result of five mice is displayed.

4.2.5.2 DIF of sera from human Dsg3-immunized HLA-DR4–tg mice

As illustrated in **Figure 4.45**, immunoblotting analysis revealed weak recognition of the analog recombinant mouse Dsg3 EC by the anti-human Dsg3-specific IgG antibodies present in the sera of HLA-DRB1*04:02–tg mice immunized (**3.2.1.2a**) with human Dsg3 protein. In order to assess whether human Dsg3-responsive IgG antibodies also target murine Dsg3 under *in vivo* conditions, tissue samples from HLA-DRB1*04:02–tg mice immunized with human Dsg3 were assayed for binding of antibodies to both tongue and palatinal mucosa using DIF microscopy (**3.2.2.3**).
As presented in **Figure 4.46**, tissue-bound IgG antibodies were present on neither mouse palate (**a**) nor tongue (**b**) epithelium. Lack of binding of anti-human Dsg3-reactive IgG antibodies to the murine Dsg3 EC may explain the absence of a blistering and erosive phenotype of HLA-DRB1*04:02–tg mice upon immunization with human Dsg3 protein.

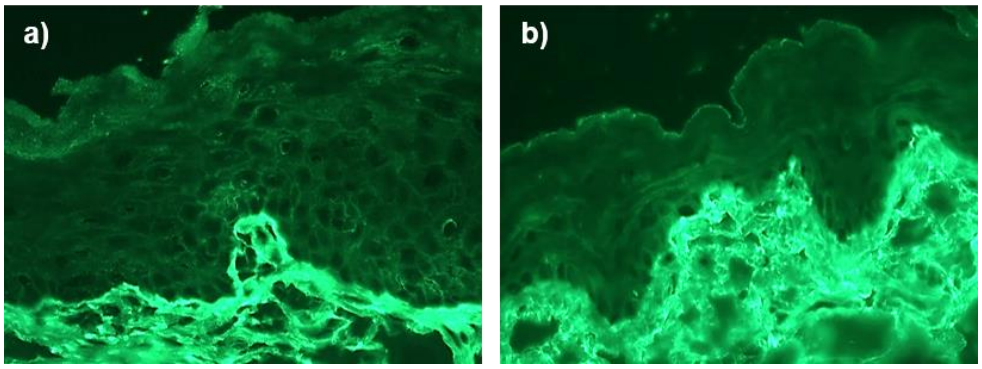

Figure 4.46: Direct immunofluorescence microscopy analysis of sera from HLA-DRB1*04:02–transgenic mice immunized with human Dsg3 protein.
Palatinal (**a**) and tongue (**b**) mucosa from HLA-DRB1*04:02–transgenic mice immunized with human Desmoglein 3 (Dsg3) protein did not display tissue deposition of Dsg3-reactive antibodies as detected by direct immunofluorescence microscopy. Representative results of 5 mice are shown.

5 Discussion

5.1 Human study

Aim of the first part of this doctoral thesis was to investigate APC and APC-derived cytokines, as well as their relation to Th cell subsets and to the auto-ab response in the pathogenesis of pemphigus.

Circulating IgG auto-ab are a characteristic hallmark of the rare, but potentially fatal autoimmune bullous skin disorder pemphigus and predominantly target the desmosomal cadherins Dsg1 and Dsg3, thereby causing loss of epidermal keratinocyte adhesion (Hertl and Veldman 2001; Amagai 2002). Therefore, pemphigus is considered as a model for an auto-ab–mediated organ-specific disorder (Amagai 2002). To date, the precise immunological events involved in the initiation and perpetuation of this disease are not yet completely understood, confining therapeutic options primarily to broad systemic immunosuppression often associated with severe side effects and comorbidities (Frew, Martin, and Murrell 2011). Numerous studies provide evidence for autoreactive Dsg-specific $CD4^+$ T cells playing a critical role in controlling self-destructive IgG auto-ab responses in the pathogenesis of pemphigus (Hertl, Amagai, et al. 1998a; Nishifuji et al. 2000; Takahashi et al. 2008; Takahashi, Kuwana, and Amagai 2009; Eming et al. 2008; Amber et al. 2013). Since the activation of $CD4^+$ T cells generally relies on the interaction with APC, the contribution of cytokines to disease development has been more and more analyzed in several studies (Giordano and Sinha 2012). However, the specific function of APC in the generation of autoreactive antibodies is largely unknown.

Cohorts of clinically well characterized pemphigus patients, as well as HC were recruited for this cross-sectional study, whereas patients affected with the neuromuscular disorder MG served as a further unrelated auto-ab–mediated and organ-specific disease control. First of all, circulating populations of DC and monocytes were phenotypically characterized in peripheral venous blood of the test subjects, including markers for activation and migration. Functional properties of the blood monocyte compartment were investigated, including their ability to synthesize and release cytokines, as well as their capacity to internalize antigen. Beside APC of the innate immune response, the distribution of $CD4^+$ T cell subpopulations, as well as serum autoantigen-specific IgG antibody titers were determined. These titers were finally related to the parameters assessed in both the APC and $CD4^+$ T cell compartment in order to uncover novel potential relationships in the process of auto-ab production in pemphigus.

The findings obtained in this study revealed altered DC behavior in active pemphigus patients, such as diminished frequencies of circulating mDC and pDC along with an increased CCR2 expression of mDC, indicating enhanced migration activities of mDC into inflamed peripheral tissues. Furthermore, increased frequencies of blood $CD16^+$ monocytes, among which the nonclassical subset was additionally elevated were found in active pemphigus patients, as well as augmented CCR2 expression of $CD14^+$ monocytes. Yet, circulating monocytes from pemphigus patients did neither display increased stimulation-induced production of the proinflammatory cytokines IL-6 and TNF-α nor differences in maximum antigen uptake capacity.

Nevertheless, active pemphigus patients exhibited enhanced plasma levels of TNF-α and the immunomodulatory IL-27, of which the latter apparently rather exerts an inflammatory function. Although in active pemphigus patients, neither elevated frequencies of circulating Th1 nor Th2 cells were detected, these patients displayed augmented populations of both Th17 cells and IL-10–synthesizing T cells.

Important for pemphigus pathology, a significant correlation was not only found between serum IgG auto-ab titers and the plasma levels of both IL-6 and TNF-α, but also between auto-ab titers and the plasma concentrations of IL-27. Furthermore, similarly augmented plasma levels of IL-21 exerting a crucial function in B cell activation possibly indicate the mechanism of an IL-27–mediated activation of IL-21–producing Tfh cells (Batten et al. 2010).

5.1.1 Role of dendritic cells in pemphigus

An initial focus was put on the APC of the innate immune system, to which the circulating subsets of DC and monocytes belong. The broad family of DC discovered by Steinman et al. in 1973 holds a central position in the immune system because they represent specialized sentinels connecting the innate and adaptive immune responses without directly engaging in effector activities, such as the eradication of pathogens (Steinman and Cohn 1973a; Ganguly et al. 2013). Following recognition and procession of the pathogen, DC instantly migrate to the T cell areas of lymphoid organs, where they present pathogen-derived antigens to the respective antigen-specific T cells (Ganguly et al. 2013).

Human circulating DC comprise two major classes with different appearance, function, cytokine profile, as well as expression of pathogen-sensing receptors and cell surface markers, the $CD14^{-}$ $CD11c^{++}$ mDC and pDC that may represent the precursors of the DC located in tissues and LN (Collin, McGovern, and Haniffa 2013). Recently, an additional highly proinflammatory subset of DC within the heterogeneous population of blood $CD16^{+}CD14^{-}$ inflammatory monocytes termed slanDC was identified by Schaekel et al. that was also found to be present in the inflammatory infiltrate in psoriasis, RA, and CD (Schakel et al. 2002; Schakel 2009).

The engagement of DC in tolerance and autoimmunity is considered to be complex and dichotomous since on the one hand, DC establish and maintain immune tolerance through various mechanisms, such as the induction of both Treg cells and T cell anergy. On the other hand, the extraordinary T cell stimulatory capacity of DC may also facilitate the priming and differentiation of autoreactive T cells, a consequence of either misguided activation stimuli or a cell-specific disruption of negative regulation. Accordingly, a potential involvement of a qualitatively or quantitatively altered DC compartment in disease pathogenesis has been implicated in several autoimmune disorders, including autoimmune myocarditis, MS, psoriasis, TID, and SLE (Eriksson, Ricci, et al. 2003; Eriksson, Kurrer, et al. 2003; Serafini et al. 2000; Greter et al. 2005; Karni et al. 2006; Lande et al. 2008; Schwab et al. 2010; Calderon et al. 2008; Tortola et al. 2012; Guiducci et al. 2010; Turley et al. 2003; Ronnblom and Alm 2001; Bave et al. 2003; Means et al. 2005).

In our study, active pemphigus patients displayed reduced frequencies of circulating mDC with significance close to the threshold (p= 0.0875) compared to HC, and significantly versus MG

controls (**Figure 4.8a**). Interestingly, this diminution of mDC concurred with a significantly augmented expression of CCR2 (**Figure 4.11a**). In addition, blood pDC were also found to be decreased in active pemphigus patients (**Figure 4.8b**), whereas slanDC numbers did not vary between the test subject groups (**Figure 4.9**).

The capacity of DC to instruct adaptive immune responses is a consequence of their localization within tissues and their specialized ability for mobilization (Randolph, Ochando, and Partida-Sanchez 2008). In order to enter nonlymphoid peripheral tissues and move within them, mDC express chemokine receptors indicating an immature immunogenicity status, such as CCR2 (Geissmann, Jung, and Littman 2003; Merad et al. 2002), CCR5 (Stumbles et al. 2001; Yamagami et al. 2005), and CCR6 (Merad et al. 2004). Upon activation induced by the recognition of PAMP or danger-associated signals, DC downregulate their responsiveness to the respective inflammatory chemokines (Alvarez, Vollmann, and von Andrian 2008). Instead they upregulate CCR7 (Forster, Davalos-Misslitz, and Rot 2008), thereby acquiring a migratory phenotype, and express structures associated with maturation, including CD40, CD80, CD86, and HLA-DR (Randolph, Ochando, and Partida-Sanchez 2008).

The reduced circulating population size and instantaneous upregulation of CCR2 of mDC in pemphigus patients is likely interpreted by mDC being recruited from the circulation into the inflamed skin. Although the CCR2 ligands, CCL2 and CCL7 are marginally expressed in resting tissues, epidermal keratinocytes of the skin produce considerable amounts of these chemokines under inflammatory conditions (Merad et al. 2004; Vanbervliet et al. 2002). In pemphigus, the scenario of CCR2-mediated skin infiltration of mDC is underpinned by the findings by Chiossi et al. of dermal DC numbers correlating with the serum IgG auto-ab titers in a cohort of Brazilian PF patients (Chiossi, Costa, and Roselino 2004). In addition, Picut et al. found the local presence of DC being associated with intraepidermal cleft formation in skin biopsies from PV patients and suggested that these DC represented functionally active APC possibly contributing to the development of lesions (Picut et al. 1987).

According to these observations in pemphigus, a study by Clarkson et al. reported of an accumulation of DC in the central nervous system (CNS) in experimental MS and this effect was dependent on CCR2. Furthermore, CCR2-expressing DC were demonstrated to be critical for effector T cell restimulation and progression of the disease (Clarkson et al. 2015). Similarly, the work by Dogan et al. observed in a murine model of EAE glia-derived CCL2 attracting TNF– and nitric oxide synthase (NOS)-expressing macrophages and mDC to the CNS (Dogan, Elhofy, and Karpus 2008). Moreover, the work by Henkel et al. demonstrated in spinal cord tissues from patients affected with amyotrophic lateral sclerosis (ALS) an enrichment of DC concurrent with an increased expression of CCL2. In addition, ALS patients with a higher extent of DC accumulation in neurological tissues exhibited an accelerated progression of the disease compared to those with a reduced local involvement of DC (Henkel et al. 2006). Apart from that, human pancreatic islets were shown to constitutively produce CCL2 being upregulated by the presence of proinflammatory cytokines, such as TNF-α and IL-1β (Piemonti et al. 2002). Owing to their expression of CCR2, blood monocytes and mDC might be directed to the islets by locally

secreted CCL2 and this way contribute to the perpetuation of the autoimmune response in TID (Penna, Sozzani, and Adorini 2001).

Interestingly, in concordance with our findings in pemphigus, a study by Nieminen et al. also observed decreasing frequencies of circulating mDC and pDC populations in recent-onset pediatric TID. Yet, they detected a reduced surface expression of CCR2 on both mDC and pDC from these patients (Nieminen et al. 2012). In the pathogenesis of SLE, Khan et al. reported of a decrease of circulating mDC in patients affected with active disease, although they did not investigate whether this reduction concurred with an altered expression of CCR2 (Khan et al. 2013). In accordance with that, further studies also found in lupus patients a decreased frequency of circulating mDC (Scheinecker et al. 2001; Migita et al. 2005; Fiore et al. 2008; Jin et al. 2008). Furthermore, in psoriasis, the work of Prignano et al. similarly identified a diminished population of peripheral DC, including mDC and pDC in untreated patients and also assumed an augmented recruitment of DC into cutaneous plaques in these patients (Prignano et al. 2012). In primary SS, the number of blood DC was as well observed to be decreased, particularly that of the $CD1a^+$ subset. Interestingly, they detected a local increase of mononuclear cells in the patients' salivary glands, again suggesting trafficking of DC into peripheral sites of inflammation (Ozaki et al. 2001). Of note, a protective function of DC-expressed CCR2 has also been observed in autoimmune conditions, such as murine RA (Quinones et al. 2004).

To summarize, our hypothesis of CCR2-mediated migration into the inflamed skin based on the detected reduced circulating frequencies of mDC and augmented expression of CCR2 in active pemphigus is supported by similar findings in other autoimmune diseases. Accordingly, not only in autoimmune disorders with mainly auto-ab–induced pathology, such as SLE (Khan et al. 2013), TID (Nieminen et al. 2012), and SS (Ozaki et al. 2001) blood mDC populations were found to be diminished, but also in psoriasis (Prignano et al. 2012). Further human and experimental studies observed an enrichment of DC populations in the respective organ targeted, i.e. the CNS in EAE (Clarkson et al. 2015; Dogan, Elhofy, and Karpus 2008) and ALS (Henkel et al. 2006), as well as the pancreatic islands in TID (Penna, Sozzani, and Adorini 2001), with migration depending on CCR2 in both EAE and TID. The found increased expression levels of CCL2 in EAE (Dogan, Elhofy, and Karpus 2008), ALS (Henkel et al. 2006), and TID (Piemonti et al. 2002) thereby provide indirect evidence for a central role of CCR2 in the migration of DC in autoimmune disease. Interestingly, the extent of DC accumulation within the affected tissue was also found to be critical for disease progression in both EAE (Clarkson et al. 2015) and ALS (Henkel et al. 2006).

Like mDC, circulating pDC are also able to enter inflamed tissues although their responsiveness towards proinflammatory chemokines, such as CCL2, CCL5, and CCL20 is considerably reduced despite their chemokine receptor profile resembling that of mDC, including CCR2, CCR5, CXCR3, CXCR4, and CCR7 (Penna, Sozzani, and Adorini 2001). In the steady state, pDC rather migrate to secondary lymphoid organs attracted by the two CCR7-related chemokines CCL19 and CCL21 (Penna, Sozzani, and Adorini 2001).

Several autoimmune disorders are associated with an accumulation of pDC in lymphoid and peripheral tissues, such as SLE, psoriasis, and RA, as well as allergic diseases (Sozzani et al.

2010). In normal skin, pDC are absent, yet, in some inflammatory dermatoses, such as lupus erythematosus (LE) (Banchereau and Pascual 2006) and psoriasis (Nestle et al. 2005), pDC infiltrate the skin, in which they orchestrate local immune responses. In LE skin lesions, pDC were found to persist throughout the whole progression of the disease and to specifically locate to the dermis and dermo-epidermal junction where epithelial damage takes place (Vermi et al. 2009). Cytotoxic damage is a key pathological event in the interface dermatitis of LE and might be perpetuated by the local release of cytotoxic molecules by pDC (Sozzani et al. 2010). However in psoriasis, the infiltration of pDC is predominantly confined to the dermis and occurs during the early phases of the disease (Nestle et al. 2005).

Apart from skin autoimmune diseases, a study by Lande et al. reported of immature pDC being recruited to both synovial fluid and tissue of RA patients. Expression of the IFN-α–inducible protein MxA in RA synovial tissue thereby suggested local production of type I IFN by mature pDC (Lande et al. 2004). In human nasal allergy, experimental challenge with allergen resulted in a pronounced increase of pDC in nasal mucosa. Moreover, adhesion molecules were found to be upregulated on pDC, normally allowing for leukocyte extravasation in organized lymphoid tissue (Jahnsen et al. 2000).

In addition, a study by Baumgart et al. identified in patients with active IBD enhanced frequencies of pDC in the inflamed colonic mucosa and mesenteric LN (Baumgart et al. 2011). Similar to pemphigus, the enrichment of gut mucosal pDC was associated with declining numbers of peripheral immature pDC that also correlated with the severity of the disease (Baumgart et al. 2005). Furthermore, patients with active IBD not only exhibited a significant drop of blood pDC, but also immature mDC. Likewise, a study by Chen et al. observed significantly reduced frequencies of pDC in the peripheral blood of TID patients (Chen et al. 2008).

Altogether, analogous to our results of decreasing circulating pDC in active pemphigus patients, a diminished population of blood pDC was similarly found in patients with TID (Chen et al. 2008) and IBD with concurrent accumulation of pDC in the colonic mucosa (Baumgart et al. 2011). In pemphigus, pDC may possibly as well infiltrate the skin and cause damage by cytokine production as it was shown for patients with LE (Vermi et al. 2009) and psoriasis (Nestle et al. 2005).

Interestingly, human pDC are weak APC, but upon activation by for instance viral nucleic acids acquire full DC morphology and phenotype (Liu 2005). Although less efficiently than classical mDC, they in this state present antigen to $CD4^+$ T cells, as well as cross-prime $CD8^+$ T cells (Villadangos and Young 2008; Hoeffel et al. 2007). The capacity of pDC to activate Dsg3-specific $CD4^+$ T cells in pemphigus has not yet been investigated.

The circulating population of slanDC was quantitatively characterized using a combination of two blood samples, and analysis of both data sets together revealed no significant differences between the test subject groups (**Figure 4.9**). Since in several autoimmune disorders, including psoriasis (Schakel 2009), MS (Thomas et al. 2014), RA (Schakel et al. 2006), and CD (de Baey et al. 2003), slanDC have been reported to be present in the inflammatory infiltrate, slanDC may likewise accumulate in the skin of pemphigus patients.

Interestingly, functional analysis indicated a close relationship of slanDC with rather nonclassical monocytes instead of DC, as they similarly possessed a superior ability to produce TNF-α (Schakel et al. 2002). In addition, treatment with glucocorticoids resulted in their depletion (Thomas et al. 2014), which we also observed in our patients (data not shown).
In order to assess the state of maturity/immunogenicity of slanDC we as well determined their cell surface expression of HLA-DR and the costimulatory molecule CD86. Against our expectations, the distribution intensities of neither HLA-DR nor CD86 were elevated on blood slanDC from active pemphigus patients (**Figure 4.12**). We suggest that activation of slanDC rather occurs at the site of inflammation, where large amounts of skin-derived autoantigens are present and is thus not detectable in the circulation. This explanation may be in line with the observed increased expression of the inflammatory CCR2 on mDC from active pemphigus patients (**Figure 4.11a**), indicating an immature immune status.

Interesting for pemphigus pathogenesis, numerous DC subsets that populate the human skin have been shown to shape both cellular and humoral immune responses in health and disease (Klechevsky 2013). Epidermal LC not only are particularly efficient at priming and cross-priming naïve $CD8^+$ T cells into potent cytotoxic T cells, but also orchestrate $CD4^+$ T cell responses (Klechevsky et al. 2008). Besides differentiating naïve T cells into cells releasing Th1 and Th2 cell signature cytokines, LC also initiate Th17 responses *in vitro* (Mathers et al. 2007). In addition, LC were demonstrated to be eminently adept at differentiating naïve $CD4^+$ T cells into IL-22– and IL-21–secreting cells (Penel-Sotirakis et al. 2012).
In contrast to LC, $CD14^+$ dermal DC can directly activate B cells into IgM-secreting plasma cells, partly by their secretion of IL-12 (Klechevsky et al. 2008). Moreover, $CD14^+$ DC differentiate $CD4^+$ T cells into T cells exhibiting a Tfh cell phenotype (Klechevsky et al. 2008).
In this study, we found in active pemphigus patients increasing frequencies of circulating Th17 cells (**Figure 4.23c**), which according to the findings above may be primed by LC of the epidermis. Correspondingly, elevated amounts of IL-21 in the plasma of active pemphigus patients (**Figure 4.29**) may originate from either epidermal LC or $CD14^+$ dermal DC primed Tfh cells. These presumptions have not yet been clarified in the pathogenesis of pemphigus.

Taken together, active pemphigus patients exhibited reduced frequencies of both circulating mDC and pDC concurrent with an increased expression of CCR2 on mDC, indicating an elevated migration activity of these cells into the inflamed skin. Finally, we did not find clears signs of blood DC playing a dominant role in pemphigus pathogenesis, in contrast to observations in the disease development of other auto-ab–mediated autoimmune disorders, such as SLE (Means et al. 2005). Of note, differing identification strategies used for a marginal blood cell population, as well as a possible impact of medication may aggravate the quantitative and phenotypic comparison of DC subsets between individual studies.

5.1.2 Role of monocytes in pemphigus

Beside DC with their unique extraordinary T cell stimulatory capacity (Steinman 2012), monocytes contribute to the maintenance of immune homeostasis and defense by various both direct and indirect effector functions (Auffray, Sieweke, and Geissmann 2009). Human monocytes constitute a heterogeneous population of accessory cells composed of several functionally discrete subsets and they are also able to both stimulate and suppress T cell responses in infectious and autoimmune diseases (Serbina et al. 2008; Evans et al. 2009; Movahedi et al. 2008; Geissmann et al. 2010).

So far, only sporadic data exist concerning the role of monocytes in the evolvement and progression of pemphigus. In 2000, González et al. claimed a correlation between the population size and the phenotype of circulating mononuclear cells and the severity of PV disease, but the study suffered from a rather small cohort of patients (Gonzalez et al. 2000). In order to specify the relevance of monocytes in pemphigus more systematically, we firstly examined the distinct decomposition of the two major functionally discrete monocyte subsets distinguished by the expression of CD16 (Auffray, Sieweke, and Geissmann 2009). Unlike the predominant subpopulation of classical monocytes being devoid of CD16, $CD16^+$ monocytes display a higher level of HLA-DR expression, entailing an enhanced APC and T cell stimulatory activity (Passlick, Flieger, and Ziegler-Heitbrock 1989) and are capable of inducing TNF-α upon stimulation with bacterial LPS (Belge et al. 2002). In the setting of nonautoimmune disease, $CD16^+$ monocytes were reported to be augmented in the blood of patients with acute inflammation and infectious diseases (Mizuno et al. 2005; Fingerle-Rowson, Auers, et al. 1998; Horelt et al. 2002).

Indeed, we also found for active pemphigus patients a significantly increased proportion of $CD16^+$ monocytes and in addition, elevated values for MG patients who were used as a further unrelated antibody-mediated autoimmune disorder control (**Figure 4.10a**). But a correlation between frequencies of $CD16^+$ monocytes and the occurrence of disease-associated subpopulations of $CD4^+$ T cells could not be found.

An increase of $CD16^+$ monocytes was similarly observed in other autoimmune inflammatory conditions, including RA (Ziegler-Heitbrock 2007). In peripheral blood of RA patients, $CD16^+$ monocyte numbers were not only found to be augmented, but also correlated with RBC sedimentation rate and plasma levels of C-reactive protein (Baeten et al. 2000). In addition, numerous studies reported of frequencies of $CD16^+$ monocytes rising with RA activity (Kawanaka et al. 2002; Hepburn, Mason, and Davies 2004; Iwahashi et al. 2004; Cooper et al. 2012; Rossol et al. 2012; Yoon et al. 2014).

Interestingly, systemic treatment of RA patients with corticosteroids resulted in a diminution of the circulating $CD16^+$ monocyte population, possibly by selective apoptosis, whilst the population of classical monocytes even increased in a dose-dependent manner (Fingerle-Rowson, Angstwurm, et al. 1998; Dayyani et al. 2003). Consequently and crucial for validation of our data, increased frequencies of $CD16^+$ monocytes in both active pemphigus patients and MG patients did not rely on the intake of immunosuppressive agents of individual patients.

Similar to RA, an expansion of blood $CD16^+$ monocytes producing TNF-α has also been observed in complication-free TID patients (Mysliwska et al. 2012), as well as in both peripheral blood and

salivary glands of SS patients (Ciccia et al. 2013). Contrariwise, SLE patients displayed reduced numbers and functionally impaired peripheral blood $CD16^+$ monocytes, possibly pointing to a different function of this cell population in disease pathogenesis (Burbano, Vasquez, and Rojas 2014).
In summary, the rise of blood $CD16^+$ monocytes in both active pemphigus and MG patients was observed in several other autoimmune disorders, including RA (Baeten et al. 2000), TID (Mysliwska et al. 2012), and SS (Ciccia et al. 2013), which points to a rather disease-unspecific, common inflammatory effect.

Regarding the function of $CD16^+$ monocytes, these cells are located within blood vessels, where they patrol healthy tissues for damage or pathogenic microbes by continually crawling long-range onto the endothelium (Auffray et al. 2007). Following extravasation into the tissue, $CD16^+$ monocytes differentiate into macrophages and elicit an innate immune response (Auffray et al. 2007). Accordingly, Hepburn et al. suggested for RA that the elevation of the circulating $CD16^+$ monocyte subset represented an activation of the monocyte/macrophage system and that these cells might possess the ability of differentiating into macrophages in the synovium, where they would act as potent producers of proinflammatory cytokines (Hepburn, Mason, and Davies 2004). In the pathogenesis of IBD, $CD16^+$ monocytes have been proposed to exert a similar function as they have been found to particularly accumulate within the active inflamed regions of the mucosa, in which high levels of TNF-α have been linked with disease progression (Koch et al. 2010; Hanai et al. 2008; Komatsu et al. 2001; Papadakis and Targan 2000). In addition, an expansion of activated $CD16^+$ monocytes has also been observed in patients affected with the demyelinating autoimmune disorder MS (Chuluundorj et al. 2014).
In active pemphigus patients, elevated plasma levels of the proinflammatory cytokines IL-6 and TNF-α (**Figure 4.19**) may as well originate from the expanded circulating population of $CD16^+$ monocytes as discussed later on.

Based on the expression level of CD14, $CD16^+$ monocytes can be further subdivided into two functionally discrete subsets with the designated nonclassical and intermediate monocytes exhibiting reduced and high levels of CD14, respectively (Grage-Griebenow, Flad, and Ernst 2001). Possibly in transition from the classical to the nonclassical subset (Ziegler-Heitbrock and Hofer 2013), $CD14^+CD16^+$ intermediate monocytes constitute a marginal population with unique properties that was observed to be expanded under inflammatory conditions (Rossol et al. 2012; Moniuszko et al. 2009).
Similarly, the population of $CD14^{dim}CD16^+$ nonclassical monocytes has been implicated in the pathogenesis of autoimmune disorders. For instance, TLR7- and TLR8-mediated recognition of immune complexes containing nucleic acids by nonclassical monocytes resulted in the production of proinflammatory cytokines, including TNF-α (Cros et al. 2010). In addition, a very recent study identified nonclassical monocytes as the primary proinflammatory $CD16^+$ monocyte subset. Upon stimulation with LPS, these cells not only secreted elevated amounts of IL-1β and TNF-α compared to the intermediate subset, but were also expanded in patients with sepsis and SLE

(Mukherjee et al. 2015). Interestingly, sepsis patients as well exhibited enhanced frequencies of intermediate monocytes, whilst an increase of the nonclassical subset was confined to patients with SLE, possibly due to their status of chronic inflammation (Grage-Griebenow, Flad, and Ernst 2001).

In our study, active pemphigus patients displayed augmented numbers of circulating $CD14^{dim}CD16^{+}$ nonclassical monocytes with significance close to the threshold (**Figure 4.10b**). This observation is in concordance with the findings by Baeten et al. of elevated frequencies of $CD16^{+}$ monocytes that expressed a specific autoantigen of RA and exhibited diminished levels of CD14 in both peripheral blood and synovial tissue from RA patients. Furthermore, the presence of these cells in synovial tissue correlated with the extent of joint destruction, suggesting a local involvement of nonclassical, but not intermediate monocytes in the pathogenesis of RA (Baeten et al. 2000). A study by Rossol et al. however identified a selective expansion of the intermediate monocyte subset in peripheral blood of RA patients (Rossol et al. 2012), so data concerning the quantitatively predominant $CD16^{+}$ monocyte subtype in RA is still controversial.

In contrast to our observation of increasing nonclassical monocytes in active pemphigus patients, augmented circulating frequencies of the intermediate subtype were reported in several other autoimmune disorders, such as CD (Grip et al. 2007) and Eales' disease (ED) (Sen et al. 2011). A possible explanation for the more frequently detected expansion of the intermediate subset in inflammatory and autoimmune conditions was very recently provided by Liu et al. Analogous to the findings in CD (Grip et al. 2007), ED (Sen et al. 2011), and RA (Rossol et al. 2012), patients with autoimmune uveitis (AU) as well displayed augmented circulating intermediate monocytes, but importantly, this effect was associated with glucocorticoid therapy (Liu et al. 2015). Moreover, according to the work by Mukherjee et al., purification of PBMC by density gradient centrifugation critically changed surface expression of CD14 and CD16 on monocytes towards a nonclassical monocyte phenotype (Mukherjee et al. 2015).

Since we only included patients with no or minimal intake of Pred in our study, as well as used whole blood for the phenotypic characterization of $CD16^{+}$ monocytes, the observed expansion of the nonclassical subset in active pemphigus patients was not induced by medication or density gradient centrifugation.

Altogether, active pemphigus patients displayed in peripheral blood a selective expansion of the nonclassical subset within $CD16^{+}$ monocytes as similarly reported in patients with RA (Baeten et al. 2000), sepsis, and SLE (Grage-Griebenow, Flad, and Ernst 2001). Importantly, therapy with glucocorticoids as well as the respective cell isolation method selected may critically impact the frequencies of nonclassical monocytes, which has to be considered upon comparison of different study results.

It has long been known that there is a local accumulation of mononuclear APC, including monocytes, macrophages, and the skin-resident LC in both lesional and perilesional skin biopsies obtained from PV patients (Nestor, Cochran, and Ahmed 1987; Mashkilleyson, Konttinen, and Visa 1989; Chiapa-Labastida et al. 2011). The migration of circulating monocytes into the periphery underlies distinct trafficking mechanisms, with the classical $CD16^{-}$ population being

recruited via the CCL2/monocyte chemoattractant protein-1 (MCP-1)–CCR2 axis, whilst $CD16^+$ monocytes are largely devoid of CCR2 (Geissmann, Jung, and Littman 2003). Under inflammatory conditions, this route enables classical monocytes to exit the blood stream and access peripheral nonlymphoid tissues (Boring et al. 1997; Kurihara et al. 1997; Maus et al. 2003; Kuziel et al. 1997). This may also explain why blocking CCR2 was effective in attenuating disease in models of atherosclerosis, MS, and RA with known monocyte-induced pathology (Viola and Luster 2008; Boring et al. 1998; Izikson et al. 2000; Gong et al. 1997).

Interestingly, CCR2 expression of monocytes was elevated in active pemphigus patients and similarly also in MG controls (**Figure 4.11b**), and the associated CCR2-mediated monocyte migration in the state of inflammation may be likewise linked to pemphigus pathogenesis. In support of this hypothesis, there is great evidence for a pathogenic role of CCL2/CCR2-mediated migration of monocytes in other autoimmune disorders.

Accordingly, CCR2-dependent infiltration of mononuclear cells induced inflammation and destruction of the retina in a spontaneous mouse model of AU. Depletion of functional CCR2 however efficiently prevented induction of the disease phenotype, probably by an impaired monocyte-mediated recruitment of autoreactive $CD4^+$ T lymphocytes (Chen et al. 2014). In experimental TID, in which monocytes infiltrating the islets contribute to disease pathogenesis, CCR2 deficiency similarly resulted in a delayed onset of diabetes pathology (Solomon, Balasa, and Sarvetnick 2010). Furthermore, loss of CCR2 also resulted in diminished renal disease and prolonged survival in experimental SLE (Perez de Lema et al. 2005).

Concerning human studies, Vestergaard et al. found in patients afflicted with the skin autoimmune diseases atopic dermatitis (AD) and psoriasis a slight, but significant overexpression of CCR2 on peripheral blood monocytes compared to HC (Vestergaard et al. 2004). Moreover, rising levels of CCL2 were detected within the colon of IBD patients that might attract CCR2-bearing monocytes into the inflamed mucosal tissue. In addition, local CCL2 expression also correlated with the activity of the disease (Banks et al. 2003).

CCL2 is a potent attractor for monocytes and DC (Nakamura, Williams, and Kupper 1995), as well as both Th1 and Th2 cells (Sebastiani et al. 2001), and was present in the basal layer of lesions from patients with AD and psoriasis (Giustizieri et al. 2001). While CCL2 was undetectable in resting keratinocytes, a study by Li et al. observed expression of CCL2 in both oral and skin keratinocytes depending on TNF-α and/or IFN-γ (Li, Farthing, and Thornhill 2000). Interestingly, the work by Feliciani et al. revealed in both lesional and perilesional skin areas from PV patients an overexpression of IL-1α and TNF-α by keratinocytes. In addition, keratinocyte synthesis of the IL-1α and TNF-α transcripts was induced by autoreactive IgG antibodies *in vitro*, and mice deficient in either cytokine exhibited reduced pemphigus pathology upon passive transfer of pemphigus (Feliciani et al. 2000). Apart from that, a study by Chiapa-Labastida et al. found in PV patients apoptotic keratinocytes to accumulate in both dermis and epidermis that induce monocytes to release TNF-α during phagocytosis of these cells (Chiapa-Labastida et al. 2011).

To summarize, active pemphigus patients displayed an upregulation of CCR2 on classical monocytes, suggesting a potential role of CCL2/CCR2-mediated migration of monocytes into the inflamed skin. This scenario is supported by several studies in other autoimmune disorders,

including experimental AU (Chen et al. 2014), TID (Solomon, Balasa, and Sarvetnick 2010), and SLE (Perez de Lema et al. 2005), in which depletion of functional CCR2 resulted in an amelioration of disease pathology. Moreover, increasing levels of the CCR2-associated CCL2 were detected in the colon of IBD patients (Banks et al. 2003), as well as lesional skin of patients with AD and psoriasis (Giustizieri et al. 2001). Interestingly, CCL2 expression of keratinocytes was found to be induced by TNF-α and/or IFN-γ (Li, Farthing, and Thornhill 2000), and IgG auto-ab from pemphigus patients induced keratinocytes to synthesize the transcripts of IL1α and TNF-α *in vitro*. Hence, auto-ab–mediated production of TNF-α of keratinocytes may stimulate the local expression of CCL2 attracting CCR2-bearing blood monocytes to the site of inflammation in pemphigus.

In order to assess the status of immunogenicity of monocytes from pemphigus patients, we specifically determined their capacity to secrete the proinflammatory cytokines IL-6 and TNF-α upon stimulation with LPS. Although the intensity of intracellular IL-6 and TNF-α expression was not significantly increased in active pemphigus patients, they displayed a gradual elevation of TNF-α synthesis (**Figure 4.15b**) and a shift to higher IL-6 and TNF-α individual values (**Figure 4.15c**). Considering the relationship between both IL-6 and TNF-α yielded an even enhanced separation between active pemphigus patients and HC (**Figure 4.15c**).
Upon analysis of proinflammatory cytokine synthesis, we did not specify $CD14^+$ monocytes by additional expression of CD16. Yet, according to the findings by Mukherjee et al. we assume that the nonclassical $CD16^+$ monocyte subset is the primary source of TNF-α that we also found to be expanded in active pemphigus patients (**Figure 4.10b**).

Several studies reported of significantly elevated levels of circulating, i.e. released TNF-α in PV patients (Ludwig and Schmidt 2009; Keskin et al. 2008; Alecu et al. 1999; Lopez-Robles et al. 2001). Moreover, D'Auria et al. detected increasing levels of both TNF-α and IL-6 in the sera of PV patients, which also correlated with the activity of the disease. In addition, systemic therapy with corticosteroids resulted in a diminution of both cytokine serum concentrations, as well as clinical manifestations (D/'Auria, Bonifati, and Mussi 1997). Furthermore, Narbutt et al. observed in both active and remittent stage of PV elevated serum concentrations of IL-6, as well as a negative correlation between TNF-α levels and IgG auto-ab titers in remittent PV patients (Narbutt et al. 2008).
In our study, the plasma concentrations of TNF-α in active pemphigus patients were significantly augmented (**Figure 4.19b**). An elevation may also be valid for the IL-6 plasma levels of the active pemphigus patients, although only a nonsignificant elevation was observed, but the significance level was possibly affected by the two extreme IL-6 values within the HC group (**Figure 4.19a**). Noteworthy, the plasma levels of both TNF-α and IL-6 of the three remittent patients assumed similar values as the HC (**Figures 4.19**). Important for pemphigus pathology, high titers of anti-Dsg IgG antibodies were found in patients with high levels of both TNF-α (**Figure 4.26a** and **b**) and IL-6 (**Figure 25a** and **b**) as revealed by the correlation of these parameters. However,

according to a recent study by Hall et al., application of the mAb Infliximab targeting TNF-α did not prove effective for the treatment of patients with PV (Hall et al. 2015).
In summary, we could not detect significant differences in IL-6 and TNF-α production by $CD14^+$ monocytes between active pemphigus patients and control groups. Yet, we observed in active pemphigus patients increasing levels of both IL-6 and TNF-α, which is in line with other respective reports in the literature (Ludwig and Schmidt 2009; D/'Auria, Bonifati, and Mussi 1997). Important for pemphigus pathology, we observed a correlation between both IL-6 and TNF-a plasma levels and serum IgG auto-ab titers in pemphigus patients, indicating a contribution of these cytokines to auto-ab induction.

The capability to take up and process antigen belongs to the most important functions of APC, including monocytes (Auffray, Sieweke, and Geissmann 2009). In order to assess whether this property is altered in pemphigus, PBMC were exposed to fluorescently labeled recombinant human Dsg3 protein, and the maximum antigen uptake capacity, as well as kinetics of take up were determined.
In active pemphigus patients, neither the antigen uptake ability of $CD16^+$ (**Figure 4.18a**) nor classical monocytes (**Figure 4.18b**) significantly differed from that of HC. In addition, no differences in the velocity of protein internalization were found between active pemphigus patients and HC (**Figure 4.18c**). Thus, we could not observe pemphigus-specific particularities concerning the antigen uptake capacity of monocytes.
In agreement with the report by Mukherjee et al., we similarly identified classical monocytes as the primary subset specialized in phagocytosis compared to $CD16^+$ monocytes (Mukherjee et al. 2015). Equipped with high expression of the scavenger receptors CD36 and CD163, as well as a high intracellular concentration of myeloperoxidase and lysozyme, classical monocytes represent excellent phagocytes (Mysliwska et al. 2012).

5.1.3 Role of $CD4^+$ T cells in pemphigus

Although the pathology of pemphigus is linked to the generation of mainly Dsg-specific IgG auto-ab, the critical role of autoreactive $CD4^+$ T cells in the induction of these humoral responses has been analyzed and highlighted in numerous studies (Hertl, Eming, and Veldman 2006).
In 1997, Lin et al. developed Dsg3-reactive $CD4^+$ T cell lines and clones from PV patients that upon stimulation secreted Th2 cell signature cytokines, and whose response to recombinant Dsg3 fusion proteins was restricted to HLA-DR (Lin, Swartz, and Lopez 1997). Using Dsg3-specific T cell lines and clones from PV patients, a study from our group found in chronic active PV patients frequencies of autoreactive Th1 cells surpassing those of autoreactive Th2 cells, as well as T cell recognition of the Dsg3 ectodomain being restricted to both HLA-DRB1*04:02 and HLA-DQB1*05:03 (Veldman et al. 2003b). In addition, Rizzo et al. observed in PV patients a significant association of Dsg3-specific Th2 activity with overt disease, whereby this Th2 activity also correlated with the Dsg3-specific IgG titers of the patients (Rizzo et al. 2005).
Considering the observed increased production of APC-derived proinflammatory cytokines in pemphigus patients (**Figures 4.19**), we as well sought to assess whether the polarization of $CD4^+$

T cells is affected. Yet, in our study, the circulating frequencies of neither Th1 nor Th2 cells significantly varied between active pemphigus patients and control groups as illustrated in **Figure 4.23a** and **b**. Zhu et al. similarly observed for Th2 cells no significant differences between HC, active and remittent PV patients, but differing from our findings a significantly reduced subset of Th1 cells in active PV patients (Zhu et al. 2012).

Based on the Th2 cell bias observed in active PV patients (Rizzo et al. 2005), we additionally determined the levels of the Th2 cell-related IL-33 in the plasma of pemphigus patients and controls. IL-33, identified as a member of the IL-1 cytokine family in 2005 (Schmitz et al. 2005) predominantly induces Th2 cell responses and has also been implicated in the pathogenesis of allergy and several autoimmune diseases, such as RA, SLE, and MS (Pei et al. 2014; Tang et al. 2013; Li, Lin, and Zheng 2014; Christophi et al. 2012). In addition, IL-33 functions as an alarmin upon cellular stress or damage (Cayrol and Girard 2014).
In active pemphigus patients, the plasma levels of IL-33 tended to be enhanced (p= 0.1687) compared to HC and additionally, MG patients also exhibited elevated (p= 0.1171) values, although without reaching significance (**Figure 4.20a**). A very recent publication by Tirado-Sanchez et al. indeed detected in the sera of PV patients significantly elevated levels of IL-33 and its respective soluble receptor sST2, with these two parameters also correlating with both Dsg3-specific IgG titers and the clinical activity of the disease (Tirado-Sanchez, Bonifaz, and Ponce-Olivera 2015).

Interestingly, active pemphigus patients exhibited a clearly elevated subpopulation of Th17 cells, which was also the case to a lower extent in MG controls (**Figure 4.23c**). Th17 cells are a discrete subset of $CD4^+$ T cells that is considered as proinflammatory and that has been implicated in the pathogenesis of various autoimmune disorders, including psoriasis, RA, IBD, and MS (Yang et al. 2014). Our finding is in accordance with the study by Xu et al. who demonstrated significantly elevated numbers of circulating Th17 cells particularly in both acute onset and chronic active PV patients (Xu et al. 2013). A recent study by Asothai et al. similarly observed increasing frequencies of Th17 cells in the blood of PV patients (Asothai et al. 2015). In addition, a study by Arakawa et al. reported of an accumulation of lesional Th17 cells in PV patients, while neither the Dsg3-specific antibody titers nor the severity of the disease correlated with the proportion of $IL\text{-}17^+$ cells present in the skin lesions (Arakawa et al. 2009). Identification of the precise function of Th17 cells in the pathogenesis of pemphigus still requires further investigation. Yet, the finding of elevated frequencies of Th17 cells in MG patients is in concordance with very recent results in an experimental animal model of MG by Schaffert et al. who demonstrated the production of AChR-specific IgG antibodies relying on Th17 cells in their *in vivo* model (Schaffert et al. 2015).
Of note, a study by Iwamoto et al. reported of TNF-α inducing Th1 and Th17 cell responses by mediating the differentiation of LPS-stimulated $CD14^+$ monocytes into mature DC (Iwamoto et al. 2007). Nonclassical $CD16^+$ monocytes being expanded in peripheral blood of both pemphigus and MG patients (**Figure 4.10b**) potently produce TNF-α upon stimulation (Mukherjee et al. 2015;

Belge et al. 2002), this way possibly contributing to the formation of Th17 cells in the pathogenesis of pemphigus and MG.

Remarkably, active pemphigus patients exhibited slightly augmented subpopulations of IL-10–producing $CD4^+$ T cells unlike MG controls showing diminished percentages of these cells (**Figure 4.23d**). Although various studies demonstrated reduced immunoregulatory $CD4^+$ T cell numbers in PV patients (Veldman, Hohne, et al. 2004; Hertl, Eming, and Veldman 2006; Sugiyama et al. 2007; Xu et al. 2013), this discrepancy might be explained by Th2 cells as well secreting IL-10, since we did not assess a suppressive function of these cells. Otherwise, the augmented proportion of IL-10–producing $CD4^+$ T cells may represent a population of Tr1 cells in transition to Th17 cells under the influence of proinflammatory cytokines, including IL-6, IL-1β, and TNF-α, as it has already been demonstrated for other skin diseases (Bovenschen et al. 2011). Alternatively, increasing frequencies of IL-10–secreting Th cells may also represent a compensatory mechanism triggered by the misguided immune response in pemphigus. Moreover, this observed rise possibly depends on IL-27, which we found to be augmented in the plasma of pemphigus patients (**Figure 4.20b**) and which is known to propagate the IL-10 secretion of effector $CD4^+$ T cells (Neumann et al. 2014; Batten et al. 2008). The potential function of IL-27 in the pathogenesis of pemphigus will be further discussed in the following section.

Altogether, since in active pemphigus patients, we did not find an increase of circulating Th2 cells, we conclude that the Th2 cell bias reported in pemphigus is rather confined to Dsg-specific $CD4^+$ T cells (Rizzo et al. 2005). Interestingly, and according to findings in the literature (Xu et al. 2013; Asothai et al. 2015), active pemphigus patients displayed a pronounced elevation of peripheral Th17 cells, which may be induced by the observed expanded population of $CD16^+$ monocytes (Mukherjee et al. 2015). Unexpectedly, active pemphigus patients as well exhibited augmented frequencies of IL-10–producing T cells, which is opposed to the findings of other respective studies (Veldman, Hohne, et al. 2004; Xu et al. 2013). Possibly, since we did not test for a suppressive capacity of these cells, this detected increase is due to Th2 cells as well secreting IL-10, transitional Th cell plasticity, immune compensatory mechanisms, or an effect depending on IL-27, which we also found to be elevated in the plasma of active pemphigus patients.

5.1.4 Role of IL-21–producing T cells in pemphigus

Neutralizing antibodies are an essential factor in the development of protective immunity to most pathogenic threats. However, B cell dysfunction can result in the generation of autoreactive antibodies and the formation of immune complexes, which induce local inflammatory reactions and tissue damage (Martin and Chan). Pemphigus is considered as a prototypical auto-ab–mediated organ-specific disorder and when monitored in individual patients, the titers of serum Dsg-reactive IgG antibodies generally correlate with disease activity (Ishii et al. 1997; Sams and Jordon 1971). In accordance with that, predominantly active PV patients of our cohort displayed elevated Dsg3-specific serum IgG titers, which varied within a wide range (**Figure 4.24**).

Interestingly, beside IgG auto-ab titers correlating with both circulating TNF-α (**Figure 4.26a** and **b**) and IL-6 (**Figure 25a** and **b**), we also found for pemphigus and solely PV patients a strong correlation between the presence of serum Dsg-responsive antibodies and the plasma levels of IL-27 (**Figure 27a** and **b**). Moreover in pemphigus patients, the plasma levels of IL-27 not only strongly correlated with the production of circulating auto-ab, but were also significantly elevated in individuals with active disease (**Figure 4.20b**). Similarly, MG patients exhibited enhanced plasma concentrations of IL-27 (**Figure 4.20b**), which yet in contrast to pemphigus patients did not relate to the serum titers of anti-AChR IgG (**Figure 4.27c**), suggesting a disease-specific function of IL-27 in the pathogenesis of pemphigus.

IL-27, a heterodimeric cytokine recently discovered by Pflanz et al. is evolutionary related to IL-12 and is mainly expressed by activated APC (Pflanz et al. 2002). IL-27 exerts key modulatory effects on both T and B cells with its regulatory activities comprising the induction of inhibitory receptor PD ligand 1 (PD-L1) upregulation and the secretion of IL-10 by several $CD4^+$ T cells. To its inflammatory properties belong the development of cytotoxic $CD8^+$ T cells, the capacity to directly support B cell production of antibodies (Hunter and Kastelein 2012), and the promotion of Tfh cells (Batten et al. 2010; Gringhuis et al. 2014). Interestingly, a disease-propagating function of IL-27 has been described in several disorders of autoimmune etiology, such as IBD (Schmidt et al. 2005; Cox et al. 2011), RA (Goldberg, Wildbaum, et al. 2004; Cao et al. 2008 ; Shen et al. 2011), EAE (Goldberg, Zohar, et al. 2004; Li et al. 2005), psoriasis (Shibata et al. 2010; Shibata et al. 2013), TID (Wang et al. 2008), and SS (Xia et al. 2012). For instance, Goldberg et al. suppressed an ongoing disease in mouse models of both RA and MS via the neutralization of the p28 subunit of IL-27 (Goldberg, Wildbaum, et al. 2004; Goldberg, Zohar, et al. 2004). In our study, we observed in pemphigus patients a strong correlation of IL-27 plasma concentrations with TNF-α, which might support an additional proinflammatory role of IL-27 in pemphigus pathogenesis (**Figure 4.28a**).

Recently, Batten et al. demonstrated that IL-27 signaling in T cells resulted in the production of IL-21 and in addition that IL-27 was able to enhance both the survival of activated $CD4^+$ T cells and the expression of the Tfh cell markers CXCR5, PD-1, and ICOS. Moreover, loss of IL-27 signaling was shown to ameliorate disease in a murine model of SLE (Batten et al. 2010). Tfh cells represent specialized and essential providers of B cell help both to form and maintain GC and to regulate the differentiation of B cells into memory B cells and antibody-secreting plasma cells. The characteristic expression of CXCR5 defines the localization of Tfh cells to B cell follicles within secondary lymphoid tissues, including GC. During the process of affinity maturation, $CD4^+CXCR5^+$ Tfh cells efficiently induce both class switching and antibody secretion of B cells (Tangye et al. 2013). Owing to their capacity to control the induction of high-affinity humoral immune responses, alterations in the peripheral Tfh cell compartment have been found in several antibody-mediated autoimmune disorders, like SLE and RA, in which the expansion of blood Tfh cells (defined as $CD4^+CXCR5^+PD\text{-}1^+ICOS^+$ T cells) could be linked to disease activity (Simpson et al. 2010; Wang et al. 2013). Moreover, a study by Gringhuis et al. observed fucose-based

stimuli inducing DC to potently produce IL-27, which can drive Tfh cell generation and T cell-dependent IgG production by B cells (Gringhuis et al. 2014).

In order to assess whether the above mentioned axis of IL-27-induced promotion of Tfh cells may also be relevant in pemphigus, we determined the plasma levels of IL-21 being robustly produced by Tfh cells (Luthje et al. 2012) and exerting an essential function in B cell differentiation into class-switched plasma cells (Linterman et al. 2010). Indeed, active pemphigus patients and MG controls displayed significantly elevated plasma concentrations of IL-21 (**Figure 4.29**). Increasing amounts of circulating IL-21 have been previously observed in MG (Yilmaz et al. 2015; Li et al. 2014) and other antibody–mediated autoimmune disorders, such as RA (Liu et al. 2012) and SLE (Dolff et al. 2011), suggesting that IL-21 is produced by autoreactive Tfh cells during an ongoing auto-ab response. Interestingly, an increase of both IL-21 levels and Tfh cells has also been found in patients with the pemphigus-related AIBD BP (Li et al. 2013).

In order to trace the cellular source of the circulating IL-21 and to underpin the hypothesis of an IL-27–mediated propagation of Tfh cells, we additionally quantified the circulating population of Tfh cells in a further cohort of pemphigus and MG patients, as well as HC (Hennerici et al. 2016). According to the findings by Morita et al., circulating counterparts of Tfh cells can be detected by the expression of CXCR5 in peripheral blood (Morita et al. 2011). Of note, a few pemphigus patients used in this analysis received systemic immunosuppressive treatment, which however was reported to induce a decline of both peripheral Tfh cells and IL-21 plasma levels (Jin et al. 2014; Liu et al. 2014).

Indeed, the frequency of $CD4^+CXCR5^+$ circulating Tfh cells was significantly increased in pemphigus patients. Yet, in contrast to previous studies in MG (Tackenberg et al. 2007; Saito et al. 2005; Luo et al. 2013), we did not detect elevated numbers of peripheral Tfh cells in our cohort of MG patients, which is possibly due to the rather mild to moderate disease activity of the study patients. In addition, since almost all MG patients were under either immunomodulatory or immunosuppressive therapy at the time of analysis, a potential therapeutic effect cannot be excluded. Furthermore, we identified in the investigated pemphigus patients Dsg3-specific IL-21–producing cells in a significantly higher frequency compared to HC by ELISPOT assay. These findings indicate that autoreactive Dsg3-specific IL-21–producing T cells are involved in the induction of Dsg3-specific auto-ab by providing help to autoreactive B cells. However, the effect of IL-27 on promoting peripheral Tfh cell rather occurs in a timely delayed manner because we could not find a correlation between circulating Tfh cells and IL-27 plasma levels.

Of note, IL-21 is also produced by Th17 cells (Zhou et al. 2007), which we noticed to be enhanced in the blood of both active pemphigus and MG patients (**Figure 4.23c**). IL-21 induces the differentiation of Th17 cells in a STAT3-depending manner, this way possibly providing autocrine stimulation for the maintenance of Th17 cells (Wei et al. 2007; Korn et al. 2007). In this respect, we conclude that IL-21 in pemphigus is partially released by activated Th17 cells, which is also confirmed by the detection of IL17/IL-21 double positive T cells in pemphigus patients. Yet, IL-21–producing Th17 cells only displayed a minor T cell population in the analyzed pemphigus patients with IL-21 single positive T cells being over 20 times more frequent.

Taken together, increasing plasma levels of IL-27, which strongly correlated with Dsg-specific IgG auto-ab titers, as well as elevated plasma levels of IL-21 concurrent with augmented frequencies of circulating Tfh and Th17 cells imply a potential contribution of IL-27 and IL-21–producing T cells to the pathogenesis of pemphigus. Hence, the antagonization of IL-27 and other factors involved in Tfh cell function, like IL-21 may represent a novel therapeutic option in pemphigus.

5.1.5 Summary

Aim of the first part of this doctoral thesis was to investigate antigen-presenting cells and APC-derived cytokines, as well as their relation to Th cell subsets and the auto-ab response in the pathogenesis of pemphigus. Concerning the APC compartment, we found quantitative alterations with the frequencies of both blood mDC and pDC being diminished in active pemphigus patients. This reduction concurred with a significantly augmented expression of CCR2 on circulating mDC, an effect that we similarly observed on $CD14^+$ monocytes, possibly suggesting an increased migration of APC into the inflamed skin. Moreover, circulating frequencies of $CD16^+$ monocytes were significantly elevated in active pemphigus patients, as well as MG controls, of which the nonclassical $CD16^+$ subset identified by a reduced expression of CD14 was specifically expanded in active pemphigus patients. With regard to the function of APC, active pemphigus patients displayed slightly and significantly augmented plasma concentrations of IL-6 and TNF-α, respectively. Moreover, plasma levels of the Th2 cell-linked IL-33 were slightly enhanced in active pemphigus patients without reaching significance. Interestingly, pemphigus patients with active disease exhibited significantly elevated circulating frequencies of Th17 cells and IL-10–producing T cells.

Furthermore, a strong correlation between IL-27 plasma levels and Dsg-specific IgG auto-ab titers, as well as elevated plasma levels of IL-21 along with increased frequencies of peripheral Tfh and Th17 cells suggest a potential implication of IL-27 and IL-21–producing T cells in the pathogenesis of pemphigus.

5.2 Mouse study

The second part of this doctoral thesis deals with a novel HLA-DRB1*04:02–tg mouse model of PV, in which we characterized Dsg3-specific cellular and humoral immune responses. This model was created based on a high prevalence of distinct HLA class II alleles in pemphigus patients, including HLA-DRB1*04:02, –DRB1*14:01, and –DQB1*05:03 (Tron et al. 2006). Detailed epidemiological analysis revealed the association of PV with HLA-DRB1*04:02 and –DQB1*05:03 being primary, whereas the increased occurrence of –DRB1*14:01 and –DQB1*03:02 is due to a linkage disequilibrium with the respective primary allele (Lee et al. 2006). The pathogenesis of pemphigus being linked to the presence of specific HLA class II alleles implies autoreactive $CD4^+$ T cells exerting a critical function in the initiation and maintenance of this disease, for which numerous studies have provided evidence (Hertl, Amagai, et al. 1998a; Nishifuji et al. 2000; Takahashi et al. 2008; Takahashi, Kuwana, and Amagai 2009; Eming et al. 2008; Amber et al. 2013).

In this study, the HLA-DRB1*04:02–tg mouse model of PV served as a means to investigate APC-mediated activation of $CD4^+$ T cells, as well as $CD4^+$ T cell-mediated induction of IgG antibody production in a Dsg3-specific manner, respecting the strong genetic association of this autoimmune disorder. In order to assess Dsg3-specific antibody production, we at first immunized these mice with human Dsg3 protein and assayed IgG reactivity against both recombinant and native Dsg3. Subsequently, we tested for the HLA-DRB1*04:02–restricted activation of Dsg3-specific $CD4^+$ T cells using APC equipped with epitopes of the Dsg3 ectodomain, which specifically bind to HLA-DRβ1*04:02. In the reverse experiment, HLA-DRB1*04:02–dependent T cell recognition of human Dsg3 was determined by *in vivo* immunization of mice with the HLA-DRβ1*04:02–binding Dsg3 epitopes and *ex vivo* restimulation of activated T cells with the full Dsg3 ectodomain. Furthermore, in order to test whether Dsg3-specific production of IgG antibodies was similarly regulated by HLA-DRB1*04:02, we additionally immunized mice with Dsg3 epitopes, which bind to HLA-DRβ1*04:02 and checked for reactivity against both recombinant and native Dsg3 protein. Finally, we analyzed whether the IgG induced in mice by immunization with the human Dsg3 ectodomain displayed cross-reactivity against recombinant and native murine Dsg3 in both *ex vivo* and *in vivo* experiments.

In order to specify the findings obtained in this study for HLA-DRB1*04:02, some of the experiments were additionally performed in mice transgenic for HLA-DRB1*04:01 by the collaboration group of J. Bäcklund. Unlike HLA-DRB1*04:02, the HLA-DRB1*04:01 allele strongly relates to the prevalence of RA.

In our HLA-DRB1*04:02**–**tg mouse model of PV, we could demonstrate that induction of human Dsg3-specific IgG antibodies depended on prior activation of Dsg3-reactive $CD4^+$ T cells. T cell activation thereby critically relied on the recognition of epitopes of the Dsg3 ectodomain, which specifically bind to HLA-DRβ1*04:02. Thus, polymorphisms of peptide-binding motifs of distinct PV-linked HLA class II alleles tightly regulated $CD4^+$ T cell-mediated induction of Dsg3-reactive IgG antibodies. However, human Dsg3-specific IgG antibodies only displayed weak cross-reactivity with the murine analogue protein *ex vivo* and did not interact with native protein in mice

immunized with human Dsg3 protein. This might explain why this mouse model did not develop the clinical PV phenotype, but reliably reproduced HLA-DRB1*04:02–restricted human Dsg3-specific immune responses on both the $CD4^+$ T cell and B cell level.

5.2.1 T cell recognition of human Dsg3 is tightly HLA-DRB1*04:02–restricted

The pathology of pemphigus is primarily caused by the presence of Dsg-specific IgG auto-ab causing loss of epidermal keratinocyte adhesion, which manifests clinically in blister and erosion formation of the skin and mucous membranes (Kneisel and Hertl 2011a; Stanley and Amagai 2008). Yet, multiple studies have provided evidence for these humoral immune responses being controlled by autoreactive $CD4^+$ T cells (Hertl, Amagai, et al. 1998a; Nishifuji et al. 2000; Takahashi et al. 2008; Takahashi, Kuwana, and Amagai 2009; Eming et al. 2008; Amber et al. 2013).

The epidemiological observation that the prevalence of pemphigus is highly associated with distinct HLA class II alleles, HLA-DRB1*04:02, –DRB1*14:01, and –DQB1*05:03 (Tron et al. 2006), is strongly indicative of the interaction between APC and T cells being critically involved in the pathogenesis of this disease. Accordingly, the work by Wucherpfennig et al. investigated the structural basis for this relation and revealed that HLA-DRβ1*04:02 displayed the physicochemical properties required for efficient positioning of antigenic Dsg3 peptides in the HLA-DR pocket. The β-chain of the HLA-DRβ1*04:02 binding groove carries the negatively charged amino acid residues aspartic acid (D70) and glutamic acid (E71) at P4, so that peptides with a positively charged amino acid at the same position were efficiently bound and preferentially presented to $CD4^+$ T cells. This facilitated activation of autoreactive $CD4^+$ T cells by this specific HLA-DR–peptide complex (Wucherpfennig et al. 1995). Upon the development of atomic models of the PV-associated HLA class II alleles, Tong et al. confirmed HLA-DRβ1*04:02 exerting a crucial function in selecting specific self-peptides (Tong et al. 2006).

Analogous to HLA-DRB1*04:02, HLA-DQB1*05:03 as well interacted with the same set of Dsg3 epitopes (Tong et al. 2006), underpinning recent experimental data from our group of both HLA class II alleles restricting T cell recognition of identical epitopes of Dsg3 (Veldman, Gebhard, et al. 2004). The finding of T cell recognition of the Dsg3 ectodomain being restricted by HLA-DRB1*04:02 and –DQB1*05:03 was already observed in former studies (Hertl, Amagai, et al. 1998a; Hertl, Karr, et al. 1998). Interestingly, data from our group not only identified in PV patients, but also healthy carriers of the pemphigus-linked alleles T cell clones that recognized the Dsg3 peptides, which bound to HLA-DRβ1*04:02, and which all shared either a positively charged lysine (K) or arginine (R) at P4 (Veldman, Gebhard, et al. 2004).

The observation of healthy individuals displaying Dsg3-specific T cell reactivity (Veldman et al. 2003b; Hertl, Amagai, et al. 1998a), as well as healthy, immediate relatives of PV patients exhibiting Dsg3-reactive IgG auto-ab (Kricheli et al. 2000) testifies of additional regulatory mechanisms preventing disease in these subjects. In this respect, a study from our group identified an increasing frequency of Dsg3-specific IL-10–producing Tr1 cells with constitutive expression of FOXP3 in healthy carriers of the PV-linked HLA class II alleles compared to PV patients (Veldman, Hohne, et al. 2004). In concordance with that, Sugiyama et al. observed in PV

patients markedly reduced circulating Treg cells along with both decreased gene and protein expression of FOXP3 in $CD4^+CD25^+$ T cells (Sugiyama et al. 2007). Moreover, Treg cells in acute onset PV patients also appeared to numerically surpass those in patients with remittent stage of disease (Xu et al. 2013). Finally, the work by Yokoyama et al. demonstrated in experimental PV that Treg cells induced in Dsg3-deficient mice prevented induction of an anti-Dsg3 IgG response upon transfer into mice with active production of auto-ab, highlighting the therapeutic potential of Treg cells in pemphigus (Yokoyama et al. 2011).

Concerning a PV-specific polarization of Th cells, a study by Lin et al. showed that upon stimulation, Dsg3-reactive $CD4^+$ T cell lines and clones from PV patients produced Th2 cell-related cytokines and that their response to Dsg3 fusion proteins was also restricted to HLA-DR (Lin, Swartz, and Lopez 1997). Furthermore, using Dsg3-responsive T cell lines and clones from PV patients, a study from our group revealed that Dsg3-specific autoreactive Th cells mainly belonged to the Th2 cell lineage, whilst those in healthy individuals predominantly represented Th1 cells (Veldman et al. 2003b). In addition, Rizzo et al. observed Dsg3-specific Th2 cell activity being significantly associated with PV patients suffering from active disease, as well as the extent of Th2 cell activity correlating with the Dsg3-specific auto-ab titers of the patients (Rizzo et al. 2005). In concordance with these findings, the IgG4 subclass prevailed in active pemphigus patients, being linked with Th2 cell activation, in contrast to IgG1 being related to a Th1 cell-dominated immune response (Ayatollahi et al. 2004; Romagnani 1992).

Apart from that, data from an active mouse model of PV provided evidence for autoreactive Th2 cells priming naïve B cells to produce pathogenic Dsg3-specific IgG. In this model, release of the Th2 cell signature cytokine IL-4 by a single Dsg3-reactive T cell clone critically contributed to the formation of pathogenic IgG auto-ab, and *in vivo* blockade of IL-4 was effective in preventing development of the pemphigus phenotype (Takahashi et al. 2008). Similarly, in an unrelated mouse model, immunization of mice with human Dsg3 resulted in the generation of Dsg3-reactive Th2 cells, which induced unprimed B cells to produce Dsg3-specific IgG (Zhu et al. 2012).

In our HLA-DRB1*04:02–tg mouse model of PV, we clearly demonstrated that T cell recognition of human Dsg3 protein is tightly restricted by HLA-DRB1*04:02 since solely T cells from mice immunized with a set of HLA-DRβ1*04:02–binding Dsg3 peptides displayed *ex vivo* reactivity against Dsg3 (**Figure 4.38**). In contrast, immunization of mice with a set of Dsg3 peptides, which do not bind to HLA-DRβ1*04:02 did not induce Dsg3-specific T cell responses (**Figure 4.38**).

As a specificity control, mice transgenic for the unrelated RA-associated HLA class II allele DRB1*04:01 were selected and as expected, immunization of these mice with T cell epitopes of human Dsg3, which bind or not bind to HLA-DRβ1*04:02, respectively, only resulted in an immune response directed against the respective peptides they were injected with (**Figure 4.39**). As investigated by Wucherpfennig et al., the peptide binding cleft of the HLA-DRβ1*04:01 protein structurally differs from the PV-associated DR4 subtype by the β-chain carrying the neutral and positively charged amino acid residues glutamine (Q70) and lysine (K71) at P4, respectively. The P4 pocket of the HLA-DR4 molecule is situated in the center of the HLA-DR peptide binding groove and the physicochemical properties of its polymorphic surface residues critically determine

the binding of antigenic peptides, whilst flanking residues function as primary TCR contact site (Wucherpfennig et al. 1995).

Similarly, in the reverse *in vitro* experiment of APC presenting the Dsg3 peptides to Dsg3-specific T cells, only presentation of a set of HLA-DRβ1*04:02–binding Dsg3 peptides yielded a proliferative response of Dsg3-specific murine $CD4^+$ T cells (**Figure 4.36a**), which is in concordance with the described peptide binding algorithm for HLA-DRB1*04:02 proposed by Wucherpfennig et al. (Wucherpfennig et al. 1995). Accordingly, stimulation of Dsg3-reactive T cells with a set of Dsg3 peptides, which do not interact with HLA-DRβ1*04:02 only resulted in a response to the extent of background proliferation (**Figure 4.36b**). Consequently, the findings obtained in our *in vitro* and *in vivo* studies documented well the high level of specificity of the HLA-DRB1*04:02–restricted $CD4^+$ T cell response to human Dsg3.

Considering an algorithm for anchor motifs of the Dsg3 peptides and the charge of critical peptide binding pockets of HLA-DRβ1*04:02, Wucherpfennig et al. defined several T cell epitopes of Dsg3, of which three located within the Dsg3 ectodomain induced activation of peripheral lymphocytes from PV patients (Wucherpfennig et al. 1995). In line with this, a study from our group demonstrated in PV patients that carried the HLA-DRB1*04:02 allele T cell reactivity against these HLA-DRβ1*04:02–binding Dsg3 peptides using Dsg3-reactive $CD4^+$ T cell clones (Veldman, Gebhard, et al. 2004). As expected, those Dsg3 peptides, which do not bind to HLA-DRβ1*04:02 did not induce a proliferative T cell response (Veldman, Gebhard, et al. 2004). According to the findings by Wucherpfennig et al. (Wucherpfennig et al. 1995), the identified T cell epitopes of Dsg3 specifically fulfilled the requirements for efficient binding to the peptide binding groove of HLA-DRβ1*04:02 and exhibited shared anchor motifs at P1, P4, and P6, including a positively charged residue at P4 (Veldman, Gebhard, et al. 2004).

Altogether, the HLA-DRB1*04:02**–**tg mouse model clearly reproduced the algorithm proposed (Wucherpfennig et al. 1995) and ascertained (Tong et al. 2006) for peptide interaction with HLA-DRβ1*04:02 as observed in PV patients (Veldman, Gebhard, et al. 2004).

5.2.2 Dsg3 peptide immunization of HLA-DRB1*04:02–tg mice induces Dsg3-specific IgG

Pathogenic Dsg3-specific IgG auto-ab, which induced dissociation of epidermal keratinocyte adhesion represents a key finding in PV (Amagai 2008). Repetitive immunization of our HLA-DRB1*04:02**–**tg mice with human Dsg3 protein resulted in a robust antigen-specific IgG antibody response, recognizing both recombinant (**Figure 4.32**) and native human Dsg3 epitopes (**Figure 4.33**). Owing to limited cross-reactivity with the analogous mouse protein, immunization of these mice with the human Dsg3 ectodomain failed to induce a clinical phenotype, which will be discussed in more detail in the following section of this discussion. However, as determined by our group, sera from human Dsg3-immunized mice caused loss of adhesion of human epidermal keratinocytes under *in vitro* conditions. Furthermore, injection of these sera into human skin specimens resulted in antiepithelial surface IgG deposits, as well as intraepidermal loss of cell

adhesion, providing clear evidence for the pathogenicity of these IgG antibodies. Interestingly, sera from HLA-DRB1*04:02–tg mice immunized with human Dsg3 protein induced dissociation of human epidermal keratinocyte monolayers to a greater extent than did the sera from Dsg3-immunized HLA-DR-B1*04:01–tg mice (Eming et al. 2014). This finding is in line with the observation of autoreactive $CD4^+$ T cells regulating production of pathogenic IgG auto-ab in the pathogenesis of PV as demonstrated by numerous studies (Hertl, Amagai, et al. 1998a; Nishifuji et al. 2000; Takahashi et al. 2008; Takahashi, Kuwana, and Amagai 2009; Eming et al. 2008; Amber et al. 2013).

In our PV model, further evidence for the generation of Dsg3-responsive IgG antibodies relying on the activation of autoreactive T cells is provided by the finding of only sera from mice immunized with the HLA-DRβ1*04:02–binding Dsg3 peptides showing reactivity against human recombinant (**Figure 4.41a**) and native monkey Dsg3 protein (**Figure 4.42a** and **b**). However, immunization of mice with a set of Dsg3 peptides, which do not efficiently bind to HLA-DRβ1*04:02 did not yield IgG antibodies reactive against both recombinant (**Figure 4.41a**) and native epitopes of Dsg3 expressed by monkey esophagus epithelium (**Figure 4.42c** and **d**). In line with the observation of defying restriction by HLA-DRB1*04:02 on the T cell level (**Figure 4.39b**), sera from mice transgenic for the RA-associated HLA-DRB1*04:01 allele did neither display reactivity against recombinant (**Figure 4.41b**) nor native (**Figure 4.43a**) Dsg3 upon immunization with the HLA-DRβ1*04:02–binding Dsg3 peptides.
Of note, sera from both HLA-DRB1*04:02–tg and –DRB1*04:01–tg mice immunized with the full Dsg3 ectodomain displayed IgG reactivity against recombinant Dsg3 (**Figure 4.41**). However, compared to HLA-DRB1*04:02–tg mice, Dsg3-reactive IgG from HLA-DRB1*04:01–tg mice were found to be reduced in pathogenicity as determined by human epidermal keratinocyte dissociation assay (Eming et al. 2014). This indicates that the human Dsg3 protein additionally contains $CD4^+$ T cell epitopes that bind to HLA-DRβ1*04:01, which finally results in the production of human Dsg3-specific IgG antibodies.

The interaction between $CD4^+$ T cells and B cells being required for the induction of human Dsg3-reactive antibodies is further demonstrated by blockade of $CD4^+$ T cells efficaciously abrogating the formation of anti-Dsg3 IgG in HLA-DRB1*04:02–transgenic mice. In addition, application of an anti-CD154 mAb disturbing T cell-mediated activation of B cells similarly impeded induction of Dsg3-specific IgG antibodies (Eming et al. 2014). Interestingly, there is also clinical proof for $CD4^+$ T cell–B cell interaction being critical in the formation of Dsg3-reactive auto-ab since application of the anti-CD20 mAb Rituximab not only entailed a rapid clearance of peripheral B cells, but additionally resulted in a swift reduction of IL-4–producing Dsg3-reactive Th2 cells in the circulation (Eming et al. 2008). Likewise, medical treatment of PV patients with Rituximab led to a significant decline of Dsg3-specific serum IgG auto-ab. Of note, the diminution of Dsg3-reactive T cells was also linked with an immediate amelioration of clinical symptoms before anti-Dsg3 serum auto-ab were reduced (Eming et al. 2014). These observations imply the relevance of autoantigen presentation by B cells in the activation of Dsg3-reactive $CD4^+$ T cells.

Interestingly, Nishifuji et al. quantitatively determined Dsg3-specific IgG auto-ab production of circulating B cells by ELISPOT assay. In order to not only detect peripheral Dsg3-reactive activated, but also memory B cells, PBMC were additionally stimulated with recombinant Dsg3 protein *ex vivo*. Importantly, the anti-Dsg3 antibody production was abolished upon depleting the PV patients' lymphocytes of $CD4^+$ T cells, highlighting the critical function of autoreactive $CD4^+$ T cells in auto-ab induction (Nishifuji et al. 2000).

The currently most established mouse model to study pathoimmunological mechanisms of PV *in vivo* was developed by Amagai et al. in 2000. In this model, active autoimmune disease can be reproduced by using a Dsg3-deficient mouse created by Koch et al. (Koch et al. 1997), which lacks acquired tolerance against Dsg3 and displays a phenotype reminiscent of PV. As the target protein is not expressed, immunization of these mice with murine Dsg3 induces a Dsg3-specific IgG response, which does not cause appearance of PV clinical symptoms. Yet, adoptive transfer of these Dsg3-reactive lymphocytes into immunodeficient $Rag2^{-/-}$ mice competent of epithelial Dsg3 expression results in the generation of anti-Dsg3 IgG auto-ab and manifestation of the PV phenotype (Amagai et al. 2000).
Using this active autoimmune disease mouse model of PV, a study by Tsunoda et al. demonstrated that Dsg3-specific loss of tolerance was required in both T cells and B cells for efficient production of pathogenic IgG auto-ab, as well as induction of PV-like clinical signs. While adoptive transfer of purified T and B cells from $Dsg3^{+/-}$ or $Dsg3^{+/+}$ mice into $Rag2^{-/-}$ mice did not induce pathogenic anti-Dsg3 IgG production, only a combination of both $Dsg3^{-/-}$ T and $Dsg3^{-/-}$ B cells was successful at causing the autoimmune state of PV. Furthermore, the single transfer of solely $Dsg3^{-/-}$ $CD4^+$ T cells or B cells into $Rag2^{-/-}$ mice was not efficient at inducing anti-Dsg3 IgG and/or a clinical phenotype, underpinning the collaboration of Dsg3-specific $CD4^+$ T cells being essential for the generation of pathogenic anti-Dsg3 auto-ab (Tsunoda et al. 2002).
Using the same animal model, Aoki-Ota et al. later provided evidence for the induction of pathogenic anti-Dsg3 IgG critically relying on CD40–CD154 interaction, as its antagonization almost entirely abrogated auto-ab production and prevented blister formation in the recipient mice (Aoki-Ota et al. 2006). T cell-mediated activation of B cells requires binding of CD154 on activated T cells with CD40 expressed on B cells (Lougaris et al. 2005). Interestingly, administration of an anti-CD154 mAb was found to expand Treg cells in allograft transplant patients (Zhai et al. 2006).
Interestingly, Takahashi et al. established in this active mouse model a novel system for the evaluation of the *in vivo* pathogenicity of Dsg3-responsive T cells at a clonal level. Therefore, Dsg3-specific $CD4^+$ T cell lines generated *in vitro* were adoptively transferred into $Rag2^{-/-}$ mice combined with primed B cells from Dsg3-immunized Dsg3-deficient mice. Analysis of pathogenic T cell lines, which induced in recipient mice an anti-Dsg3 IgG response and a clinical phenotype identified the capacity of IL-4 production as a potential factor linked with pathogenicity (Takahashi et al. 2008). In a following study, Takahashi et al. demonstrated in this model that a single potent Dsg3-reactive T cell clone sufficed to render naïve B cells capable of secreting pathogenic anti-Dsg3 IgG auto-ab, which induced the clinical PV phenotype (Takahashi, Kuwana, and Amagai 2009).

Although the active disease model reliably imitates ongoing autoimmune responses in PV (Amagai 2008), it does not take into account the strong association of distinct HLA class II alleles in pemphigus patients. Thus, we show in our HLA-DRB1*04:02–tg mouse model for the first time that pathogenic anti-Dsg3 IgG antibody generation tightly depends on activation of autoreactive T cells under the immunogenetic restriction by HLA-DRB1*04:02.

5.2.3 IgG cross-reactivity of sera from human Dsg3-immunized HLA-DR4–tg mice

In the HLA-DRB1*04:02–tg mouse model of PV, we demonstrated that the activation of Dsg3-specific $CD4^+$ T cells and B cells leading to the production of pathogenic Dsg3-reactive IgG antibodies is tightly restricted by HLA-DRB1*04:02. Accordingly, immunization of these mice with T cell epitopes of human Dsg3, which bind to HLA-DRβ1*04:02 was efficient at inducing a marked anti-Dsg3 IgG response reactive against both recombinant (**Figure 4.41a**) and native Dsg3 protein (**Figure 4.42a** and **b**).

Since mice immunized with human Dsg3 did not display a clinical phenotype, we further assessed cross-reactivity of human Dsg3-specific IgG against murine Dsg3. Apparently, sera from these mice only displayed a diminished interaction with murine Dsg3 *ex vivo* (**Figure 4.45**), while DIF microscopy revealed lack of binding of IgG antibodies to both palatinal (**Figure 4.46a**) and tongue (**Figure 4.46b**) mucosa. Comparative analysis of mouse and human Dsg3 amino acid sequences yielded an overall homology of 85.6%, indicating a relatively high level of conservation. Particularly, the proportion of identical sequences between the ectodomains of Dsg3 rises from the carboxy-terminal EC5 domain (56%) to the amino-terminal EC1 and EC2 domains (86–89%) (Ishikawa et al. 2000). Of note, the amino-terminal adhesive surface of Dsg3 was identified to provide the dominant pathogenic auto-ab epitopes in PV patients (Sekiguchi et al. 2001; Futei et al. 2000; Yokouchi et al. 2009). However, anti-human Dsg3-reactive IgG failed to efficiently bind to epithelial Dsg3 *in vivo*, which might explain the absence of a blistering phenotype in our HLA-DRB1*04:02–tg mouse model of PV. A possible explanation may be the amino acid sequences of human and mouse Dsg3 significantly differing at the sites critical for $CD4^+$ T cell activation. Insufficient activation of Dsg3-reactive $CD4^+$ T cells may critically hamper activation of autoreactive B cells being responsible for the production of pathogenic antibodies (Eming et al. 2014).

To summarize, because of a missing clinical phenotype in HLA-DRB1*04:02–tg mice due to limited cross-reactivity of human Dsg3-specific antibodies against murine Dsg3, this model does not comprehensively reproduce human PV. Yet, this model efficiently displays immunopathological mechanisms on both the $CD4^+$ T cell and B cell level of PV considering the important restriction by HLA-DRB1*04:02. Thus, our HLA-DRB1*04:02–tg mouse model represents a promising tool for further investigations of pathoimmunological mechanisms.

5.2.4 Summary

In our HLA-DRB1*04:02–tg mouse model of PV we investigated Dsg3-specific cellular and humoral immune responses under the immunogenetic restriction by HLA-DRB1*04:02 based on the prevalence of PV being associated with distinct HLA class II alleles, including HLA-DRB1*04:02, –DRB1*14:01, and –DQB1*05:03. Our model convincingly confirmed the basic mechanisms of HLA-dependent and Dsg3-specific induction of $CD4^+$ T and B cell responses observed in PV patients. Accordingly, the generation of human Dsg3-reactive IgG antibody responses highly depended on prior activation of Dsg3-reactive $CD4^+$ T cells. In addition, APC-mediated activation of T cells critically relied on the recognition of epitopes of the Dsg3 ectodomain displaying strong binding affinity to the PV-linked HLA class II allele HLA-DRB1*04:02.

A limitation of this PV model is the found restricted cross-reactivity of Dsg3-specific IgG antibodies against the mouse analogue protein explaining lack of a clinical PV phenotype upon immunization of HLA-DRB1*04:02–tg mice with human Dsg3 protein. Yet, this model accurately reproduces that polymorphisms of peptide-binding motifs of specific PV-related HLA-class II alleles tightly regulate $CD4^+$ T cell-mediated induction of Dsg3-reactive IgG antibodies. Consequently, our novel mouse model represents a promising tool for further studies of PV pathoimmunological mechanisms *in vivo* considering the strong HLA class II association of the disease.

6 References

Abram, C. L., G. L. Roberge, L. I. Pao, B. G. Neel, and C. A. Lowell. 2013. 'Distinct roles for neutrophils and dendritic cells in inflammation and autoimmunity in motheaten mice', *Immunity*, 38: 489-501.

Aggarwal, S., N. Ghilardi, M. H. Xie, F. J. de Sauvage, and A. L. Gurney. 2003. 'Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17', *J Biol Chem*, 278: 1910-4.

Ahmed, A. R., E. J. Yunis, K. Khatri, R. Wagner, G. Notani, Z. Awdeh, and C. A. Alper. 1990. 'Major histocompatibility complex haplotype studies in Ashkenazi Jewish patients with pemphigus vulgaris', *Proc Natl Acad Sci U S A*, 87: 7658-62.

Alecu, M., S. Alecu, G. Coman, E. Galatescu, and C. Ursaciuc. 1999. 'ICAM-1, ELAM-1, TNF-alpha and IL-6 in serum and blister liquid of pemphigus vulgaris patients', *Roum Arch Microbiol Immunol*, 58: 121-30.

Allen, Jennifer S., Karl Pang, Ania Skowera, Richard Ellis, Chloe Rackham, Biliana Lozanoska-Ochser, Timothy Tree, R. David G. Leslie, Jennifer M. Tremble, Colin M. Dayan, and Mark Peakman. 2009. 'Plasmacytoid Dendritic Cells Are Proportionally Expanded at Diagnosis of Type 1 Diabetes and Enhance Islet Autoantigen Presentation to T-Cells Through Immune Complex Capture', *Diabetes*, 58: 138-45.

Allman, D., and S. Pillai. 2008. 'Peripheral B cell subsets', *Curr Opin Immunol*, 20: 149-57.

Alpsoy, E., A. Akman-Karakas, and S. Uzun. 2015. 'Geographic variations in epidemiology of two autoimmune bullous diseases: pemphigus and bullous pemphigoid', *Arch Dermatol Res*, 307: 291-8.

Alvarez, David, Elisabeth H. Vollmann, and Ulrich H. von Andrian. 2008. 'Mechanisms and Consequences of Dendritic Cell Migration', *Immunity*, 29: 325.

Amagai, M. 2002. 'Pemphigus as a paradigm of autoimmunity and cell adhesion', *Keio J Med*, 51: 133-9.

Amagai, M. 2008. 'Pemphigus vulgaris and its active disease mouse model', *Curr Dir Autoimmun*, 10: 167-81.

Amagai, M., S. Karpati, R. Prussick, V. Klaus-Kovtun, and J. R. Stanley. 1992. 'Autoantibodies against the amino-terminal cadherin-like binding domain of pemphigus vulgaris antigen are pathogenic', *J Clin Invest*, 90: 919-26.

Amagai, M., V. Klaus-Kovtun, and J. R. Stanley. 1991. 'Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion', *Cell*, 67: 869-77.

Amagai, M., P. J. Koch, T. Nishikawa, and Stanley. 1996. 'Pemphigus vulgaris antigen (desmoglein 3) is localized in the lower epidermis, the site of blister formation in patients', *J Invest Dermatol*, 106: 351-55.

Amagai, M., A. Komai, T. Hashimoto, Y. Shirakata, K. Hashimoto, T. Yamada, Y. Kitajima, K. Ohya, H. Iwanami, and T. Nishikawa. 1999. 'Usefulness of enzyme-linked immunosorbent assay using recombinant desmogleins 1 and 3 for serodiagnosis of pemphigus', *Br J Dermatol*, 140: 351-7.

Amagai, M., K. Tsunoda, H. Suzuki, K. Nishifuji, S. Koyasu, and T. Nishikawa. 2000. 'Use of autoantigen-knockout mice in developing an active autoimmune disease model for pemphigus', *J Clin Invest*, 105: 625-31.

Amagai, M., K. Tsunoda, D. Zillikens, T. Nagai, and T. Nishikawa. 1999. 'The clinical phenotype of pemphigus is defined by the anti-desmoglein autoantibody profile', *J Am Acad Dermatol*, 40: 167-70.

Amagai, Masayuki, and John R. Stanley. 2012. 'Desmoglein as a Target in Skin Disease and Beyond', *J Invest Dermatol*, 132: 776-84.

Amber, K. T., P. Staropoli, M. I. Shiman, G. W. Elgart, and M. Hertl. 2013. 'Autoreactive T cells in the immune pathogenesis of pemphigus vulgaris', *Exp Dermatol*, 22: 699-704.

Anhalt, G. J., R. S. Labib, and Voorhes. 1982. 'Induction of pemphigus in neonatal mice by passive trasfer of IgG from patients with disease', *N Engl J Med*, 306: 1189-96.

Anzai, H., Y. Fujii, K. Nishifuji, M. Aoki-Ota, T. Ota, M. Amagai, and T. Nishikawa. 2004. 'Conformational epitope mapping of antibodies against desmoglein 3 in experimental murine pemphigus vulgaris', *J Dermatol Sci*, 35: 133-42.

Aoki-Ota, M., M. Kinoshita, T. Ota, K. Tsunoda, T. Iwasaki, S. Tanaka, S. Koyasu, T. Nishikawa, and M. Amagai. 2006. 'Tolerance induction by the blockade of CD40/CD154 interaction in pemphigus vulgaris mouse model', *J Invest Dermatol*, 126: 105-13.

Arakawa, M., T. Dainichi, S. Yasumoto, and T. Hashimoto. 2009. 'Lesional Th17 cells in pemphigus vulgaris and pemphigus foliaceus', *J Dermatol Sci*, 53: 228-31.

Asothai, R., V. Anand, D. Das, P. S. Antil, S. Khandpur, V. K. Sharma, and A. Sharma. 2015. 'Distinctive Treg associated CCR4-CCL22 expression profile with altered frequency of Th17/Treg cell in the immunopathogenesis of Pemphigus Vulgaris', *Immunobiology*, 220: 1129-35.

Auffray, C., D. Fogg, M. Garfa, G. Elain, O. Join-Lambert, S. Kayal, S. Sarnacki, A. Cumano, G. Lauvau, and F. Geissmann. 2007. 'Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior', *Science*, 317: 666-70.

Auffray, Cedric, Michael H. Sieweke, and Frederic Geissmann. 2009. 'Blood Monocytes: Development, Heterogeneity, and Relationship with Dendritic Cells', *Annual Review of Immunology Annu. Rev. Immunol.*, 27: 669-92.

Avalos-Diaz, E., M. Olague-Marchan, A. Lopez-Swiderski, R. Herrera-Esparza, and L. A. Diaz. 2000. 'Transplacental passage of maternal pemphigus foliaceus autoantibodies induces neonatal pemphigus', *J Am Acad Dermatol*, 43: 1130-4.

Ayatollahi, M., S. Joubeh, H. Mortazavi, R. Jefferis, and A. Ghaderi. 2004. 'IgG4 as the predominant autoantibody in sera from patients with active state of pemphigus vulgaris', *J Eur Acad Dermatol Venereol*, 18: 241-2.

Backlund, J., S. Carlsen, T. Hoger, B. Holm, L. Fugger, J. Kihlberg, H. Burkhardt, and R. Holmdahl. 2002. 'Predominant selection of T cells specific for the glycosylated collagen type II epitope (263-270) in humanized transgenic mice and in rheumatoid arthritis', *Proc Natl Acad Sci U S A*, 99: 9960-5.

Baeten, D., A. M. Boots, P. G. Steenbakkers, D. Elewaut, E. Bos, G. F. Verheijden, G. Berheijden, A. M. Miltenburg, A. W. Rijnders, E. M. Veys, and F. De Keyser. 2000. 'Human cartilage gp-39+,CD16+ monocytes in peripheral blood and synovium: correlation with joint destruction in rheumatoid arthritis', *Arthritis Rheum*, 43: 1233-43.

Banchereau, J., and V. Pascual. 2006. 'Type I interferon in systemic lupus erythematosus and other autoimmune diseases', *Immunity*, 25: 383-92.

Banks, C., A. Bateman, R. Payne, P. Johnson, and N. Sheron. 2003. 'Chemokine expression in IBD. Mucosal chemokine expression is unselectively increased in both ulcerative colitis and Crohn's disease', *J Pathol*, 199: 28-35.

Bar-On, L., T. Birnberg, K. W. Kim, and S. Jung. 2011. 'Dendritic cell-restricted CD80/86 deficiency results in peripheral regulatory T-cell reduction but is not associated with lymphocyte hyperactivation', *Eur J Immunol*, 41: 291-8.

Batsalova, T., B. Dzhambazov, P. Merky, A. Backlund, and J. Backlund. 2010. 'Breaking T cell tolerance against self type II collagen in HLA-DR4-transgenic mice and development of autoimmune arthritis', *Arthritis Rheum*, 62: 1911-20.

Batten, M., N. M. Kljavin, J. Li, M. J. Walter, F. J. de Sauvage, and N. Ghilardi. 2008. 'Cutting edge: IL-27 is a potent inducer of IL-10 but not FoxP3 in murine T cells', *J Immunol*, 180: 2752-6.

Batten, Marcel, Nandhini Ramamoorthi, Noelyn M Kljavin, Cindy S Ma, Jennifer H Cox, Hart S Dengler, Dimitry M Danilenko, Patrick Caplazi, Melanie Wong, David A Fulcher, Matthew C Cook, Cecile King, Stuart G Tangye, Frederic J de Sauvage, and Nico Ghilardi. 2010. 'IL-

27 supports germinal center function by enhancing IL-21 production and the function of T follicular helper cells', *J Exp Med*, 207: 2895-906.

Baum, Sharon, Nicole Sakka, Ofir Artsi, Henri Trau, and Aviv Barzilai. 2014. 'Diagnosis and classification of autoimmune blistering diseases', *Autoimmun Rev*, 13: 482-89.

Baumgart, D. C., D. Metzke, O. Guckelberger, A. Pascher, C. Grotzinger, I. Przesdzing, Y. Dorffel, J. Schmitz, and S. Thomas. 2011. 'Aberrant plasmacytoid dendritic cell distribution and function in patients with Crohn's disease and ulcerative colitis', *Clin Exp Immunol*, 166: 46-54.

Baumgart, D. C., D. Metzke, J. Schmitz, A. Scheffold, A. Sturm, B. Wiedenmann, and A. U. Dignass. 2005. 'Patients with active inflammatory bowel disease lack immature peripheral blood plasmacytoid and myeloid dendritic cells', *Gut*, 54: 228-36.

Bave, U., M. Magnusson, M. L. Eloranta, A. Perers, G. V. Alm, and L. Ronnblom. 2003. 'Fc gamma RIIa is expressed on natural IFN-alpha-producing cells (plasmacytoid dendritic cells) and is required for the IFN-alpha production induced by apoptotic cells combined with lupus IgG', *J Immunol*, 171: 3296-302.

Belge, K. U., F. Dayyani, A. Horelt, M. Siedlar, M. Frankenberger, B. Frankenberger, T. Espevik, and L. Ziegler-Heitbrock. 2002. 'The Proinflammatory CD14+CD16+DR++ Monocytes Are a Major Source of TNF', *The Journal of Immunology*, 168: 3536-42.

Belkaid, Y., and K. Tarbell. 2009. 'Regulatory T cells in the control of host-microorganism interactions (*)', *Annu Rev Immunol*, 27: 551-89.

Bennett, L., A. K. Palucka, E. Arce, V. Cantrell, J. Borvak, J. Banchereau, and V. Pascual. 2003. 'Interferon and granulopoiesis signatures in systemic lupus erythematosus blood', *J Exp Med*, 197: 711-23.

Bhol, K. C., A. I. Rojas, I. U. Khan, and A. R. Ahmed. 2000. 'Presence of interleukin 10 in the serum and blister fluid of patients with pemphigus vulgaris and pemphigoid', *Cytokine*, 12: 1076-83.

Birnberg, T., L. Bar-On, A. Sapoznikov, M. L. Caton, L. Cervantes-Barragan, D. Makia, R. Krauthgamer, O. Brenner, B. Ludewig, D. Brockschnieder, D. Riethmacher, B. Reizis, and S. Jung. 2008. 'Lack of conventional dendritic cells is compatible with normal development and T cell homeostasis, but causes myeloid proliferative syndrome', *Immunity*, 29: 986-97.

Blair, Paul A., Lina Yassin Noreña, Fabian Flores-Borja, David J. Rawlings, David A. Isenberg, Michael R. Ehrenstein, and Claudia Mauri. 'CD19+CD24hiCD38hi B Cells Exhibit Regulatory Capacity in Healthy Individuals but Are Functionally Impaired in Systemic Lupus Erythematosus Patients', *Immunity*, 32: 129-40.

Bogdanos, D. P., D. S. Smyk, E. I. Rigopoulou, M. G. Mytilinaiou, M. A. Heneghan, C. Selmi, and M. E. Gershwin. 2012. 'Twin studies in autoimmune disease: genetics, gender and environment', *J Autoimmun*, 38: J156-69.

Bonasio, R., M. L. Scimone, P. Schaerli, N. Grabie, A. H. Lichtman, and U. H. von Andrian. 2006. 'Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus', *Nat Immunol*, 7: 1092-100.

Bonilla, F. A., and H. C. Oettgen. 2010. 'Adaptive immunity', *J Allergy Clin Immunol*, 125: S33-40.

Boring, L., J. Gosling, S. W. Chensue, S. L. Kunkel, R. V. Farese, Jr., H. E. Broxmeyer, and I. F. Charo. 1997. 'Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice', *J Clin Invest*, 100: 2552-61.

Boring, L., J. Gosling, M. Cleary, and I. F. Charo. 1998. 'Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis', *Nature*, 394: 894-7.

Bovenschen, H. J., P. C. van de Kerkhof, P. E. van Erp, R. Woestenenk, I. Joosten, and H. J. Koenen. 2011. 'Foxp3+ regulatory T cells of psoriasis patients easily differentiate into IL-17A-producing cells and are found in lesional skin', *J Invest Dermatol*, 131: 1853-60.

Broussard, JoshuaA, Spiro Getsios, and KathleenJ Green. 2015. 'Desmosome regulation and signaling in disease', *Cell and Tissue Research*: 1-12.

Brubaker, S. W., K. S. Bonham, I. Zanoni, and J. C. Kagan. 2015a. 'Innate immune pattern recognition: a cell biological perspective', *Annu Rev Immunol*, 33: 257-90.

Brubaker, S. W. 2015b. 'Innate Immune Pattern Recognition: A Cell Biological Perspective', *Annu Rev Immunol*.

Brzustewicz, E., and E. Bryl. 2015. 'The role of cytokines in the pathogenesis of rheumatoid arthritis - Practical and potential application of cytokines as biomarkers and targets of personalized therapy', *Cytokine*.

Burbano, C., G. Vasquez, and M. Rojas. 2014. 'Modulatory effects of CD14+CD16++ monocytes on CD14++CD16- monocytes: a possible explanation of monocyte alterations in systemic lupus erythematosus', *Arthritis Rheumatol*, 66: 3371-81.

Bystryn, Jean-Claude, and Jennifer L. Rudolph. 'Pemphigus', *The Lancet*, 366: 61-73.

Calderon, B., A. Suri, M. J. Miller, and E. R. Unanue. 2008. 'Dendritic cells in islets of Langerhans constitutively present beta cell-derived peptides bound to their class II MHC molecules', *Proc Natl Acad Sci U S A*, 105: 6121-6.

Campa, M., C. Ryan, and A. Menter. 2015. 'An overview of developing TNF-alpha targeted therapy for the treatment of psoriasis', *Expert Opin Investig Drugs*: 1-12.

Cao, Yanxia, Paul D. Doodes, Tibor T. Glant, and Alison Finnegan. 2008 'IL-27 Induces a Th1 Immune Response and Susceptibility to Experimental Arthritis 10.4049/jimmunol.180.2.922 ', *The Journal of Immunology*, 180 922-30

Carsetti, R., M. M. Rosado, and H. Wardmann. 2004. 'Peripheral development of B cells in mouse and man', *Immunol Rev*, 197: 179-91.

Cayrol, Corinne, and Jean-Philippe Girard. 2014. 'IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy', *Current Opinion in Immunology Autoimmunity * Allergy and hypersensitivity*, 31: 31-37.

Chaplin, D. D. 2010. 'Overview of the immune response', *J Allergy Clin Immunol*, 125: S3-23.

Chen, Xueqin, Levi H. C. Makala, Yulan Jin, Diane Hopkins, Andy Muir, Nikhil Garge, Robert H. Podolsky, and Jin-Xiong She. 2008. 'Type 1 diabetes patients have significantly lower frequency of plasmacytoid dendritic cells in the peripheral blood', *Clin Immunol*, 129: 413-18.

Chen, Y. F., D. Zhou, T. Metzger, M. Gallup, M. Jeanne, D. B. Gould, M. S. Anderson, and N. A. McNamara. 2014. 'Spontaneous development of autoimmune uveitis Is CCR2 dependent', *Am J Pathol*, 184: 1695-705.

Chiapa-Labastida, M., A. Zentella-Dehesa, G. Leon-Dorantes, and I. Becker. 2011. 'Pemphigus vulgaris: accumulation of apoptotic cells in dermis and epidermis possibly relates to pathophysiology through TNF-alpha production by phagocytes', *Eur J Dermatol*, 21: 874-88.

Chiossi, M. P., R. S. Costa, and A. M. Roselino. 2004. 'Dermal dendritic cell number correlates with serum autoantibody titers in Brazilian pemphigus foliaceus patients', *Braz J Med Biol Res*, 37: 337-41.

Cho, J. H. 2008. 'The genetics and immunopathogenesis of inflammatory bowel disease', *Nat Rev Immunol*, 8: 458-66.

Cho, Judy H., and Marc Feldman. 2015. 'Heterogeneity of autoimmune diseases: pathophysiologic insights from genetics and implications for new therapies', *Nat Med*, 21: 730-38.

Cho, M. J., A. S. Lo, X. Mao, A. R. Nagler, C. T. Ellebrecht, E. M. Mukherjee, C. M. Hammers, E. J. Choi, P. M. Sharma, M. Uduman, H. Li, A. H. Rux, S. A. Farber, C. B. Rubin, S. H. Kleinstein, B. S. Sachais, M. R. Posner, L. A. Cavacini, and A. S. Payne. 2014. 'Shared VH1-46 gene usage by pemphigus vulgaris autoantibodies indicates common humoral immune responses among patients', *Nat Commun*, 5: 4167.

Cho, Michael Jeffrey, Christoph T. Ellebrecht, and Aimee S. Payne. 'The dual nature of interleukin-10 in pemphigus vulgaris', *Cytokine*.

Chow, Andrew, Brian D. Brown, and Miriam Merad. 2011. 'Studying the mononuclear phagocyte system in the molecular age', *Nat Rev Immunol*, 11: 788-98.

Christophi, G. P., R. C. Gruber, M. Panos, R. L. Christophi, B. Jubelt, and P. T. Massa. 2012. 'Interleukin-33 upregulation in peripheral leukocytes and CNS of multiple sclerosis patients', *Clin Immunol*, 142: 308-19.

Chuluundorj, D., S. A. Harding, D. Abernethy, and A. C. La Flamme. 2014. 'Expansion and preferential activation of the CD14(+)CD16(+) monocyte subset during multiple sclerosis', *Immunol Cell Biol*, 92: 509-17.

Ciccia, F., R. Alessandro, V. Rodolico, G. Guggino, S. Raimondo, C. Guarnotta, A. Giardina, G. Sireci, G. Campisi, G. De Leo, and G. Triolo. 2013. 'IL-34 is overexpressed in the inflamed salivary glands of patients with Sjogren's syndrome and is associated with the local expansion of pro-inflammatory CD14(bright)CD16+ monocytes', *Rheumatology (Oxford)*, 52: 1009-17.

Cirillo, N., E. Cozzani, M. Carrozzo, and S. A. Grando. 2012. 'Urban legends: pemphigus vulgaris', *Oral Dis*, 18: 442-58.

Clarkson, B. D., A. Walker, M. G. Harris, A. Rayasam, M. Sandor, and Z. Fabry. 2015. 'CCR2-dependent dendritic cell accumulation in the central nervous system during early effector experimental autoimmune encephalomyelitis is essential for effector T cell restimulation in situ and disease progression', *J Immunol*, 194: 531-41.

Collin, M., N. McGovern, and M. Haniffa. 2013. 'Human dendritic cell subsets', *Immunology*, 140: 22-30.

Collins, C. B., C. M. Aherne, E. N. McNamee, M. D. Lebsack, H. Eltzschig, P. Jedlicka, and J. Rivera-Nieves. 2012. 'Flt3 ligand expands CD103(+) dendritic cells and FoxP3(+) T regulatory cells, and attenuates Crohn's-like murine ileitis', *Gut*, 61: 1154-62.

Congia, M., S. Patel, A. P. Cope, S. De Virgiliis, and G. Sonderstrup. 1998. 'T cell epitopes of insulin defined in HLA-DR4 transgenic mice are derived from preproinsulin and proinsulin', *Proc Natl Acad Sci U S A*, 95: 3833-8.

Cooper, D. L., S. G. Martin, J. I. Robinson, S. L. Mackie, C. J. Charles, J. Nam, J. D. Isaacs, P. Emery, and A. W. Morgan. 2012. 'FcgammaRIIIa expression on monocytes in rheumatoid arthritis: role in immune-complex stimulated TNF production and non-response to methotrexate therapy', *PLoS One*, 7: e28918.

Cope, A. P., S. D. Patel, F. Hall, M. Congia, H. A. Hubers, G. F. Verheijden, A. M. Boots, R. Menon, M. Trucco, A. W. Rijnders, and G. Sonderstrup. 1999. 'T cell responses to a human cartilage autoantigen in the context of rheumatoid arthritis-associated and nonassociated HLA-DR4 alleles', *Arthritis Rheum*, 42: 1497-507.

Cox, J. H., N. M. Kljavin, N. Ramamoorthi, L. Diehl, M. Batten, and N. Ghilardi. 2011. 'IL-27 promotes T cell-dependent colitis through multiple mechanisms', *J Exp Med*, 208: 115-23.

Criswell, L. A., K. A. Pfeiffer, R. F. Lum, B. Gonzales, J. Novitzke, M. Kern, K. L. Moser, A. B. Begovich, V. E. Carlton, W. Li, A. T. Lee, W. Ortmann, T. W. Behrens, and P. K. Gregersen. 2005. 'Analysis of families in the multiple autoimmune disease genetics consortium (MADGC) collection: the PTPN22 620W allele associates with multiple autoimmune phenotypes', *Am J Hum Genet*, 76: 561-71.

Cros, J., N. Cagnard, K. Woollard, N. Patey, S. Y. Zhang, B. Senechal, A. Puel, S. K. Biswas, D. Moshous, C. Picard, J. P. Jais, D. D'Cruz, J. L. Casanova, C. Trouillet, and F. Geissmann. 2010. 'Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors', *Immunity*, 33: 375-86.

Crotty, S. 2014. 'T follicular helper cell differentiation, function, and roles in disease', *Immunity*, 41: 529-42.

Culton, D. A., Y. Qian, N. Li, D. Rubenstein, V. Aoki, G. H. Filhio, E. A. Rivitti, and L. A. Diaz. 2008. 'Advances in pemphigus and its endemic pemphigus foliaceus (Fogo Selvagem) phenotype: a paradigm of human autoimmunity', *J Autoimmun*, 31: 311-24.

D/'Auria, L., C. Bonifati, and A. Mussi. 1997. 'Cytokines in the sera of patients with pemphigus vulgaris: interleukin-6 and tumor necrosis factor-alfa levels are significantly increased as compared to healthy subjects and correlate with disease activity', *Eur Cytokine Netw*, 8: 383-87.

Darrasse-Jeze, G., S. Deroubaix, H. Mouquet, G. D. Victora, T. Eisenreich, K. H. Yao, R. F. Masilamani, M. L. Dustin, A. Rudensky, K. Liu, and M. C. Nussenzweig. 2009. 'Feedback control of regulatory T cell homeostasis by dendritic cells in vivo', *J Exp Med*, 206: 1853-62.

Dayyani, F., K. U. Belge, M. Frankenberger, M. Mack, T. Berki, and L. Ziegler-Heitbrock. 2003. 'Mechanism of glucocorticoid-induced depletion of human CD14+CD16+ monocytes', *J Leukoc Biol*, 74: 33-9.

de Baey, A., I. Mende, G. Baretton, A. Greiner, W. H. Hartl, P. A. Baeuerle, and H. M. Diepolder. 2003. 'A subset of human dendritic cells in the T cell area of mucosa-associated lymphoid tissue with a high potential to produce TNF-alpha', *J Immunol*, 170: 5089-94.

Dean, Phillip N., and Robert A. Hoffman. 2001. 'Overview of Flow Cytometry Instrumentation.' in, *Current Protocols in Cytometry* (John Wiley & Sons, Inc.).

Delgado, J. C., A. Hameed, J. J. Yunis, K. Bhol, A. I. Rojas, S. B. Rehman, A. A. Khan, M. Ahmad, C. A. Alper, A. R. Ahmed, and E. J. Yunis. 1997. 'Pemphigus vulgaris autoantibody response is linked to HLA-DQB1*0503 in Pakistani patients', *Hum Immunol*, 57: 110-9.

Di Meglio, Paola, Gayathri K Perera, and Frank O Nestle. 'The Multitasking Organ: Recent Insights into Skin Immune Function', *Immunity*, 35: 857-69.

Dick, H. M. 1978. 'HLA and disease. Introductory review', *Br Med Bull*, 34: 271-4.

Dogan, Rukiye-Nazan E., Adam Elhofy, and William J. Karpus. 2008. 'Production of CCL2 by Central Nervous System Cells Regulates Development of Murine Experimental Autoimmune Encephalomyelitis through the Recruitment of TNF- and iNOS-Expressing Macrophages and Myeloid Dendritic Cells', *The Journal of Immunology*, 180: 7376-84.

Dolff, S., W. H. Abdulahad, J. Westra, B. Doornbos-van der Meer, P. C. Limburg, C. G. Kallenberg, and M. Bijl. 2011. 'Increase in IL-21 producing T-cells in patients with systemic lupus erythematosus', *Arthritis Res Ther*, 13: R157.

Donaldson, J. G. 2001. 'Immunofluorescence staining', *Curr Protoc Cell Biol*, Chapter 4: Unit 4 3.

Eming, R. 2015. '[Pemphigus : Model disease for targeted therapy]', *Hautarzt*, 66: 574-82.

Eming, R., T. Hennerici, J. Backlund, C. Feliciani, K. C. Visconti, S. Willenborg, J. Wohde, R. Holmdahl, G. Sonderstrup, and M. Hertl. 2014. 'Pathogenic IgG antibodies against desmoglein 3 in pemphigus vulgaris are regulated by HLA-DRB1*04:02-restricted T cells', *J Immunol*, 193: 4391-9.

Eming, R., and M. Hertl. 2006. 'Immunoadsorption in pemphigus', *Autoimmunity*, 39: 609-16.

Eming, R., A. Nagel, S. Wolff-Franke, E. Podstawa, D. Debus, and M. Hertl. 2008. 'Rituximab exerts a dual effect in pemphigus vulgaris', *J Invest Dermatol*, 128: 2850-8.

Eriksson, U., M. O. Kurrer, I. Sonderegger, G. Iezzi, A. Tafuri, L. Hunziker, S. Suzuki, K. Bachmaier, R. M. Bingisser, J. M. Penninger, and M. Kopf. 2003. 'Activation of dendritic cells through the interleukin 1 receptor 1 is critical for the induction of autoimmune myocarditis', *J Exp Med*, 197: 323-31.

Eriksson, U., R. Ricci, L. Hunziker, M. O. Kurrer, G. Y. Oudit, T. H. Watts, I. Sonderegger, K. Bachmaier, M. Kopf, and J. M. Penninger. 2003. 'Dendritic cell-induced autoimmune heart failure requires cooperation between adaptive and innate immunity', *Nat Med*, 9: 1484-90.

Evangelista, F., D. A. Dasher, L. A. Diaz, P. S. Prisayanh, and N. Li. 2008. 'E-cadherin is an additional immunological target for pemphigus autoantibodies', *J Invest Dermatol*, 128: 1710-8.

Evans, H. G., N. J. Gullick, S. Kelly, C. Pitzalis, G. M. Lord, B. W. Kirkham, and L. S. Taams. 2009. 'In vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses', *Proc Natl Acad Sci U S A*, 106: 6232-7.

Feliciani, C., P. Toto, P. Amerio, S. M. Pour, G. Coscione, G. Shivji, B. Wang, and D. N. Sauder. 2000. 'In vitro and in vivo expression of interleukin-1alpha and tumor necrosis factor-alpha mRNA in pemphigus vulgaris: interleukin-1alpha and tumor necrosis factor-alpha are involved in acantholysis', *J Invest Dermatol*, 114: 71-7.

Fingerle-Rowson, G., M. Angstwurm, R. Andreesen, and H. W. Ziegler-Heitbrock. 1998. 'Selective depletion of CD14+ CD16+ monocytes by glucocorticoid therapy', *Clin Exp Immunol*, 112: 501-6.

Fingerle-Rowson, G., J. Auers, E. Kreuzer, P. Fraunberger, M. Blumenstein, and L. H. Ziegler-Heitbrock. 1998. 'Expansion of CD14+CD16+ monocytes in critically ill cardiac surgery patients', *Inflammation*, 22: 367-79.

Fiore, N., G. Castellano, A. Blasi, C. Capobianco, A. Loverre, V. Montinaro, S. Netti, D. Torres, C. Manno, G. Grandaliano, E. Ranieri, F. P. Schena, and L. Gesualdo. 2008. 'Immature myeloid and plasmacytoid dendritic cells infiltrate renal tubulointerstitium in patients with lupus nephritis', *Mol Immunol*, 45: 259-65.

Forster, Reinhold, Ana Clara Davalos-Misslitz, and Antal Rot. 2008. 'CCR7 and its ligands: balancing immunity and tolerance', *Nat Rev Immunol*, 8: 362-71.

Foster, B., C. Prussin, F. Liu, J. K. Whitmire, and J. L. Whitton. 2007. 'Detection of intracellular cytokines by flow cytometry', *Curr Protoc Immunol*, Chapter 6: Unit 6 24.

Frew, J. W., L. K. Martin, and D. F. Murrell. 2011. 'Evidence-based treatments in pemphigus vulgaris and pemphigus foliaceus', *Dermatol Clin*, 29: 599-606.

Fuss, I. J., M. E. Kanof, P. D. Smith, and H. Zola. 2009. 'Isolation of whole mononuclear cells from peripheral blood and cord blood', *Curr Protoc Immunol*, Chapter 7: Unit7 1.

Futei, Y., M. Amagai, M. Sekiguchi, K. Nishifuji, Y. Fujii, and T. Nishikawa. 2000. 'Use of domain-swapped molecules for conformational epitope mapping of desmoglein 3 in pemphigus vulgaris', *J Invest Dermatol*, 115: 829-34.

Gallagher, S. R., S. E. Winston, S. A. Fuller, and J. G. Hurrell. 2001. 'Immunoblotting and immunodetection', *Curr Protoc Cell Biol*, Chapter 6: Unit 6 2.

Gallegos, A. M., and M. J. Bevan. 2004. 'Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation', *J Exp Med*, 200: 1039-49.

Ganguly, Dipyaman, Stefan Haak, Vanja Sisirak, and Boris Reizis. 2013. 'The role of dendritic cells in autoimmunity', *Nat Rev Immunol*, 13: 566-77.

Geissmann, F., S. Jung, and D. R. Littman. 2003. 'Blood monocytes consist of two principal subsets with distinct migratory properties', *Immunity*, 19: 71-82.

Geissmann, F., M. G. Manz, S. Jung, M. H. Sieweke, M. Merad, and K. Ley. 2010. 'Development of monocytes, macrophages, and dendritic cells', *Science*, 327: 656-61.

Giordano, C. N., and A. A. Sinha. 2012. 'Cytokine networks in Pemphigus vulgaris: An integrated viewpoint', *Autoimmunity*, 45: 427-39.

Giustizieri, M. L., F. Mascia, A. Frezzolini, O. De Pita, L. M. Chinni, A. Giannetti, G. Girolomoni, and S. Pastore. 2001. 'Keratinocytes from patients with atopic dermatitis and psoriasis show a distinct chemokine production profile in response to T cell-derived cytokines', *J Allergy Clin Immunol*, 107: 871-7.

Goldberg, R., G. Wildbaum, Y. Zohar, G. Maor, and N. Karin. 2004. 'Suppression of ongoing adjuvant-induced arthritis by neutralizing the function of the p28 subunit of IL-27', *J Immunol*, 173: 1171-8.

Goldberg, R., Y. Zohar, G. Wildbaum, Y. Geron, G. Maor, and N. Karin. 2004. 'Suppression of ongoing experimental autoimmune encephalomyelitis by neutralizing the function of the p28 subunit of IL-27', *J Immunol*, 173: 6465-71.

Gong, Jiang-Hong, Leslie G. Ratkay, J. Douglas Waterfield, and Ian Clark-Lewis. 1997. 'An Antagonist of Monocyte Chemoattractant Protein 1 (MCP-1) Inhibits Arthritis in the MRL-lpr Mouse Model', *J Exp Med*, 186: 131-37.

Gonzalez, A., A. Espana, M. J. Lopez-Zabalza, B. Pelacho, I. Sanchez-Carpintero, E. Santiago, and N. Lopez-Moratalla. 2000. 'Correlation between profile of circulating mononuclear cells and clinical manifestations in patients with pemphigus vulgaris', *Autoimmunity*, 32: 115-28.

Goon, Anthony Teik-Jin, and Suat-Hoon Tan. 2001. 'Comparative study of pemphigus vulgaris and pemphigus foliaceus in Singapore', *Australasian Journal of Dermatology*, 42: 172-75.

Grage-Griebenow, E., H.-D. Flad, and M. Ernst. 2001. 'Heterogeneity of human peripheral blood monocyte subsets', *J Leukoc Biol*, 69: 11-20.

Grando, S. A. 2000. 'Autoimmunity to keratinocyte acetylcholine receptors in pemphigus', *Dermatology*, 201: 290-5.

Greter, M., F. L. Heppner, M. P. Lemos, B. M. Odermatt, N. Goebels, T. Laufer, R. J. Noelle, and B. Becher. 2005. 'Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis', *Nat Med*, 11: 328-34.

Griffiths, C. E., B. E. Strober, P. van de Kerkhof, V. Ho, R. Fidelus-Gort, N. Yeilding, C. Guzzo, Y. Xia, B. Zhou, S. Li, L. T. Dooley, N. H. Goldstein, and A. Menter. 2010. 'Comparison of ustekinumab and etanercept for moderate-to-severe psoriasis', *N Engl J Med*, 362: 118-28.

Gringhuis, Sonja I., Tanja M. Kaptein, Brigitte A. Wevers, Michiel van der Vlist, Elsenoor J. Klaver, Irma van Die, Lianne E. M. Vriend, Marein A. W. P. de Jong, and Teunis B. H. Geijtenbeek. 2014. 'Fucose-based PAMPs prime dendritic cells for follicular T helper cell polarization via DC-SIGN-dependent IL-27 production', 5.

Grip, O., A. Bredberg, S. Lindgren, and G. Henriksson. 2007. 'Increased subpopulations of CD16(+) and CD56(+) blood monocytes in patients with active Crohn's disease', *Inflamm Bowel Dis*, 13: 566-72.

Guiducci, C., C. Tripodo, M. Gong, S. Sangaletti, M. P. Colombo, R. L. Coffman, and F. J. Barrat. 2010. 'Autoimmune skin inflammation is dependent on plasmacytoid dendritic cell activation by nucleic acids via TLR7 and TLR9', *J Exp Med*, 207: 2931-42.

Hall, R. P., 3rd, J. Fairley, D. Woodley, V. P. Werth, D. Hannah, R. D. Streilein, J. McKillip, J. Okawa, M. Rose, L. L. Keyes-Elstein, A. Pinckney, A. Overington, J. Wedgwood, L. Ding, and B. Welch. 2015. 'A multicentre randomized trial of the treatment of patients with pemphigus vulgaris with infliximab and prednisone compared with prednisone alone', *Br J Dermatol*, 172: 760-8.

Hammer, G. E., E. E. Turer, K. E. Taylor, C. J. Fang, R. Advincula, S. Oshima, J. Barrera, E. J. Huang, B. Hou, B. A. Malynn, B. Reizis, A. DeFranco, L. A. Criswell, M. C. Nakamura, and A. Ma. 2011. 'Expression of A20 by dendritic cells preserves immune homeostasis and prevents colitis and spondyloarthritis', *Nat Immunol*, 12: 1184-93.

Hanai, H., T. Iida, K. Takeuchi, F. Watanabe, M. Yamada, M. Kikuyama, Y. Maruyama, Y. Iwaoka, K. Hirayama, S. Nagata, and K. Takai. 2008. 'Adsorptive depletion of elevated proinflammatory CD14+CD16+DR++ monocytes in patients with inflammatory bowel disease', *Am J Gastroenterol*, 103: 1210-6.

Harlow, L., I. Fernandez, M. Soejima, W. M. Ridgway, and D. P. Ascherman. 2012. 'Characterization of TLR4-mediated auto-antibody production in a mouse model of histidyl-tRNA synthetase-induced myositis', *Innate Immun*, 18: 876-85.

Hawiger, D., K. Inaba, Y. Dorsett, M. Guo, K. Mahnke, M. Rivera, J. V. Ravetch, R. M. Steinman, and M. C. Nussenzweig. 2001. 'Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo', *J Exp Med*, 194: 769-79.

Hawiger, D., R. F. Masilamani, E. Bettelli, V. K. Kuchroo, and M. C. Nussenzweig. 2004. 'Immunological unresponsiveness characterized by increased expression of CD5 on peripheral T cells induced by dendritic cells in vivo', *Immunity*, 20: 695-705.

Henkel, J. S., D. R. Beers, L. Siklos, and S. H. Appel. 2006. 'The chemokine MCP-1 and the dendritic and myeloid cells it attracts are increased in the mSOD1 mouse model of ALS', *Mol Cell Neurosci*, 31: 427-37.

Hennerici, T., R. Pollmann, T. Schmidt, M. Seipelt, B. Tackenberg, C. Mobs, K. Ghoreschi, M. Hertl, and R. Eming. 2016. 'Increased Frequency of T Follicular Helper Cells and Elevated Interleukin-27 Plasma Levels in Patients with Pemphigus', *PLoS One*, 11: e0148919.

Hepburn, A. L., J. C. Mason, and K. A. Davies. 2004. 'Expression of Fcgamma and complement receptors on peripheral blood monocytes in systemic lupus erythematosus and rheumatoid arthritis', *Rheumatology (Oxford)*, 43: 547-54.

Hertl, M. 2000. 'Humoral and cellular autoimmunity in autoimmune bullous skin disorders', *Int Arch Allergy Immunol*, 122: 91-100.

Hertl, M., M. Amagai, H. Sundaram, J. Stanley, K. Ishii, and Katz. 1998a. 'Recognition of desmoglein-3 by autoreactive T cells in pemphigus vulgaris patients and normals', *J Invest Dermatol*, 110: 62-66.

Hertl, M., M. Amagai, H. Sundaram, J. Stanley, K. Ishii, and S. I. Katz. 1998b. 'Recognition of desmoglein 3 by autoreactive T cells in pemphigus vulgaris patients and normals', *J Invest Dermatol*, 110: 62-6.

Hertl, M., R. Eming, and C. Veldman. 2006. 'T cell control in autoimmune bullous skin disorders', *J Clin Invest*, 116: 1159-66.

Hertl, M., and C. Veldman. 2001. 'Pemphigus--paradigm of autoantibody-mediated autoimmunity', *Skin Pharmacol Appl Skin Physiol*, 14: 408-18.

Hertl, Michael, Robert W. Karr, Masayuki Amagai, and Stephen I. Katz. 1998. 'Heterogeneous MHC II Restriction Pattern of Autoreactive Desmoglein 3 Specific T Cell Responses in Pemphigus Vulgaris Patients and Normals', *J Investig Dermatol*, 110: 388-92.

Hill, J. A., S. Southwood, A. Sette, A. M. Jevnikar, D. A. Bell, and E. Cairns. 2003. 'Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule', *J Immunol*, 171: 538-41.

Hoebe, Kasper, Edith Janssen, and Bruce Beutler. 2004. 'The interface between innate and adaptive immunity', *Nat Immunol*, 5: 971-74.

Hoeffel, G., A. C. Ripoche, D. Matheoud, M. Nascimbeni, N. Escriou, P. Lebon, F. Heshmati, J. G. Guillet, M. Gannage, S. Caillat-Zucman, N. Casartelli, O. Schwartz, H. De la Salle, D. Hanau, A. Hosmalin, and C. Maranon. 2007. 'Antigen crosspresentation by human plasmacytoid dendritic cells', *Immunity*, 27: 481-92.

Horelt, A., K. U. Belge, B. Steppich, J. Prinz, and L. Ziegler-Heitbrock. 2002. 'The CD14+CD16+ monocytes in erysipelas are expanded and show reduced cytokine production', *Eur J Immunol*, 32: 1319-27.

Hornbeck, Peter V. 2001. 'Enzyme-Linked Immunosorbent Assays.' in, *Current Protocols in Immunology* (John Wiley & Sons, Inc.).

Hradetzky, S., H. Balaji, L. M. Roesner, A. Heratizadeh, I. Mittermann, R. Valenta, and T. Werfel. 2013. 'The human skin-associated autoantigen alpha-NAC activates monocytes and dendritic cells via TLR-2 and primes an IL-12-dependent Th1 response', *J Invest Dermatol*, 133: 2289-92.

Hubert, F. X., S. A. Kinkel, G. M. Davey, B. Phipson, S. N. Mueller, A. Liston, A. I. Proietto, P. Z. Cannon, S. Forehan, G. K. Smyth, L. Wu, C. C. Goodnow, F. R. Carbone, H. S. Scott, and W. R. Heath. 2011. 'Aire regulates the transfer of antigen from mTECs to dendritic cells for induction of thymic tolerance', *Blood*, 118: 2462-72.

Hultqvist, M., P. Olofsson, J. Holmberg, B. T. Backstrom, J. Tordsson, and R. Holmdahl. 2004. 'Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced

oxidative burst due to a mutation in the Ncf1 gene', *Proc Natl Acad Sci U S A*, 101: 12646-51.

Hunter, Christopher A, and Rob Kastelein. 2012. 'Interleukin-27: Balancing Protective and Pathological Immunity', *Immunity*, 37: 960-69.

Ishii, K., M. Amagai, R. P. Hall, T. Hashimoto, A. Takayanagi, S. Gamou, N. Shimizu, and T. Nishikawa. 1997. 'Characterization of autoantibodies in pemphigus using antigen-specific enzyme-linked immunosorbent assays with baculovirus-expressed recombinant desmogleins', *J Immunol*, 159: 2010-7.

Ishikawa, H., K. Li, K. Tamai, D. Sawamura, and J. Uitto. 2000. 'Cloning of the mouse desmoglein 3 gene (Dsg3): interspecies conservation within the cadherin superfamily', *Exp Dermatol*, 9: 229-39.

Iwahashi, M., M. Yamamura, T. Aita, A. Okamoto, A. Ueno, N. Ogawa, S. Akashi, K. Miyake, P. J. Godowski, and H. Makino. 2004. 'Expression of Toll-like receptor 2 on CD16+ blood monocytes and synovial tissue macrophages in rheumatoid arthritis', *Arthritis Rheum*, 50: 1457-67.

Iwamoto, S., S. Iwai, K. Tsujiyama, C. Kurahashi, K. Takeshita, M. Naoe, A. Masunaga, Y. Ogawa, K. Oguchi, and A. Miyazaki. 2007. 'TNF-alpha drives human CD14+ monocytes to differentiate into CD70+ dendritic cells evoking Th1 and Th17 responses', *J Immunol*, 179: 1449-57.

Iwasaki, Akiko, and Ruslan Medzhitov. 2015. 'Control of adaptive immunity by the innate immune system', *Nat Immunol*, 16: 343-53.

Iwata, Yohei, Takashi Matsushita, Mayuka Horikawa, David J. DiLillo, Koichi Yanaba, Guglielmo M. Venturi, Paul M. Szabolcs, Steven H. Bernstein, Cynthia M. Magro, Armistead D. Williams, Russell P. Hall, E. William St Clair, and Thomas F. Tedder. 2011. 'Characterization of a rare IL-10–competent B-cell subset in humans that parallels mouse regulatory B10 cells', *Blood*, 117: 530-41.

Izikson, L., R. S. Klein, I. F. Charo, H. L. Weiner, and A. D. Luster. 2000. 'Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR)2', *J Exp Med*, 192: 1075-80.

Jackson, S. M., P. C. Wilson, J. A. James, and J. D. Capra. 2008. 'Human B cell subsets', *Adv Immunol*, 98: 151-224.

Jahnsen, F. L., F. Lund-Johansen, J. F. Dunne, L. Farkas, R. Haye, and P. Brandtzaeg. 2000. 'Experimentally induced recruitment of plasmacytoid (CD123high) dendritic cells in human nasal allergy', *J Immunol*, 165: 4062-8.

Janeway, C. A., Jr. 1989. 'Approaching the asymptote? Evolution and revolution in immunology', *Cold Spring Harb Symp Quant Biol*, 54 Pt 1: 1-13.

Janeway, Charles A., and Ruslan Medzhitov. 2002. 'INNATE IMMUNE RECOGNITION', *Annu Rev Immunol*, 20: 197-216.

Jedlickova, H., R. Muller, D. Castiglia, M. Kovacevic, and J. Feit. 2012. 'Dystrophic epidermolysis bullosa pruriginosa with autoantibodies against collagen VII', *Eur J Dermatol*, 22: 541-2.

Jin, L., D. Yu, X. Li, N. Yu, X. Li, Y. Wang, and Y. Wang. 2014. 'CD4+CXCR5+ follicular helper T cells in salivary gland promote B cells maturation in patients with primary Sjogren's syndrome', *Int J Clin Exp Pathol*, 7: 1988-96.

Jin, O., S. Kavikondala, L. Sun, R. Fu, M. Y. Mok, A. Chan, J. Yeung, and C. S. Lau. 2008. 'Systemic lupus erythematosus patients have increased number of circulating plasmacytoid dendritic cells, but decreased myeloid dendritic cells with deficient CD83 expression', *Lupus*, 17: 654-62.

Joly, P., H. Mouquet, J. C. Roujeau, M. D'Incan, D. Gilbert, S. Jacquot, M. L. Gougeon, C. Bedane, R. Muller, B. Dreno, M. S. Doutre, E. Delaporte, C. Pauwels, N. Franck, F. Caux, C. Picard, E. Tancrede-Bohin, P. Bernard, F. Tron, M. Hertl, and P. Musette. 2007. 'A single cycle of rituximab for the treatment of severe pemphigus', *N Engl J Med*, 357: 545-52.

Jones, E. Y., L. Fugger, J. L. Strominger, and C. Siebold. 2006. 'MHC class II proteins and disease: a structural perspective', *Nat Rev Immunol*, 6: 271-82.

Kaneko, T., Y. Saito, T. Kotani, H. Okazawa, H. Iwamura, M. Sato-Hashimoto, Y. Kanazawa, S. Takahashi, K. Hiromura, S. Kusakari, Y. Kaneko, Y. Murata, H. Ohnishi, Y. Nojima, K. Takagishi, and T. Matozaki. 2012. 'Dendritic cell-specific ablation of the protein tyrosine phosphatase Shp1 promotes Th1 cell differentiation and induces autoimmunity', *J Immunol*, 188: 5397-407.

Kanno, Y., G. Vahedi, K. Hirahara, K. Singleton, and J. J. O'Shea. 2012. 'Transcriptional and epigenetic control of T helper cell specification: molecular mechanisms underlying commitment and plasticity', *Annu Rev Immunol*, 30: 707-31.

Karni, A., M. Abraham, A. Monsonego, G. Cai, G. J. Freeman, D. Hafler, S. J. Khoury, and H. L. Weiner. 2006. 'Innate immunity in multiple sclerosis: myeloid dendritic cells in secondary progressive multiple sclerosis are activated and drive a proinflammatory immune response', *J Immunol*, 177: 4196-202.

Kawanaka, N., M. Yamamura, T. Aita, Y. Morita, A. Okamoto, M. Kawashima, M. Iwahashi, A. Ueno, Y. Ohmoto, and H. Makino. 2002. 'CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis', *Arthritis Rheum*, 46: 2578-86.

Keskin, D. B., J. N. Stern, M. Fridkis-Hareli, and A. Razzaque Ahmed. 2008. 'Cytokine profiles in pemphigus vulgaris patients treated with intravenous immunoglobulins as compared to conventional immunosuppressive therapy', *Cytokine*, 41: 315-21.

Khan, Shaukat Ali, Johannes Nowatzky, Sonia Jiménez-Branda, Jeffrey David Greenberg, Robert Clancy, Jill Buyon, and Nina Bhardwaj. 2013. 'Active systemic lupus erythematosus is associated with decreased blood conventional dendritic cells', *Experimental and Molecular Pathology*, 95: 121-23.

Killeen, N., S. Sawada, and D. R. Littman. 1993. 'Regulated expression of human CD4 rescues helper T cell development in mice lacking expression of endogenous CD4', *EMBO J*, 12: 1547-53.

Kim, S. J., and B. Diamond. 2015. 'Modulation of tolerogenic dendritic cells and autoimmunity', *Semin Cell Dev Biol*, 41: 49-58.

Kim, S. J., Y. R. Zou, J. Goldstein, B. Reizis, and B. Diamond. 2011. 'Tolerogenic function of Blimp-1 in dendritic cells', *J Exp Med*, 208: 2193-9.

Kitajima, Y. 2013. 'New insights into desmosome regulation and pemphigus blistering as a desmosome-remodeling disease', *Kaohsiung J Med Sci*, 29: 1-13.

Klechevsky, E. 2013. 'Human dendritic cells - stars in the skin', *Eur J Immunol*, 43: 3147-55.

Klechevsky, Eynav, Rimpei Morita, Maochang Liu, Yanying Cao, Sebastien Coquery, LuAnn Thompson-Snipes, Francine Briere, Damien Chaussabel, Gerard Zurawski, A. Karolina Palucka, Yoram Reiter, Jacques Banchereau, and Hideki Ueno. 2008. 'Functional Specializations of Human Epidermal Langerhans Cells and CD14(+) Dermal Dendritic Cells', *Immunity*, 29: 497-510.

Klinman, D. 2008. 'ELISPOT assay to detect cytokine-secreting murine and human cells', *Curr Protoc Immunol*, Chapter 6: Unit 6 19.

Kneisel, A., and M. Hertl. 2011a. 'Autoimmune bullous skin diseases. Part 1: Clinical manifestations', *J Dtsch Dermatol Ges*, 9: 844-56; quiz 57.

Kneisel, A., and M. Hertl. 2011b. 'Autoimmune bullous skin diseases. Part 2: diagnosis and therapy', *J Dtsch Dermatol Ges*, 9: 927-47.

Knight, S. C., J. Mertin, A. Stackpoole, and J. Clark. 1983. 'Induction of immune responses in vivo with small numbers of veiled (dendritic) cells', *Proc Natl Acad Sci U S A*, 80: 6032-5.

Koch, P. J., M. G. Mahoney, H. Ishikawa, L. Pulkkinen, J. Uitto, L. Shultz, G. F. Murphy, D. Whitaker-Menezes, and J. R. Stanley. 1997. 'Targeted disruption of the pemphigus vulgaris antigen (desmoglein 3) gene in mice causes loss of keratinocyte cell adhesion with a phenotype similar to pemphigus vulgaris', *J Cell Biol*, 137: 1091-102.

Koch, S., T. Kucharzik, J. Heidemann, A. Nusrat, and A. Luegering. 2010. 'Investigating the role of proinflammatory CD16+ monocytes in the pathogenesis of inflammatory bowel disease', *Clin Exp Immunol*, 161: 332-41.

Komatsu, M., D. Kobayashi, K. Saito, D. Furuya, A. Yagihashi, H. Araake, N. Tsuji, S. Sakamaki, Y. Niitsu, and N. Watanabe. 2001. 'Tumor necrosis factor-alpha in serum of patients with inflammatory bowel disease as measured by a highly sensitive immuno-PCR', *Clin Chem*, 47: 1297-301.

Kometani, K., R. Nakagawa, R. Shinnakasu, T. Kaji, A. Rybouchkin, S. Moriyama, K. Furukawa, H. Koseki, T. Takemori, and T. Kurosaki. 2013. 'Repression of the transcription factor Bach2 contributes to predisposition of IgG1 memory B cells toward plasma cell differentiation', *Immunity*, 39: 136-47.

Kool, M., G. van Loo, W. Waelput, S. De Prijck, F. Muskens, M. Sze, J. van Praet, F. Branco-Madeira, S. Janssens, B. Reizis, D. Elewaut, R. Beyaert, H. Hammad, and B. N. Lambrecht. 2011. 'The ubiquitin-editing protein A20 prevents dendritic cell activation, recognition of apoptotic cells, and systemic autoimmunity', *Immunity*, 35: 82-96.

Korn, T., E. Bettelli, W. Gao, A. Awasthi, A. Jager, T. B. Strom, M. Oukka, and V. K. Kuchroo. 2007. 'IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells', *Nature*, 448: 484-7.

Korn, Thomas, Estelle Bettelli, Mohamed Oukka, and Vijay K. Kuchroo. 2009. 'IL-17 and Th17 Cells', *Annu Rev Immunol*, 27: 485-517.

Kricheli, D., M. David, M. Frusic-Zlotkin, D. Goldsmith, M. Rabinov, J. Sulkes, and Y. Milner. 2000. 'The distribution of pemphigus vulgaris-IgG subclasses and their reactivity with desmoglein 3 and 1 in pemphigus patients and their first-degree relatives', *Br J Dermatol*, 143: 337-42.

Kurihara, T., G. Warr, J. Loy, and R. Bravo. 1997. 'Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor', *J Exp Med*, 186: 1757-62.

Kuziel, W. A., S. J. Morgan, T. C. Dawson, S. Griffin, O. Smithies, K. Ley, and N. Maeda. 1997. 'Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2', *Proc Natl Acad Sci U S A*, 94: 12053-8.

Lande, R., V. Gafa, B. Serafini, E. Giacomini, A. Visconti, M. E. Remoli, M. Severa, M. Parmentier, G. Ristori, M. Salvetti, F. Aloisi, and E. M. Coccia. 2008. 'Plasmacytoid dendritic cells in multiple sclerosis: intracerebral recruitment and impaired maturation in response to interferon-beta', *J Neuropathol Exp Neurol*, 67: 388-401.

Lande, R., E. Giacomini, B. Serafini, B. Rosicarelli, G. D. Sebastiani, G. Minisola, U. Tarantino, V. Riccieri, G. Valesini, and E. M. Coccia. 2004. 'Characterization and recruitment of plasmacytoid dendritic cells in synovial fluid and tissue of patients with chronic inflammatory arthritis', *J Immunol*, 173: 2815-24.

Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. 'IL-23 drives a pathogenic T cell population that induces autoimmune inflammation', *J Exp Med*, 201: 233-40.

Latek, R. R., A. Suri, S. J. Petzold, C. A. Nelson, O. Kanagawa, E. R. Unanue, and D. H. Fremont. 2000. 'Structural basis of peptide binding and presentation by the type I diabetes-associated MHC class II molecule of NOD mice', *Immunity*, 12: 699-710.

Lee, E., K. A. Lendas, S. Chow, Y. Pirani, D. Gordon, R. Dionisio, D. Nguyen, A. Spizuoco, M. Fotino, Y. Zhang, and A. A. Sinha. 2006. 'Disease relevant HLA class II alleles isolated by genotypic, haplotypic, and sequence analysis in North American Caucasians with pemphigus vulgaris', *Hum Immunol*, 67: 125-39.

Li, J., P. M. Farthing, and M. H. Thornhill. 2000. 'Oral and skin keratinocytes are stimulated to secrete monocyte chemoattractant protein-1 by tumour necrosis factor-alpha and interferon-gamma', *J Oral Pathol Med*, 29: 438-44.

Li, J., B. Gran, G. X. Zhang, A. Rostami, and M. Kamoun. 2005. 'IL-27 subunits and its receptor (WSX-1) mRNAs are markedly up-regulated in inflammatory cells in the CNS during experimental autoimmune encephalomyelitis', *J Neurol Sci*, 232: 3-9.

Li, P., W. Lin, and X. Zheng. 2014. 'IL-33 neutralization suppresses lupus disease in lupus-prone mice', *Inflammation*, 37: 824-32.

Li, Q., Z. Liu, E. Dang, L. Jin, Z. He, L. Yang, X. Shi, and G. Wang. 2013. 'Follicular helper T Cells (Tfh) and IL-21 involvement in the pathogenesis of bullous pemphigoid', *PLoS One*, 8: e68145.

Li, Y., V. K. Rauniyar, W. F. Yin, B. Hu, S. Ouyang, B. Xiao, and H. Yang. 2014. 'Serum IL-21 levels decrease with glucocorticoid treatment in myasthenia gravis', *Neurol Sci*, 35: 29-34.

Lin, M. S., S. J. Swartz, and A. Lopez. 1997. 'Development and characterization of desmoglein-3 specific T cells from patients with pemphigus vulgaris', *J Clin Invest*, 99: 31-40.

Linterman, M. A., L. Beaton, D. Yu, R. R. Ramiscal, M. Srivastava, J. J. Hogan, N. K. Verma, M. J. Smyth, R. J. Rigby, and C. G. Vinuesa. 2010. 'IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses', *J Exp Med*, 207: 353-63.

Litman, Gary W., Jonathan P. Rast, and Sebastian D. Fugmann. 2010. 'The origins of vertebrate adaptive immunity', *Nat Rev Immunol*, 10: 543-53.

Liu, B., A. Dhanda, S. Hirani, E. L. Williams, H. N. Sen, F. Martinez Estrada, D. Ling, I. Thompson, M. Casady, Z. Li, H. Si, W. Tucker, L. Wei, S. Jawad, A. Sura, J. Dailey, S. Hannes, P. Chen, J. L. Chien, S. Gordon, R. W. Lee, and R. B. Nussenblatt. 2015. 'CD14++CD16+ Monocytes Are Enriched by Glucocorticoid Treatment and Are Functionally Attenuated in Driving Effector T Cell Responses', *J Immunol*, 194: 5150-60.

Liu, R., Q. Wu, D. Su, N. Che, H. Chen, L. Geng, J. Chen, W. Chen, X. Li, and L. Sun. 2012. 'A regulatory effect of IL-21 on T follicular helper-like cell and B cell in rheumatoid arthritis', *Arthritis Res Ther*, 14: R255.

Liu, Y. J. 2005. 'IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors', *Annu Rev Immunol*, 23: 275-306.

Liu, Y., P. Zhao, Z. Qu, D. A. Ayana, and Y. Jiang. 2014. 'Frequency of CD4+CXCR5+ TFH cells in patients with hepatitis b virus-associated membranous nephropathy', *Int Immunopharmacol*, 22: 98-106.

Lombardi, M. L., O. Mercuro, V. Ruocco, A. Lo Schiavo, V. Lombari, V. Guerrera, G. Pirozzi, and C. Manzo. 1999. 'Common human leukocyte antigen alleles in pemphigus vulgaris and pemphigus foliaceus Italian patients', *J Invest Dermatol*, 113: 107-10.

Lombardi, M. L., O. Mercuro, G. Tecame, C. Fusco, V. Ruocco, A. Salerno, G. Pirozzi, and C. Manzo. 1996. 'Molecular analysis of HLA DRB1 and DQB1 in Italian patients with pemphigus vulgaris', *Tissue Antigens*, 47: 228-30.

Lopez-Robles, E., E. Avalos-Diaz, E. Vega-Memije, T. Hojyo-Tomoka, R. Villalobos, S. Fraire, L. Domiguez-Soto, and R. Herrera-Esparza. 2001. 'TNFalpha and IL-6 are mediators in the blistering process of pemphigus', *Int J Dermatol*, 40: 185-8.

Lougaris, V., R. Badolato, S. Ferrari, and A. Plebani. 2005. 'Hyper immunoglobulin M syndrome due to CD40 deficiency: clinical, molecular, and immunological features', *Immunol Rev*, 203: 48-66.

Ludwig, R. J., and E. Schmidt. 2009. 'Cytokines in autoimmune bullous skin diseases. Epiphenomena or contribution to pathogenesis?', *G Ital Dermatol Venereol*, 144: 339-49.

Luo, C., Y. Li, W. Liu, H. Feng, H. Wang, X. Huang, L. Qiu, and J. Ouyang. 2013. 'Expansion of circulating counterparts of follicular helper T cells in patients with myasthenia gravis', *J Neuroimmunol*, 256: 55-61.

Luthje, K., A. Kallies, Y. Shimohakamada, G. T. Belz, A. Light, D. M. Tarlinton, and S. L. Nutt. 2012. 'The development and fate of follicular helper T cells defined by an IL-21 reporter mouse', *Nat Immunol*, 13: 491-8.

Lutz, Manfred B., Nicole Kukutsch, Alexandra L. J. Ogilvie, Susanne Rößner, Franz Koch, Nikolaus Romani, and Gerold Schuler. 1999. 'An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow', *J Immunol Methods*, 223: 77-92.

MacLennan, I. C., K. M. Toellner, A. F. Cunningham, K. Serre, D. M. Sze, E. Zuniga, M. C. Cook, and C. G. Vinuesa. 2003. 'Extrafollicular antibody responses', *Immunol Rev*, 194: 8-18.

Maldonado, R. A., and U. H. von Andrian. 2010. 'How tolerogenic dendritic cells induce regulatory T cells', *Adv Immunol*, 108: 111-65.

Marchenko, S., A. I. Chernyavsky, J. Arredondo, V. Gindi, and S. A. Grando. 2010. 'Antimitochondrial autoantibodies in pemphigus vulgaris: a missing link in disease pathophysiology', *J Biol Chem*, 285: 3695-704.

Martin, Flavius, and Andrew C. Chan. 'Pathogenic Roles of B Cells in Human Autoimmunity', *Immunity*, 20: 517-27.

Martin. 2006. 'B CELL IMMUNOBIOLOGY IN DISEASE: Evolving Concepts from the Clinic', *Annu Rev Immunol*, 24: 467-96.

Martin, L. K., V. Werth, E. Villanueva, J. Segall, and D. F. Murrell. 2009. 'Interventions for pemphigus vulgaris and pemphigus foliaceus', *Cochrane Database Syst Rev*: Cd006263.

Mashkilleyson, N., Y. T. Konttinen, and K. Visa. 1989. 'Local involvement of antigen-presenting cells and activated T cells in perilesional and clinically uninvolved skin in pemphigus vulgaris', *Acta Derm Venereol*, 69: 424-8.

Mathers, A. R., O. A. Tckacheva, B. M. Janelsins, W. J. Shufesky, A. E. Morelli, and A. T. Larregina. 2007. 'In vivo signaling through the neurokinin 1 receptor favors transgene expression by Langerhans cells and promotes the generation of Th1- and Tc1-biased immune responses', *J Immunol*, 178: 7006-17.

Maus, U. A., K. Waelsch, W. A. Kuziel, T. Delbeck, M. Mack, T. S. Blackwell, J. W. Christman, D. Schlondorff, W. Seeger, and J. Lohmeyer. 2003. 'Monocytes are potent facilitators of alveolar neutrophil emigration during lung inflammation: role of the CCL2-CCR2 axis', *J Immunol*, 170: 3273-8.

Means, T. K., E. Latz, F. Hayashi, M. R. Murali, D. T. Golenbock, and A. D. Luster. 2005. 'Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9', *J Clin Invest*, 115: 407-17.

Melillo, J. A., L. Song, G. Bhagat, A. B. Blazquez, C. R. Plumlee, C. Lee, C. Berin, B. Reizis, and C. Schindler. 2010. 'Dendritic cell (DC)-specific targeting reveals Stat3 as a negative regulator of DC function', *J Immunol*, 184: 2638-45.

Merad, M., P. Hoffmann, E. Ranheim, S. Slaymaker, M. G. Manz, S. A. Lira, I. Charo, D. N. Cook, I. L. Weissman, S. Strober, and E. G. Engleman. 2004. 'Depletion of host Langerhans cells before transplantation of donor alloreactive T cells prevents skin graft-versus-host disease', *Nat Med*, 10: 510-7.

Merad, M., M. G. Manz, H. Karsunky, A. Wagers, W. Peters, I. Charo, I. L. Weissman, J. G. Cyster, and E. G. Engleman. 2002. 'Langerhans cells renew in the skin throughout life under steady-state conditions', *Nat Immunol*, 3: 1135-41.

Merad, Miriam, Priyanka Sathe, Julie Helft, Jennifer Miller, and Arthur Mortha. 2013. 'The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting', *Annu Rev Immunol*, 31: 563-604.

Michael, D., and S. A. Grando. 2008. 'Novel mechanism for therapeutic action of IVIg in autoimmune blistering dermatoses', *Curr Dir Autoimmun*, 10: 333-43.

Migita, K., T. Miyashita, Y. Maeda, H. Kimura, M. Nakamura, H. Yatsuhashi, H. Ishibashi, and K. Eguchi. 2005. 'Reduced blood BDCA-2+ (lymphoid) and CD11c+ (myeloid) dendritic cells in systemic lupus erythematosus', *Clin Exp Immunol*, 142: 84-91.

Mimouni, D., H. Bar, M. Gdalevich, V. Katzenelson, and M. David. 2008. 'Pemphigus--analysis of epidemiological factors in 155 patients', *J Eur Acad Dermatol Venereol*, 22: 1232-5.

Miyadera, H., and K. Tokunaga. 2015. 'Associations of human leukocyte antigens with autoimmune diseases: challenges in identifying the mechanism', *J Hum Genet*.

Miyagawa, S., I. Higashimine, T. Iida, Y. Yamashina, T. Fukumoto, and T. Shirai. 1997. 'HLA-DRB1*04 and DRB1*14 alleles are associated with susceptibility to pemphigus among Japanese', *J Invest Dermatol*, 109: 615-8.

Mizuno, K., T. Toma, H. Tsukiji, H. Okamoto, H. Yamazaki, K. Ohta, K. Ohta, Y. Kasahara, S. Koizumi, and A. Yachie. 2005. 'Selective expansion of CD16highCCR2- subpopulation of circulating monocytes with preferential production of haem oxygenase (HO)-1 in response to acute inflammation', *Clin Exp Immunol*, 142: 461-70.

Mobini, N., E. J. Yunis, C. A. Alper, J. J. Yunis, J. C. Delgado, D. E. Yunis, A. Firooz, Y. Dowlati, K. Bahar, P. K. Gregersen, and A. R. Ahmed. 1997. 'Identical MHC markers in non-Jewish Iranian and Ashkenazi Jewish patients with pemphigus vulgaris: possible common central Asian ancestral origin', *Hum Immunol*, 57: 62-7.

Moniuszko, M., A. Bodzenta-Lukaszyk, K. Kowal, D. Lenczewska, and M. Dabrowska. 2009. 'Enhanced frequencies of CD14++CD16+, but not CD14+CD16+, peripheral blood monocytes in severe asthmatic patients', *Clin Immunol*, 130: 338-46.

Morita, R., N. Schmitt, S. E. Bentebibel, R. Ranganathan, L. Bourdery, G. Zurawski, E. Foucat, M. Dullaers, S. Oh, N. Sabzghabaei, E. M. Lavecchio, M. Punaro, V. Pascual, J. Banchereau, and H. Ueno. 2011. 'Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion', *Immunity*, 34: 108-21.

Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. 'Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins', *J Immunol*, 136: 2348-57.

Movahedi, K., M. Guilliams, J. Van den Bossche, R. Van den Bergh, C. Gysemans, A. Beschin, P. De Baetselier, and J. A. Van Ginderachter. 2008. 'Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity', *Blood*, 111: 4233-44.

Mukherjee, R., P. Kanti Barman, P. Kumar Thatoi, R. Tripathy, B. Kumar Das, and B. Ravindran. 2015. 'Non-Classical monocytes display inflammatory features: Validation in Sepsis and Systemic Lupus Erythematous', *Sci Rep*, 5: 13886.

Muller, R., C. Dahler, C. Mobs, E. Wenzel, R. Eming, G. Messer, A. Niedermeier, and M. Hertl. 2010. 'T and B cells target identical regions of the non-collagenous domain 1 of type VII collagen in epidermolysis bullosa acquisita', *Clin Immunol*, 135: 99-107.

Murphy, Kenneth, Paul Travers, Mark Walport, and Charles Janeway. 2012. *Janeway's immunobiology* (Garland Science: New York).

Murrell, D. F., S. Dick, A. R. Ahmed, M. Amagai, M. A. Barnadas, L. Borradori, J. C. Bystryn, G. Cianchini, L. Diaz, D. Fivenson, R. Hall, K. E. Harman, T. Hashimoto, M. Hertl, N. Hunzelmann, P. Iranzo, P. Joly, M. F. Jonkman, Y. Kitajima, N. J. Korman, L. K. Martin, D. Mimouni, A. G. Pandya, A. S. Payne, D. Rubenstein, H. Shimizu, A. A. Sinha, D. Sirois, D. Zillikens, and V. P. Werth. 2008. 'Consensus statement on definitions of disease, end points, and therapeutic response for pemphigus', *J Am Acad Dermatol*, 58: 1043-6.

Mutasim, D. F., N. J. Pelc, and G. J. Anhalt. 1993. 'Drug-induced pemphigus', *Dermatol Clin*, 11: 463-71.

Mysliwska, J., M. Smardzewski, N. Marek-Trzonkowska, M. Mysliwiec, and K. Raczynska. 2012. 'Expansion of CD14+CD16+ monocytes producing TNF-alpha in complication-free diabetes type 1 juvenile onset patients', *Cytokine*, 60: 309-17.

Nakamura, K., I. R. Williams, and T. S. Kupper. 1995. 'Keratinocyte-derived monocyte chemoattractant protein 1 (MCP-1): analysis in a transgenic model demonstrates MCP-1 can recruit dendritic and Langerhans cells to skin', *J Invest Dermatol*, 105: 635-43.

Narbutt, Joanna, Jolanta Lukamowicz, Jarosław Bogaczewicz, Anna Sysa-Jedrzejowska, Jolanta Dorota Torzecka, and Aleksandra Lesiak. 2008. 'Serum Concentration of Interleukin-6 Is

Increased Both in Active and Remission Stages of Pemphigus Vulgaris', *Mediators Inflamm*, 2008: 875394.

Nestle, F. O., C. Conrad, A. Tun-Kyi, B. Homey, M. Gombert, O. Boyman, G. Burg, Y. J. Liu, and M. Gilliet. 2005. 'Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production', *J Exp Med*, 202: 135-43.

Nestor, M. S., A. J. Cochran, and A. R. Ahmed. 1987. 'Mononuclear cell infiltrates in bullous disease', *J Invest Dermatol*, 88: 172-5.

Neumann, C., F. Heinrich, K. Neumann, V. Junghans, M. F. Mashreghi, J. Ahlers, M. Janke, C. Rudolph, N. Mockel-Tenbrinck, A. A. Kuhl, M. M. Heimesaat, C. Esser, S. H. Im, A. Radbruch, S. Rutz, and A. Scheffold. 2014. 'Role of Blimp-1 in programing Th effector cells into IL-10 producers', *J Exp Med*, 211: 1807-19.

Nieminen, J. K., J. Vakkila, H. M. Salo, N. Ekstrom, T. Harkonen, J. Ilonen, M. Knip, and O. Vaarala. 2012. 'Altered phenotype of peripheral blood dendritic cells in pediatric type 1 diabetes', *Diabetes Care*, 35: 2303-10.

Nishifuji, K., M. Amagai, M. Kuwana, T. Iwasaki, and T. Nishikawa. 2000. 'Detection of antigen-specific B cells in patients with pemphigus vulgaris by enzyme-linked immunospot assay: requirement of T cell collaboration for autoantibody production', *J Invest Dermatol*, 114: 88-94.

Nuñez, Rafael. 2001. 'Assessment of Surface Markers and Functionality of Dendritic Cells (DCs).' in, *Current Protocols in Cytometry* (John Wiley & Sons, Inc.).

Nutt, Stephen L., Philip D. Hodgkin, David M. Tarlinton, and Lynn M. Corcoran. 2015. 'The generation of antibody-secreting plasma cells', *Nat Rev Immunol*, 15: 160-71.

Ohl, Lars, Mariette Mohaupt, Niklas Czeloth, Gabriele Hintzen, Ziba Kiafard, Jörg Zwirner, Thomas Blankenstein, Golo Henning, and Reinhold Förster. 2004. 'CCR7 Governs Skin Dendritic Cell Migration under Inflammatory and Steady-State Conditions', *Immunity*, 21: 279-88.

Ohnmacht, C., A. Pullner, S. B. King, I. Drexler, S. Meier, T. Brocker, and D. Voehringer. 2009. 'Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity', *J Exp Med*, 206: 549-59.

Ozaki, Yoshio, Ryuichi Amakawa, Tomoki Ito, Hiroshi Iwai, Kenichirou Tajima, Kazutaka Uehira, Hideo Kagawa, Yoshiko Uemura, Toshio Yamashita, and Shirou Fukuhara. 2001. 'Alteration of peripheral blood dendritic cells in patients with primary Sjögren's syndrome', *Arthritis & Rheumatism*, 44: 419-31.

Papadakis, K. A., and S. R. Targan. 2000. 'Role of cytokines in the pathogenesis of inflammatory bowel disease', *Annu Rev Med*, 51: 289-98.

Pasparakis, M., I. Haase, and F. O. Nestle. 2014. 'Mechanisms regulating skin immunity and inflammation', *Nat Rev Immunol*, 14: 289-301.

Passlick, B., D. Flieger, and H. W. Ziegler-Heitbrock. 1989. 'Identification and characterization of a novel monocyte subpopulation in human peripheral blood', *Blood*, 74: 2527-34.

Payne, A. S., Y. Hanakawa, M. Amagai, and J. R. Stanley. 2004. 'Desmosomes and disease: pemphigus and bullous impetigo', *Curr Opin Cell Biol*, 16: 536-43.

Pei, C., M. Barbour, K. J. Fairlie-Clarke, D. Allan, R. Mu, and H. R. Jiang. 2014. 'Emerging role of interleukin-33 in autoimmune diseases', *Immunology*, 141: 9-17.

Pelchen-Matthews, A, I J Parsons, and M Marsh. 1993 'Phorbol ester-induced downregulation of CD4 is a multistep process involving dissociation from p56lck, increased association with clathrin-coated pits, and altered endosomal sorting. 10.1084/jem.178.4.1209 ', *J Exp Med*, 178 1209-22

Penel-Sotirakis, K., E. Simonazzi, J. Peguet-Navarro, and A. Rozieres. 2012. 'Differential capacity of human skin dendritic cells to polarize CD4+ T cells into IL-17, IL-21 and IL-22 producing cells', *PLoS One*, 7: e45680.

Penna, G., S. Sozzani, and L. Adorini. 2001. 'Cutting edge: selective usage of chemokine receptors by plasmacytoid dendritic cells', *J Immunol*, 167: 1862-6.

Perez de Lema, G., H. Maier, T. J. Franz, M. Escribese, S. Chilla, S. Segerer, N. Camarasa, H. Schmid, B. Banas, S. Kalaydjiev, D. H. Busch, K. Pfeffer, F. Mampaso, D. Schlondorff, and B. Luckow. 2005. 'Chemokine receptor Ccr2 deficiency reduces renal disease and prolongs survival in MRL/lpr lupus-prone mice', *J Am Soc Nephrol*, 16: 3592-601.

Pflanz, S., J. C. Timans, J. Cheung, R. Rosales, H. Kanzler, J. Gilbert, L. Hibbert, T. Churakova, M. Travis, E. Vaisberg, W. M. Blumenschein, J. D. Mattson, J. L. Wagner, W. To, S. Zurawski, T. K. McClanahan, D. M. Gorman, J. F. Bazan, R. de Waal Malefyt, D. Rennick, and R. A. Kastelein. 2002. 'IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+ T cells', *Immunity*, 16: 779-90.

Phelan, Mary C., and Gretchen Lawler. 2001. 'Cell Counting.' in, *Current Protocols in Cytometry* (John Wiley & Sons, Inc.).

Picut, C., J. Meunier, C. Lee, and R. Lewis. 1987. 'Expression of HLA-DR and OKT6 antigens on keratinocytes and dendritic cells in pemphigus', *Arch Dermatol Res*, 279: 516-20.

Piemonti, L., B. E. Leone, R. Nano, A. Saccani, P. Monti, P. Maffi, G. Bianchi, A. Sica, G. Peri, R. Melzi, L. Aldrighetti, A. Secchi, V. Di Carlo, P. Allavena, and F. Bertuzzi. 2002. 'Human pancreatic islets produce and secrete MCP-1/CCL2: relevance in human islet transplantation', *Diabetes*, 51: 55-65.

Pieper, K., B. Grimbacher, and H. Eibel. 2013. 'B-cell biology and development', *J Allergy Clin Immunol*, 131: 959-71.

Pisanti, S., Y. Sharav, E. Kaufman, and L. N. Posner. 1974. 'Pemphigus vulgaris: incidence in Jews of different ethnic groups, according to age, sex, and initial lesion', *Oral Surg Oral Med Oral Pathol*, 38: 382-7.

Prignano, F., F. Ricceri, M. Beccatti, P. Di Gennaro, C. Fiorillo, T. Lotti, and N. Taddei. 2012. 'Circulating dendritic cell subsets in psoriatic patients before and after biologic therapy', *J Dermatol*, 39: 274-5.

Proietto, A. I., S. van Dommelen, P. Zhou, A. Rizzitelli, A. D'Amico, R. J. Steptoe, S. H. Naik, M. H. Lahoud, Y. Liu, P. Zheng, K. Shortman, and L. Wu. 2008. 'Dendritic cells in the thymus contribute to T-regulatory cell induction', *Proc Natl Acad Sci U S A*, 105: 19869-74.

Proksch, Ehrhardt, Johanna M. Brandner, and Jens-Michael Jensen. 2008. 'The skin: an indispensable barrier', *Exp Dermatol*, 17: 1063-72.

Quah, Benjamin J. C., and Christopher R. Parish. 2012. 'New and improved methods for measuring lymphocyte proliferation in vitro and in vivo using CFSE-like fluorescent dyes', *Journal of Immunological Methods*, 379: 1-14.

Quinones, Marlon P., Sunil K. Ahuja, Fabio Jimenez, Jason Schaefer, Edgar Garavito, Arun Rao, George Chenaux, Robert L. Reddick, William A. Kuziel, and Seema S. Ahuja. 2004. 'Experimental arthritis in CC chemokine receptor 2–null mice closely mimics severe human rheumatoid arthritis', *J Clin Invest*, 113: 856-66.

Radbruch, A., G. Muehlinghaus, E. O. Luger, A. Inamine, K. G. Smith, T. Dorner, and F. Hiepe. 2006. 'Competence and competition: the challenge of becoming a long-lived plasma cell', *Nat Rev Immunol*, 6: 741-50.

Rafei, David, Ralf Müller, Norito Ishii, Maria Llamazares, Takashi Hashimoto, Michael Hertl, and Rüdiger Eming. 2011. 'IgG Autoantibodies Against Desmocollin 3 in Pemphigus Sera Induce Loss of Keratinocyte Adhesion', *Am J Pathol*, 178: 718-23.

Randolph, G. J., J. Ochando, and S. Partida-Sanchez. 2008. 'Migration of dendritic cell subsets and their precursors', *Annu Rev Immunol*, 26: 293-316.

Randolph, G. J., G. Sanchez-Schmitz, R. M. Liebman, and K. Schakel. 2002. 'The CD16(+) (FcgammaRIII(+)) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting', *J Exp Med*, 196: 517-27.

Riley, J. K., and M. X. Sliwkowski. 2000. 'CD20: a gene in search of a function', *Semin Oncol*, 27: 17-24.

Rizzo, C., M. Fotino, Y. Zhang, S. Chow, A. Spizuoco, and A. A. Sinha. 2005. 'Direct characterization of human T cells in pemphigus vulgaris reveals elevated autoantigen-specific Th2 activity in association with active disease', *Clin Exp Dermatol*, 30: 535-40.

Romagnani, S. 1992. 'Human TH1 and TH2 subsets: regulation of differentiation and role in protection and immunopathology', *Int Arch Allergy Immunol*, 98: 279-85.

Ronnblom, L., and G. V. Alm. 2001. 'A pivotal role for the natural interferon alpha-producing cells (plasmacytoid dendritic cells) in the pathogenesis of lupus', *J Exp Med*, 194: F59-63.

Ross, G. L., P. Barland, and A. I. Grayzel. 1978. 'The immunoglobulin class of anti-DNA antibodies: detection by a fluorometric immunoassay: clinical and pathological correlations in SLE', *J Rheumatol*, 5: 373-83.

Rossol, M., S. Kraus, M. Pierer, C. Baerwald, and U. Wagner. 2012. 'The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population', *Arthritis Rheum*, 64: 671-7.

Ruocco, V., E. Ruocco, A. Lo Schiavo, G. Brunetti, L. P. Guerrera, and R. Wolf. 2013. 'Pemphigus: etiology, pathogenesis, and inducing or triggering factors: facts and controversies', *Clin Dermatol*, 31: 374-81.

Saha, P., and F. Geissmann. 2011. 'Toward a functional characterization of blood monocytes', *Immunol Cell Biol*, 89: 2-4.

Saito, R., H. Onodera, H. Tago, Y. Suzuki, M. Shimizu, Y. Matsumura, T. Kondo, and Y. Itoyama. 2005. 'Altered expression of chemokine receptor CXCR5 on T cells of myasthenia gravis patients', *J Neuroimmunol*, 170: 172-8.

Sakaguchi, S., D. A. Vignali, A. Y. Rudensky, R. E. Niec, and H. Waldmann. 2013. 'The plasticity and stability of regulatory T cells', *Nat Rev Immunol*, 13: 461-7.

Sams, W. M., Jr., and R. E. Jordon. 1971. 'Correlation of pemphigoid and pemphigus antibody titres with activity of disease', *Br J Dermatol*, 84: 7-13.

Satyam, A., S. Khandpur, V. K. Sharma, and A. Sharma. 2009. 'Involvement of T(H)1/T(H)2 cytokines in the pathogenesis of autoimmune skin disease-Pemphigus vulgaris', *Immunol Invest*, 38: 498-509.

Scally, S. W., J. Petersen, S. C. Law, N. L. Dudek, H. J. Nel, K. L. Loh, L. C. Wijeyewickrema, S. B. Eckle, J. van Heemst, R. N. Pike, J. McCluskey, R. E. Toes, N. L. La Gruta, A. W. Purcell, H. H. Reid, R. Thomas, and J. Rossjohn. 2013. 'A molecular basis for the association of the HLA-DRB1 locus, citrullination, and rheumatoid arthritis', *J Exp Med*, 210: 2569-82.

Schaffert, H., A. Pelz, A. Saxena, M. Losen, A. Meisel, A. Thiel, and S. Kohler. 2015. 'IL-17-producing CD4(+) T cells contribute to the loss of B-cell tolerance in experimental autoimmune myasthenia gravis', *Eur J Immunol*, 45: 1339-47. Schakel, K. 2009. 'Dendritic cells--why can they help and hurt us', *Exp Dermatol*, 18: 264-73.

Schakel, K., R. Kannagi, B. Kniep, Y. Goto, C. Mitsuoka, J. Zwirner, A. Soruri, M. von Kietzell, and E. Rieber. 2002. '6-Sulfo LacNAc, a novel carbohydrate modification of PSGL-1, defines an inflammatory type of human dendritic cells', *Immunity*, 17: 289-301.

Schakel, K., M. von Kietzell, A. Hansel, A. Ebling, L. Schulze, M. Haase, C. Semmler, M. Sarfati, A. N. Barclay, G. J. Randolph, M. Meurer, and E. P. Rieber. 2006. 'Human 6-sulfo LacNAc-expressing dendritic cells are principal producers of early interleukin-12 and are controlled by erythrocytes', *Immunity*, 24: 767-77.

Scheinecker, C., B. Zwolfer, M. Koller, G. Manner, and J. S. Smolen. 2001. 'Alterations of dendritic cells in systemic lupus erythematosus: phenotypic and functional deficiencies', *Arthritis Rheum*, 44: 856-65.

Schmidt, C., T. Giese, B. Ludwig, I. Mueller-Molaian, T. Marth, S. Zeuzem, S. C. Meuer, and A. Stallmach. 2005. 'Expression of interleukin-12-related cytokine transcripts in inflammatory bowel disease: elevated interleukin-23p19 and interleukin-27p28 in Crohn's disease but not in ulcerative colitis', *Inflamm Bowel Dis*, 11: 16-23.

Schmitz, Jochen, Alexander Owyang, Elizabeth Oldham, Yaoli Song, Erin Murphy, Terril K. McClanahan, Gerard Zurawski, Mehrdad Moshrefi, Jinzhong Qin, Xiaoxia Li, Daniel M.

Gorman, J. Fernando Bazan, and Robert A. Kastelein. 2005. 'IL-33, an Interleukin-1-like Cytokine that Signals via the IL-1 Receptor-Related Protein ST2 and Induces T Helper Type 2-Associated Cytokines', *Immunity*, 23: 479-90.

Schroeder, H. W., Jr., and L. Cavacini. 2010. 'Structure and function of immunoglobulins', *J Allergy Clin Immunol*, 125: S41-52.

Schwab, N., A. L. Zozulya, B. C. Kieseier, K. V. Toyka, and H. Wiendl. 2010. 'An imbalance of two functionally and phenotypically different subsets of plasmacytoid dendritic cells characterizes the dysfunctional immune regulation in multiple sclerosis', *J Immunol*, 184: 5368-74.

Sebastiani, S., P. Allavena, C. Albanesi, F. Nasorri, G. Bianchi, C. Traidl, S. Sozzani, G. Girolomoni, and A. Cavani. 2001. 'Chemokine receptor expression and function in CD4+ T lymphocytes with regulatory activity', *J Immunol*, 166: 996-1002.

Sekiguchi, M., Y. Futei, Y. Fujii, T. Iwasaki, T. Nishikawa, and M. Amagai. 2001. 'Dominant autoimmune epitopes recognized by pemphigus antibodies map to the N-terminal adhesive region of desmogleins', *J Immunol*, 167: 5439-48.

Sela, U., P. Olds, A. Park, S. J. Schlesinger, and R. M. Steinman. 2011. 'Dendritic cells induce antigen-specific regulatory T cells that prevent graft versus host disease and persist in mice', *J Exp Med*, 208: 2489-96.

Semerano, L., A. Thiolat, E. Minichiello, G. Clavel, N. Bessis, and M. C. Boissier. 2014. 'Targeting IL-6 for the treatment of rheumatoid arthritis: Phase II investigational drugs', *Expert Opin Investig Drugs*, 23: 979-99.

Sen, A., I. H. Chowdhury, D. Mukhopadhyay, S. K. Paine, A. Mukherjee, L. K. Mondal, M. Chatterjee, and B. Bhattacharya. 2011. 'Increased Toll-like receptor-2 expression on nonclassic CD16+ monocytes from patients with inflammatory stage of Eales' disease', *Invest Ophthalmol Vis Sci*, 52: 6940-8.

Serafini, B., S. Columba-Cabezas, F. Di Rosa, and F. Aloisi. 2000. 'Intracerebral recruitment and maturation of dendritic cells in the onset and progression of experimental autoimmune encephalomyelitis', *Am J Pathol*, 157: 1991-2002.

Serbina, N. V., T. Jia, T. M. Hohl, and E. G. Pamer. 2008. 'Monocyte-mediated defense against microbial pathogens', *Annu Rev Immunol*, 26: 421-52.

Sharma, Preety M., Eun Jung Choi, Keiko Kuroda, Takahisa Hachiya, Ken Ishii, and Aimee S. Payne. 2009. 'Pathogenic Anti-Desmoglein MAbs Show Variable ELISA Activity because of Preferential Binding of Mature versus Proprotein Isoforms of Desmoglein 3', *J Invest Dermatol*, 129: 2309-12.

Shen, H., L. Xia, W. Xiao, and J. Lu. 2011. 'Increased levels of interleukin-27 in patients with rheumatoid arthritis', *Arthritis Rheum*, 63: 860-1.

Shi, C., and E. G. Pamer. 2011. 'Monocyte recruitment during infection and inflammation', *Nat Rev Immunol*, 11: 762-74.

Shibata, S., Y. Tada, Y. Asano, K. Yanaba, M. Sugaya, T. Kadono, N. Kanda, S. Watanabe, and S. Sato. 2013. 'IL-27 activates Th1-mediated responses in imiquimod-induced psoriasis-like skin lesions', *J Invest Dermatol*, 133: 479-88.

Shibata, S., Y. Tada, N. Kanda, K. Nashiro, M. Kamata, M. Karakawa, T. Miyagaki, H. Kai, H. Saeki, Y. Shirakata, S. Watanabe, K. Tamaki, and S. Sato. 2010. 'Possible roles of IL-27 in the pathogenesis of psoriasis', *J Invest Dermatol*, 130: 1034-9.

Shirakata, Y., M. Amagai, Y. Hanakawa, T. Nishikawa, and K. Hashimoto. 1998. 'Lack of mucosal involvement in pemphigus foliaceus may be due to low expression of desmoglein 1', *J Invest Dermatol*, 110: 76-8.

Shlomchik, M. J., and F. Weisel. 2012. 'Germinal center selection and the development of memory B and plasma cells', *Immunol Rev*, 247: 52-63.

Simpson, N., P. A. Gatenby, A. Wilson, S. Malik, D. A. Fulcher, S. G. Tangye, H. Manku, T. J. Vyse, G. Roncador, G. A. Huttley, C. C. Goodnow, C. G. Vinuesa, and M. C. Cook. 2010. 'Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype

that identifies a subset of severe systemic lupus erythematosus', *Arthritis Rheum*, 62: 234-44.

Slevin, S. M., and L. J. Egan. 2015. 'New Insights into the Mechanisms of Action of Anti-Tumor Necrosis Factor-alpha Monoclonal Antibodies in Inflammatory Bowel Disease', *Inflamm Bowel Dis*.

Solomon, M., B. Balasa, and N. Sarvetnick. 2010. 'CCR2 and CCR5 chemokine receptors differentially influence the development of autoimmune diabetes in the NOD mouse', *Autoimmunity*, 43: 156-63.

Sozzani, S., W. Vermi, A. Del Prete, and F. Facchetti. 2010. 'Trafficking properties of plasmacytoid dendritic cells in health and disease', *Trends Immunol*, 31: 270-7.

Sozzani, Silvano. 2005. 'Dendritic cell trafficking: More than just chemokines', *Cytokine & Growth Factor Reviews Chemokines*, 16: 581-92.

Stanford, S. M., and N. Bottini. 2014. 'PTPN22: the archetypal non-HLA autoimmunity gene', *Nat Rev Rheumatol*, 10: 602-11.

Stanley, J. R., and M. Amagai. 2008. 'Autoimmune bullous diseases: historical perspectives', *J Invest Dermatol*, 128: E16-8.

Steinman, R. M., and Z. A. Cohn. 1973a. 'Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution', *J Exp Med*, 137: 1142-62.

Steinman, Ralph M. 2012. 'Decisions About Dendritic Cells: Past, Present, and Future', *Annu Rev Immunol*, 30: 1-22.

Steinman, Ralph M., and Zanvil A. Cohn. 1973b. 'IDENTIFICATION OF A NOVEL CELL TYPE IN PERIPHERAL LYMPHOID ORGANS OF MICE : I. MORPHOLOGY, QUANTITATION, TISSUE DISTRIBUTION', *J Exp Med*, 137: 1142-62.

Sticherling, Michael, and Cornelia Erfurt-Berge. 2012. 'Autoimmune blistering diseases of the skin', *Autoimmun Rev*, 11: 226-30.

Strober, Warren. 2001. 'Trypan Blue Exclusion Test of Cell Viability.' in, *Current Protocols in Immunology* (John Wiley & Sons, Inc.).

Stumbles, P. A., D. H. Strickland, C. L. Pimm, S. F. Proksch, A. M. Marsh, A. S. McWilliam, A. Bosco, I. Tobagus, J. A. Thomas, S. Napoli, A. E. Proudfoot, T. N. Wells, and P. G. Holt. 2001. 'Regulation of dendritic cell recruitment into resting and inflamed airway epithelium: use of alternative chemokine receptors as a function of inducing stimulus', *J Immunol*, 167: 228-34.

Sugiyama, H., H. Matsue, A. Nagasaka, Y. Nakamura, K. Tsukamoto, N. Shibagaki, T. Kawamura, R. Kitamura, N. Ando, and S. Shimada. 2007. 'CD4+CD25high regulatory T cells are markedly decreased in blood of patients with pemphigus vulgaris', *Dermatology*, 214: 210-20.

Swee, L. K., N. Bosco, B. Malissen, R. Ceredig, and A. Rolink. 2009. 'Expansion of peripheral naturally occurring T regulatory cells by Fms-like tyrosine kinase 3 ligand treatment', *Blood*, 113: 6277-87.

Tackenberg, B., J. Kruth, J. E. Bartholomaeus, K. Schlegel, W. H. Oertel, N. Willcox, B. Hemmer, and N. Sommer. 2007. 'Clonal expansions of CD4+ B helper T cells in autoimmune myasthenia gravis', *Eur J Immunol*, 37: 849-63.

Takahashi, H., M. Amagai, T. Nishikawa, Y. Fujii, Y. Kawakami, and M. Kuwana. 2008. 'Novel System Evaluating In Vivo Pathogenicity of Desmoglein 3-Reactive T Cell Clones Using Murine Pemphigus Vulgaris', *The Journal of Immunology*, 181: 1526-35.

Takahashi, H., M. Kuwana, and M. Amagai. 2009. 'A single helper T cell clone is sufficient to commit polyclonal naive B cells to produce pathogenic IgG in experimental pemphigus vulgaris', *J Immunol*, 182: 1740-5.

Tang, S., H. Huang, F. Hu, W. Zhou, J. Guo, H. Jiang, R. Mu, and Z. Li. 2013. 'Increased IL-33 in synovial fluid and paired serum is associated with disease activity and autoantibodies in rheumatoid arthritis', *Clin Dev Immunol*, 2013: 985301.

Tangye, S. G., C. S. Ma, R. Brink, and E. K. Deenick. 2013. 'The good, the bad and the ugly - TFH cells in human health and disease', *Nat Rev Immunol*, 13: 412-26.

Thomas, K., K. Dietze, R. Wehner, I. Metz, H. Tumani, T. Schultheiss, C. Gunther, K. Schakel, H. Reichmann, W. Bruck, M. Schmitz, and T. Ziemssen. 2014. 'Accumulation and therapeutic modulation of 6-sulfo LacNAc(+) dendritic cells in multiple sclerosis', *Neurol Neuroimmunol Neuroinflamm*, 1: e33.

Thomason, H. A., A. Scothern, S. McHarg, and D. R. Garrod. 2010. 'Desmosomes: adhesive strength and signalling in health and disease', *Biochem J*, 429: 419-33.

Tirado-Sanchez, A., A. Bonifaz, and R. M. Ponce-Olivera. 2015. 'Elevated IL-33 and sST2 levels in sera of patients with pemphigus vulgaris. Correlation with clinical and immunological parameters during follow up', *Br J Dermatol*.

Tong, J. C., J. Bramson, D. Kanduc, S. Chow, A. A. Sinha, and S. Ranganathan. 2006. 'Modeling the bound conformation of Pemphigus vulgaris-associated peptides to MHC Class II DR and DQ alleles', *Immunome Res*, 2: 1.

Tortola, L., E. Rosenwald, B. Abel, H. Blumberg, M. Schafer, A. J. Coyle, J. C. Renauld, S. Werner, J. Kisielow, and M. Kopf. 2012. 'Psoriasiform dermatitis is driven by IL-36-mediated DC-keratinocyte crosstalk', *J Clin Invest*, 122: 3965-76.

Travis, M. A., B. Reizis, A. C. Melton, E. Masteller, Q. Tang, J. M. Proctor, Y. Wang, X. Bernstein, X. Huang, L. F. Reichardt, J. A. Bluestone, and D. Sheppard. 2007. 'Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice', *Nature*, 449: 361-5.

Tron, F., D. Gilbert, P. Joly, H. Mouquet, L. Drouot, M. B. Ayed, M. Sellami, H. Masmoudi, and S. Makni. 2006. 'Immunogenetics of pemphigus: an update', *Autoimmunity*, 39: 531-9.

Tsai, Sue, and Pere Santamaria. 2013. 'MHC Class II Polymorphisms, Autoreactive T-Cells, and Autoimmunity', *Front Immunol*, 4: 321.

Tsunoda, K., T. Ota, H. Suzuki, M. Ohyama, T. Nagai, T. Nishikawa, M. Amagai, and S. Koyasu. 2002. 'Pathogenic autoantibody production requires loss of tolerance against desmoglein 3 in both T and B cells in experimental pemphigus vulgaris', *Eur J Immunol*, 32: 627-33.

Turley, S., L. Poirot, M. Hattori, C. Benoist, and D. Mathis. 2003. 'Physiological beta cell death triggers priming of self-reactive T cells by dendritic cells in a type-1 diabetes model', *J Exp Med*, 198: 1527-37.

Vanbervliet, B., B. Homey, I. Durand, C. Massacrier, S. Ait-Yahia, O. de Bouteiller, A. Vicari, and C. Caux. 2002. 'Sequential involvement of CCR2 and CCR6 ligands for immature dendritic cell recruitment: possible role at inflamed epithelial surfaces', *Eur J Immunol*, 32: 231-42.

Veldman, C., A. Hohne, D. Dieckmann, G. Schuler, and M. Hertl. 2004. 'Type I Regulatory T Cells Specific for Desmoglein 3 Are More Frequently Detected in Healthy Individuals than in Patients with Pemphigus Vulgaris', *The Journal of Immunology*, 172: 6468-75.

Veldman, C. M., K. L. Gebhard, W. Uter, R. Wassmuth, J. Grotzinger, E. Schultz, and M. Hertl. 2004. 'T cell recognition of desmoglein 3 peptides in patients with pemphigus vulgaris and healthy individuals', *J Immunol*, 172: 3883-92.

Veldman, C., A. Stauber, R. Wassmuth, W. Uter, G. Schuler, and M. Hertl. 2003a. 'Dichotomy of autoreactive Th1 and Th2 cell responses to desmoglein 3 in patients with pemphigus vulgaris (PV) and healthy carriers of PV-associated HLA class II alleles', *J Immunol*, 170: 635-42.

Veldman, Christian, Angelika Stauber, Ralf Wassmuth, Wolfgang Uter, Gerold Schuler, and Michael Hertl. 2003b. 'Dichotomy of Autoreactive Th1 and Th2 Cell Responses to Desmoglein 3 in Patients with Pemphigus Vulgaris (PV) and Healthy Carriers of PV-Associated HLA Class II Alleles', *The Journal of Immunology*, 170: 635-42.

Vermi, W., S. Lonardi, M. Morassi, C. Rossini, R. Tardanico, M. Venturini, R. Sala, A. Tincani, P. L. Poliani, P. G. Calzavara-Pinton, L. Cerroni, A. Santoro, and F. Facchetti. 2009. 'Cutaneous

distribution of plasmacytoid dendritic cells in lupus erythematosus. Selective tropism at the site of epithelial apoptotic damage', *Immunobiology*, 214: 877-86.

Vestergaard, C., H. Just, J. Baumgartner Nielsen, K. Thestrup-Pedersen, and M. Deleuran. 2004. 'Expression of CCR2 on monocytes and macrophages in chronically inflamed skin in atopic dermatitis and psoriasis', *Acta Derm Venereol*, 84: 353-8.

Villadangos, J. A., and L. Young. 2008. 'Antigen-presentation properties of plasmacytoid dendritic cells', *Immunity*, 29: 352-61.

Viola, Antonella, and Andrew D. Luster. 2008. 'Chemokines and Their Receptors: Drug Targets in Immunity and Inflammation', *Annual Review of Pharmacology and Toxicology*, 48: 171-97.

Wang, J., Y. Shan, Z. Jiang, J. Feng, C. Li, L. Ma, and Y. Jiang. 2013. 'High frequencies of activated B cells and T follicular helper cells are correlated with disease activity in patients with new-onset rheumatoid arthritis', *Clin Exp Immunol*, 174: 212-20.

Wang, R., G. Han, J. Wang, G. Chen, R. Xu, L. Wang, X. Li, B. Shen, and Y. Li. 2008. 'The pathogenic role of interleukin-27 in autoimmune diabetes', *Cell Mol Life Sci*, 65: 3851-60.

Waschke, J. 2008. 'The desmosome and pemphigus', *Histochem Cell Biol*, 130: 21-54.

Watson, James V. 1991. *Introduction to flow cytometry* (Cambridge University Press: Cambridge England ; New York).

Wei, L., A. Laurence, K. M. Elias, and J. J. O'Shea. 2007. 'IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner', *J Biol Chem*, 282: 34605-10.

Wong, K. L., W. H. Yeap, J. J. Tai, S. M. Ong, T. M. Dang, and S. C. Wong. 2012. 'The three human monocyte subsets: implications for health and disease', *Immunol Res*, 53: 41-57.

Wucherpfennig, K. W., B. Yu, K. Bhol, D. S. Monos, E. Argyris, R. W. Karr, A. R. Ahmed, and J. L. Strominger. 1995. 'Structural basis for major histocompatibility complex (MHC)-linked susceptibility to autoimmunity: charged residues of a single MHC binding pocket confer selective presentation of self-peptides in pemphigus vulgaris', *Proc Natl Acad Sci U S A*, 92: 11935-9.

Xia, L., H. Shen, L. Zhao, and J. Lu. 2012. 'Elevated levels of interleukin-27 in patients with Sjogren's syndrome', *Scand J Rheumatol*, 41: 73-4.

Xu, R. C., H. Q. Zhu, W. P. Li, X. Q. Zhao, H. J. Yuan, J. Zheng, and M. Pan. 2013. 'The imbalance of Th17 and regulatory T cells in pemphigus patients', *Eur J Dermatol*, 23: 795-802.

Yamagami, S., P. Hamrah, K. Miyamoto, D. Miyazaki, I. Dekaris, T. Dawson, B. Lu, C. Gerard, and M. R. Dana. 2005. 'CCR5 chemokine receptor mediates recruitment of MHC class II-positive Langerhans cells in the mouse corneal epithelium', *Invest Ophthalmol Vis Sci*, 46: 1201-7.

Yamazaki, S., T. Iyoda, K. Tarbell, K. Olson, K. Velinzon, K. Inaba, and R. M. Steinman. 2003. 'Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells', *J Exp Med*, 198: 235-47.

Yang, Jianfei, Mark S. Sundrud, Jill Skepner, and Tetsuya Yamagata. 2014. 'Targeting Th17 cells in autoimmune diseases', *Trends in Pharmacological Sciences*, 35: 493-500.

Yilmaz, V., P. Oflazer, F. Aysal, H. Durmus, K. Poulas, S. P. Yentur, Y. Gulsen-Parman, S. Tzartos, A. Marx, E. Tuzun, F. Deymeer, and G. Saruhan-Direskeneli. 2015. 'Differential Cytokine Changes in Patients with Myasthenia Gravis with Antibodies against AChR and MuSK', *PLoS One*, 10: e0123546.

Yin, Qian, Tian-Min Fu, Jixi Li, and Hao Wu. 2015. 'Structural Biology of Innate Immunity', *Annu Rev Immunol*, 33: 393-416.

Yogev, N., F. Frommer, D. Lukas, K. Kautz-Neu, K. Karram, D. Ielo, E. von Stebut, H. C. Probst, M. van den Broek, D. Riethmacher, T. Birnberg, T. Blank, B. Reizis, T. Korn, H. Wiendl, S. Jung, M. Prinz, F. C. Kurschus, and A. Waisman. 2012. 'Dendritic cells ameliorate autoimmunity in the CNS by controlling the homeostasis of PD-1 receptor(+) regulatory T cells', *Immunity*, 37: 264-75.

Yokouchi, Mariko, Marwah Adly Saleh, Keiko Kuroda, Takahisa Hachiya, John R. Stanley, Masayuki Amagai, and Ken Ishii. 2009. 'Pathogenic Epitopes of Autoantibodies in Pemphigus Reside in the Amino-Terminal Adhesive Region of Desmogleins Which Are Unmasked by Proteolytic Processing of Prosequence', *J Invest Dermatol*, 129: 2156-66.

Yokoyama, T., and M. Amagai. 2010. 'Immune dysregulation of pemphigus in humans and mice', *J Dermatol*, 37: 205-13.

Yokoyama, Tomoaki, Satoshi Matsuda, Yujiro Takae, Naoko Wada, Takeji Nishikawa, Masayuki Amagai, and Shigeo Koyasu. 2011. 'Antigen-independent development of Foxp3+ regulatory T cells suppressing autoantibody production in experimental pemphigus vulgaris', *Int Immunol*, 23: 365-73.

Yokoyama, W. M., M. L. Thompson, and R. O. Ehrhardt. 2012. 'Cryopreservation and thawing of cells', *Curr Protoc Immunol*, Appendix 3: 3G.

Yoon, B. R., S. J. Yoo, Yh Choi, Y. H. Chung, J. Kim, I. S. Yoo, S. W. Kang, and W. W. Lee. 2014. 'Functional phenotype of synovial monocytes modulating inflammatory T-cell responses in rheumatoid arthritis (RA)', *PLoS One*, 9: e109775.

Zhai, Y., L. Meng, F. Gao, Y. Wang, R. W. Busuttil, and J. W. Kupiec-Weglinski. 2006. 'CD4+ T regulatory cell induction and function in transplant recipients after CD154 blockade is TLR4 independent', *J Immunol*, 176: 5988-94.

Zhou, L., M. M. Chong, and D. R. Littman. 2009. 'Plasticity of CD4+ T cell lineage differentiation', *Immunity*, 30: 646-55.

Zhou, L., Ivanov, II, R. Spolski, R. Min, K. Shenderov, T. Egawa, D. E. Levy, W. J. Leonard, and D. R. Littman. 2007. 'IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways', *Nat Immunol*, 8: 967-74.

Zhu, H., Y. Chen, Y. Zhou, Y. Wang, J. Zheng, and M. Pan. 2012. 'Cognate Th2-B cell interaction is essential for the autoantibody production in pemphigus vulgaris', *J Clin Immunol*, 32: 114-23.

Zhu, H. Q., R. C. Xu, Y. Y. Chen, H. J. Yuan, H. Cao, X. Q. Zhao, J. Zheng, Y. Wang, and M. Pan. 2015. 'Impaired function of CD19+CD24hiCD38hi regulatory B cells in patients with pemphigus', *British Journal of Dermatology*, 172: 101-10.

Zhu, J., and W. E. Paul. 2008. 'CD4 T cells: fates, functions, and faults', *Blood*, 112: 1557-69.

Zhu, Jinfang, Hidehiro Yamane, and William E. Paul. 2010. 'Differentiation of Effector CD4 T Cell Populations*', *Annu Rev Immunol*, 28: 445-89.

Ziegler-Heitbrock, H. W. 2000. 'Definition of human blood monocytes', *J Leukoc Biol*, 67: 603-6.

Ziegler-Heitbrock, L. 2007. 'The CD14+ CD16+ blood monocytes: their role in infection and inflammation', *J Leukoc Biol*, 81: 584-92.

Ziegler-Heitbrock, L., P. Ancuta, S. Crowe, M. Dalod, V. Grau, D. N. Hart, P. J. Leenen, Y. J. Liu, G. MacPherson, G. J. Randolph, J. Scherberich, J. Schmitz, K. Shortman, S. Sozzani, H. Strobl, M. Zembala, J. M. Austyn, and M. B. Lutz. 2010. 'Nomenclature of monocytes and dendritic cells in blood', *Blood*, 116: e74-80.

Ziegler-Heitbrock, L., and T. P. Hofer. 2013. 'Toward a refined definition of monocyte subsets', *Front Immunol*, 4: 23.

7 Appendix

A Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel *Characterization of cellular and humoral immune responses in pemphigus patients and an HLA-transgenic mouse model* in der Klinik für Allergologie und Dermatologie unter Leitung von PD Dr. R. Eming ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.
Ich versichere, dass ich sämtliche wörtliche oder sinngemäße Übernahmen und Zitate kenntlich gemacht habe.
Mit dem Einsatz von Software zur Erkennung von Plagiaten bin ich einverstanden.

Vorliegende Arbeit wurde in folgenden Publikationsorganen *Journal of Immunology* und *PLoS One* veröffentlicht.

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Ort, Datum, Unterschrift

Die Hinweise zur Erkennung von Plagiaten habe ich zur Kenntnis genommen, die Angebote der Philipps-Universität zur Plagiatserkennung (Plagiatssoftware zu beziehen über das Hochschulrechenzentrum) sind mir bekannt.

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Ort, Datum, Unterschrift Betreuer

B Curriculum vitae

C Verzeichnis der akademischen Lehrer

Meine akademischen Lehrer waren die Damen und Herren
Professoren, Doktoren und Dozenten
In Marburg:

Bauer S., Bauer U.-M., Becker, Brehm, Bremer, Buchholz, Cetin, Cherkasov, Czubayko, Daut, Dehnen, Eickmann, Elsässer, Eming, Feuser, Garten, Greiner, Grzeschik, Hertl, Heverhagen, Huber, Irle, Jacob, Kaufmann, Käuser, Lill, Lohöfer, Lohoff, Maisner, Matrosovich, McCormick, Milani, Müller, Müller-Brüsselbach, Nain, Pfefferle, Plant, Preisig-Müller, Schäfer, Steinhoff, Suske, Visekruna, Vollmer, Westermann, Wrocklage, Yu.

In Gießen:
Gruber, Schütz, Verhoff.

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