PMNs, naRNA-LL37 complexes and platelets – a vicious inflammatory 'trio' in psoriasis

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Summary

Psoriasis is an autoinflammatory skin disease with high incidence (3% of adults) in Western countries, accompanied by personal and socioeconomic burden. Psoriatic skin lesions are characterized by hyperproliferating keratinocytes, by vasodilatation in the dermis and most importantly by skin infiltration of leukocytes, dominated by neutrophils (PMNs). Several studies implicate a major role of T helper 17 (Th17) cells and plasmacytoid dendritic cells (pDCs) in psoriatic autoinflammatory response but only little is known about the impact of PMNs on the pathology of the disease. Intriguingly, the initial trigger for immune infiltration and inflammation in psoriasis is also so far unknown. In this thesis two different mechanisms which might play a role in innate immune responses in psoriasis were addressed: first the impact of nucleic acid and LL37 complexes on PMN activation (part I) and second the impact of platelet-PMN aggregates on the pathogenesis of psoriasis (part II).

Part I: Psoriatic lesions are associated with overexpression of the antimicrobial peptide LL37. Due to its positive charge, LL37 forms complexes with nucleic acids (RNA and DNA) and mediates their uptake by pDCs. Subsequently, nucleic acids are recognized by endosomal Toll-like receptors (TLRs) which induces the release of IFN-α by pDCs. The physiological source of nucleic acids and LL37 and the triggers which induce inflammatory responses in psoriasis are unknown. Importantly, as PMNs dominate the immune infiltrates in psoriasis and are sources of LL37 and nucleic acids (e.g. due to release of neutrophil extracellular traps (NETs)), they might fuel a self-sustaining inflammatory loop in psoriasis. This study shows that primary human and murine PMNs efficiently respond to RNA-LL37 complexes rather than to DNA-LL37 by releasing cytokines, chemokines and NETs. Interestingly, PMNs from psoriasis patients are more prone to RNA-LL37 stimulation, potentially due to a higher abundance of PMN-derived LL37. Further experiments revealed that LL37 and unexpectedly also RNA is present in NETs *in vitro* and in psoriatic lesions but not in healthy skin. Importantly, isolated NET material induces NET release in PMNs, again containing DNA, RNA and LL37. The receptors binding RNA-LL37 complexes are TLR13 and TLR8 in mice and humans, respectively. This result is validated by TLR8 inhibitory oligodeoxynucleotides that effectively block RNA-LL37-mediated cytokine/chemokine production and NETosis of PMNs *in vitro*. The present data show that in PMNs, RNA-LL37 complexes activate TLRs (TLR8 or TLR13) which can induce a self-propagating vicious cycle of inflammation. Most importantly, this study for the first time identifies NET-associated RNA (naRNA) as a NET component that can potentially fuel the vicious cycle.

Part II: Psoriasis is characterized by massive skin infiltration of PMNs and is often accompanied by cardiovascular comorbidities. The major triggers for PMN skin homing in psoriasis are not identified yet. In order to find surface antigens on PMNs which explain skin homing in psoriatic lesions, whole blood from psoriasis patients and healthy controls (five each) was screened for the expression of 332 surface antigens. These experiments identified a platelet antigen signature for circulating PMNs from psoriasis patients because of increased aggregation of

PMNs with platelets in patients' blood. Results were confirmed in an imiquimod (IMQ)-induced mouse model of psoriasis which revealed higher amounts of PMN-platelet aggregates in the blood of IMQ-treated mice compared to mock controls. In blood samples from psoriasis patients, platelet blood counts are significantly elevated and platelet plaques are found in direct contact with PMNs in the lesions of psoriasis patients which is absent in healthy skin. Importantly, in mice, depletion of platelets *in vivo* drastically ameliorates disease severity, evidenced by decreased ear thickness, epidermal thickening and reduced leukocyte infiltration. Also, in the skin of IMQ-treated mice, PMNs are present in close proximity to platelets, while depletion of platelets completely abolishes PMN infiltration. This indicates a causal function of platelets in PMN infiltration which is relevant for psoriasis pathology and disease severity. The role of platelets in psoriasis potentially explains psoriasis-associated cardiovascular comorbidities and opens a new venue for the treatment of psoriasis.

Zusammenfassung

Psoriasis ist eine autoinflammatorische Hautkrankheit, welche sich durch eine hohe Inzidenz (3% der Erwachsenen) in westlichen Ländern auszeichnet und von persönlichen und sozioökonomischen Belastungen begleitet wird. Psoriatische Hautläsionen sind gekennzeichnet durch hyperproliferierende Keratinozyten, durch Vasodilatation in der Dermis und vor allem durch Hautinfiltration von Leukozyten, die von neutrophilen Granulozyten (PMNs) dominiert werden. Mehrere Studien sprachen T Helfer 17 (Th17) Zellen und plasmazytoiden dendritischen Zellen (pDCs) eine wichtige Rolle bei der psoriatischen autoinflammatorischen Reaktion zu, es ist jedoch wenig über die Bedeutung von PMNs für die Pathologie der Krankheit bekannt. Interessanterweise ist auch der initiale Auslöser für Immuninfiltration und Entzündung bei Psoriasis bisher unbekannt. In dieser Arbeit wurden zwei unterschiedliche Mechanismen untersucht, über die das angeborene Immunsystem die Pathologie der Psoriasis vermitteln könnte: erstens der Einfluss von Nukleinsäure- und LL37-Komplexen auf die PMN-Aktivierung (Teil I) und zweitens der Einfluss von Plättchen-PMN-Aggregaten auf die Pathogenese der Psoriasis (Teil II).

Teil I: Psoriatische Läsionen sind durch Überexpression des antimikrobiellen Peptids LL37 gekennzeichnet. Aufgrund seiner positiven Ladung, komplexiert LL37 Nukleinsäuren (RNA und DNA) und vermittelt die Aufnahme von diesen durch pDCs. Anschließend werden die Nukleinsäuren von endosomalen Toll-like-Rezeptoren (TLRs) erkannt, was zur Freisetzung von IFN-α durch pDCs führt. Die physiologische Quelle von Nukleinsäuren und LL37 und die initialen Auslöser, die bei der Psoriasis entzündliche Reaktionen hervorrufen sind unbekannt. Da PMNs die Immuninfiltrate bei der Psoriasis dominieren und selbst Quellen von LL37 und Nukleinsäuren sind (z.B. durch Freisetzung neutrophiler extrazellulärer Netze (*Neutrophil extracellular traps NETs*)), könnten sie eine selbsterhaltende Entzündungsschleife bei der Psoriasis induzieren. Diese Studie zeigt, dass primäre menschliche und murine PMNs effizient auf RNA-LL37-Komplexe und nicht auf DNA-LL37 reagieren, indem sie Zytokine, Chemokine und NETs freisetzen. Interessanterweise sind PMNs von Psoriasis Patienten anfälliger für die RNA-LL37-Stimulation, möglicherweise aufgrund einer höheren Menge an PMN-generiertem LL37. Weitere Experimente zeigten, dass LL37 und unerwarteterweise auch RNA in NETs *in vitro* und auch in psoriatischen Läsionen vorhanden sind, aber nicht in gesunder Haut. Wichtig ist, dass isoliertes NET-Material in PMNs die NET-Freisetzung induziert, welche wiederum DNA, RNA und LL37 enthalten. Die Rezeptoren, die RNA-LL37-Komplexe binden, sind TLR13 und TLR8 bei Mäusen bzw. Menschen. Dieses Ergebnis wird durch TLR8-hemmende Oligodeoxynukleotide bestätigt, die die RNA-LL37-vermittelte Zytokin-/Chemokin-Produktion und NETose von PMNs *in vitro* wirksam blockieren. Die vorliegenden Daten zeigen, dass in PMNs, RNA-LL37-Komplexe TLRs (TLR8 oder TLR13) aktivieren, die einen selbstfortpflanzenden Entzündungskreislauf induzieren können. Diese Studie identifiziert außerdem erstmalig NET-assoziierte RNA (naRNA) als eine NET-Komponente, die potenziell

den Entzündungskreislauf in der Psoriasis und anderen autoinflammatorischen Erkrankungen vermitteln kann.

Teil II: Psoriasis ist durch eine massive Hautinfiltration von PMNs gekennzeichnet und wird oft von kardiovaskulären Komorbiditäten begleitet. Die Hauptauslöser für die PMN-Hautinfiltration bei Psoriasis sind noch nicht identifiziert. Um Oberflächenantigene PMNs zu finden, welche die Hautinfiltration bei psoriatischen Läsionen erklären, wurde Vollblut von Psoriasis Patienten und gesunden Kontrollen (je fünf) auf die Expression von 332 Oberflächenantigenen untersucht. Diese Experimente identifizierten eine Blutplättchen-Marker-Signatur für zirkulierende PMNs von Psoriasis Patienten. Die Blutplättchen-Signatur der PMNs wurde durch die erhöhte Aggregation von PMNs mit Blutplättchen im Blut der Patienten hervorgerufen. Diese Ergebnisse wurden in einem Imiquimod (IMQ)-induzierten Mausmodell der Psoriasis bestätigt, bei welchem, im Vergleich zu Kontrollen, höhere Mengen an PMN-Blutplättchen-Aggregaten im Blut von IMQ-behandelten Mäuse nachgewiesen wurden. Blutproben von Psoriasis Patienten zeigten außerdem signifikant erhöhte Anzahl an Blutplättchen und in Hautläsionen von Psoriasis Patienten, nicht aber in gesunder Haut, wurden Thrombozytenplaques in direktem Kontakt mit PMNs gefunden. Durch die Depletion von Blutplättchen in Mäusen wurde *in vivo* die Schwere der Erkrankung drastisch reduziert, was sich in einer geringeren Ohrdicke, reduzierter Verdickung der Epidermis und einer reduzierten Leukozyten-Infiltration zeigte. Auch in der Haut IMQ-behandelter Mäuse sind PMNs in unmittelbarer Nähe von Blutplättchen zu finden, wohingegen die Depletion der Blutplättchen die PMN-Infiltration vollständig verhinderte. Dies deutet auf eine kausale Rolle der Blutplättchen bei der PMN-Infiltration hin, die für die Psoriasis-Pathologie und den Schweregrad der Erkrankung relevant ist. Die Rolle der Thrombozyten bei der Psoriasis könnte zudem die mit der Psoriasis einhergehenden kardiovaskulären Komorbiditäten erklären und eröffnet neue Wege zur therapeutischen Behandlung der Psoriasis.

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Abbreviations

AD	Atopic Dermatitis
AF488	AlexaFluor488
AF647	AlexaFluor647
ANCA	Anti-Neutrophil cytoplasmic antibody
APC	Antigen presenting cells
APC (dye)	Allophycocyanin
BCR	B cell receptor
BM-PMNs	Bone marrow-derived polymorpho-nuclear leukocytes
bRNA	Bacterial RNA
BV421	Brilliant violet 421
CBA	Cytometric bead array
CD	Cluster of differentiation
CLRs	C-type-lectin-receptors
CpG	Cytosine and guanosine rich DNA
CQ	Chloroquine
CRAMP	Cathelin related antimicrobial peptide
CTLs	Cytotoxic T lymphocytes
CVD	Cardiovascular disease
CXCL4	Synonym for platelet-factor 4 (PF4)
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FPR2	Formyl-peptide receptor 2
H&E	Hematoxylin and eosin
HD	Healthy donor
HLA	Human leukocyte antigen
HSP	Heat-shock protein
ICs	Immune complexes
IFN	Interferon
IL	Interleukin
IMQ	Imiquimod
LPS	Lipopolysaccharide
MAMPs	Microbe-associated-molecular-patterns
M-CSF	Macrophage colony-stimulating factor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MIP-1β	Macrophage Inflammatory Protein-1 beta
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary response protein 88
NADPH	Nicotinamid-adenin-dinucleotid-phosphat
naRNA	NET-associated-RNA
NE	Neutrophil elastase
NETosis	NET induced cell death
NETs	Neutrophil-extracellular-traps
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NLRs	NOD-like receptors
PAD4	Protein arginine deaminase 4
PASI	Psoriasis area and severity index
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principle component analysis
pDCs	Plasmacytoid dendritic cells
PE	Phycoerythrin
PF4	Platelet-factor 4
PLTs	platelets
PMA	Phorbol myristate acetate
PMNs	Polymorpho-nuclear leukocytes
PRRs	Pattern-recognition-receptors
PSGL-1	P-selectin glycoprotein ligand 1
PsorP	Psoriasis patient
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid
ROS	reactive oxygen species
RT	Room temperature
SDF-1α	Stromal-cell-derived factor 1
sGAS	Cyclic GMP-AMP synthase
SLE	Systemic Lupus Erythematodes
STING	Stimulator of interferon genes
TCR	T cell receptor
Th cells	T helper cells
TLRs	Toll-like-receptors
TNF	Tumor necrosis factor
vWf	Von Willebrand factor
WHO	World Health Organization
WT	Wildtype
Ψ-U	Pseudouridine

Introduction

1.1. The Immune System

To fend off microbial infection, the human immune system relies on three primary components: barriers, innate and adaptive immunity. The first obstacles a pathogen has to overcome are barriers which prevent the entry into the host, including physical barriers like the skin, chemical barriers inhibiting the growth of the pathogen (e.g. a certain pH) and also the composition of the host's microbiome creating a protective environment (Chaplin 2010) (Gallo and Nizet 2008).

Innate and adaptive immune system comprise complex networks of different cell types and mediators with the major task to protect the host from microbes, toxins or other harmful substances interfering with normal homeostasis. Indispensable for host defense is the ability to distinguish between non-self and self-derived structures. In mammals, two different systems which differ in specificity and speed of immune reaction have developed over time; they are also better known as innate and adaptive immunity. Despite their classification as two different systems, they often function as one union, where cross-talks and interactions happen to provide an efficient immune response (Chaplin 2010).

1.1.1. Innate Immunity

If a pathogen was able to invade the host by overcoming physical barriers like the skin, an immediate response is induced by the innate immune system. It consists of soluble (humoral), e.g. the complement system, and a number of cellular components, i.e. different cell types, such as professional phagocytes (monocytes, neutrophils and macrophages), cytotoxic cells (natural killer cells/NK cells) and antigen presenting cells (dendritic cells/DCs). These cells recognize structures that are common to pathogens by conserved receptors which are typically also found in lower organisms including plants and insects. In response to pathogen recognition, receptor signaling induces initial defense mechanisms such as phagocytosis and killing-mechanisms to neutralize invaders, cytokine release to signal further responses, activation of a cellular anti-viral state and opsonizing mechanisms like activation of the complement system etc. (Chaplin 2010).

Charles Janeway Jr. first postulated in 1989, that innate immune cells are able to recognize microbe-associated molecular patterns (MAMPs) by so-called pattern-recognition receptors (PRRs) in a non-specific way (Janeway 1989). Later, it was shown that PRRs are diverse in their structure and also in their way of recognizing foreign components. PRRs were shown to sense a variety of different pathogenic mediators, ranging from polysaccharides, to lipids or even nucleic acids. Different PRR families have been characterized so far: the most extensively studied ones are called Toll-like receptors (TLRs). Each of these receptors binds and senses distinct microbial structures. As pathogens colonize the host extra- and intracellularly, respective PRR receptors, recognizing different structures, are found on the surface of cells,

or in intracellular compartments (see below 1.2.1.) (Iwasaki and Medzhitov 2015). Another mechanism, in addition to the recognition of microbe-derived structures, is the "altered-self-recognition" (Karre *et al.* 1986). NK cells for example use this strategy to scan for infected cells: Viral infection or any form of stress associated with infection may change the surface antigens of a cell and mark it for NK cell-mediated lysis (Raulet 2006).

Additionally, after invasion of pathogens, skin resident innate immune cells recognize the invader and secrete pro-inflammatory cytokines and chemokines which attract other immune cells from the circulation. Usually, the first cells that are recruited to a site of inflammation are granulocytes, especially neutrophils. Activation of neutrophils stimulates phagocytosis of the pathogen, degranulation, release of reactive oxygen species (ROS) and eventually Neutrophil extracellular trap (NET) formation or "NETosis". The process of NETosis involves ejection of cellular DNA that traps the pathogen in place and facilitates efficient killing of the invader (Thomas and Schroder 2013) (also see 1.3.2.). When intracellular pathogens such as viruses are sensed by cytosolic PRRs, secretion of type I interferons is induced creating an "anti-viral" environment which inhibits viral spread (Iwasaki and Medzhitov 2015). Each of these mechanisms is important to combat infection and to activate adaptive immune responses.

1.1.2. Adaptive Immunity

Unlike the relatively unspecific immediate response of the innate immune system, the response of the adaptive immune system is delayed owing to the fact that it is far more specific. Adaptive immunity is therefore specifically found in higher developed organisms like mammals. The adaptive immune system can build on the early immune responses of the innate immune system and mount an antigen-specific immune response by activation of so-called T- and B-lymphocytes. T cell development takes place in the thymus where gene rearrangement generates clonally distributed individual T cell receptors (TCRs). The resulting T cell population with a variety of individual receptors provides the primary T cell repertoire. The arranged TCRs are then tested for cross-reactivity with the host, resulting in positive or negative selection. The remaining non-self-reactive TCRs form the functional T cell repertoire. Cells of the functional T cell repertoire can be activated by binding to a specific antigen presented by antigen-presenting cells (APCs) (Parkin and Cohen 2001) (Viret and Janeway 1999) (Krueger *et al.* 2017). B cell development starts in the bone marrow. Here, immunoglobulin-gene-rearrangement takes place and produces a large number of B cell clones with specific B cell receptors. Failure in any step to produce a functional B cell receptor leads to the death of the immature B cell. Thus, exclusively B cells with a functional B cell receptor (BCR) leave the bone marrow to circulate in blood and secondary lymphoid tissues. When a B cell encounters its specific antigen and is stimulated by T cells, this specific B cell can undergo isotype switching and somatic hypermutation (affinity maturation). When the immune response is shut down, the B cell undergoes apoptosis (programmed cell death) or becomes a long-lasting memory B cell which is pre-selected to produce antibodies against a specific antigen (Chaplin 2010) (Parkin and Cohen 2001) (Melchers 2015).

In contrast to B cells that bind a free antigen via their B cell receptor, conventional T cells heavily rely on antigen-presentation by APCs. These APCs include different phagocytic cells such as certain dendritic cell subsets and macrophages. APCs process internalized antigens which are then exposed on their cell surface bound to major histocompatibility complexes (MHC) class I or II. The antigen/MHC complex is bound by the specific TCR with the help of co-receptors, either CD4 (for MHC class II) or CD8 (for MHC class I) thus mediating their activation and differentiation of the respective T cell. The two T cell subsets ($CD4^+$ or $CD8^+$) are activated by different mechanisms: $CD4^+$ T cells, also known as T-helper (Th) cells, are activated by extracellular pathogens-derived MHC II ligands, whereas $CD8^+$ T cells, also known cytotoxic T cells (CTLs), are activated by antigens derived from intracellular pathogens complexed with MHC I (Chaplin 2010) (Parkin and Cohen 2001). Hence, CTLs recognize infected or invaded cells and induce apoptosis by granzymes, perforins or by binding to death inducing molecules (e.g. Fas-FasL). Th cells can be subdivided in several subtypes with Th1 and Th2 as classical representatives. Th1 cells produce cytokines like IL-2 which is crucial for T cell proliferation in general and cytotoxicity by CTLs. Th1 cells importantly release IFN-γ and thus activate macrophages and enhance their capacity to kill engulfed pathogens. On the other hand, Th2 cells release IL-4, 5, 6 and 10, and mainly promote antibody production by B cells (Parkin and Cohen 2001). So far, further T-helper subsets were characterized, namely Th17, T-follicular helper cells (TFHs), regulatory T cells (Treg) or T helper 22 (Th22) cells (Hirahara and Nakayama 2016) (Jia and Wu 2014) which all display important functions in adaptive immunity but will not be discussed in further detail.

As mentioned before: the major task of B cells is to produce antibodies against pathogenic invaders. Antibodies represent the humoral part of adaptive immunity and are important for sensitizing infected cells for complement (CDCC) or antibody-dependent cellular cytotoxicity (ADCC) mediated by NK cells; antibodies also bind to and cover the surface (opsonize) of pathogens labeling them for phagocytosis. Furthermore, B cells can also act as APCs for T cells: when an antigen is recognized by a BCR, this antigen is internalized, processed and presented on MHC class II of the B cell. Recognition of this antigen-MHC class II complex by a TCR activates the respective Th cell and mediates a Th2 response, i.e. the Th cell produces cytokines which induce B cell proliferation and maturation of antibody secreting plasma cells (Parkin and Cohen 2001).

1.2. Toll-like Receptors (TLRs)

Until the discovery of Toll-like receptors (TLRs), innate immune responses were thought to be unspecific and primitive. Now it is appreciated that TLRs recognize a variety of different microbe associated molecular patterns (MAMPs) from bacteria, viruses, fungi and protozoa quite specifically and induce microbe-specific immune responses. Recognition of self-derived structures can also lead to autoinflammation.

1.2.1. TLRs and their respective ligands and signaling pathways

The Toll story started in 1985, when Christiane Nüsslein-Volhard discovered that the Toll protein was important for embryonic segmentation of *Drosophila* (Anderson *et al.* 1985). Approximately one decade later, Toll was identified to play a role in the immune system of *Drosophila* (Lemaitre *et al.* 1996). Shortly thereafter, a human homologue for Toll was identified (Taguchi *et al.* 1996) leading finally to the characterization of the first Toll-like receptor in humans (Medzhitov *et al.* 1997).

Toll-like receptors (TLRs) are germline-encoded pattern-recognition-receptors (PRRs) which induce immune activation upon recognition of different components derived from pathogens (also called microbe-associated-molecular-pattern (MAMPs)) and are expressed on a variety of innate immune cells, like granulocytes, macrophages or dendritic cells (Kawai and Akira 2011) but also some tissue-resident cells like epithelial or endothelial cells and fibroblasts. This knowledge is based on a seminal publication of Janeway in 1989, postulating that there have to be receptors recognizing certain molecular structures of microbes to link innate and adaptive immunity (Janeway 1989). Although TLRs play an important role in innate immunity and often bridge immune responses of innate and adaptive immunity, they are not the only PRRs inducing immune activation. As PRRs gained more attention in recent decades, additional receptors were characterized, e.g. C-type lectins (CLRs) recognizing MAMPs from fungi (Brown *et al.* 2018), RIG-I-like receptors (RLRs) sensing viral RNA (Loo and Gale 2011), cytosolic NOD-like receptors (NLRs) which bind bacterial peptides and respond to other cellular stress signals (Kim *et al.* 2016) or, most recently, cytosolic DNA sensors like cGAS-STING (Kawai and Akira 2011) (Chen *et al.* 2016).

To date, 10 TLRs in humans and 13 TLRs in mice have been described. Each of them recognizes certain microbial molecular structures (see Table 1.1) (Kawai and Akira 2011) (O'Neill *et al.* 2013).

Table 1.1: Toll-like receptors and their ligands

TLR	localization	Type of ligand	Ligands*
TLR1 with 2	surface	bacterial	Triacylated lipopeptides
		synthetic	Pam3CSK4
		fungal	Chitin (Fuchs *et al.* 2018)
TLR2	surface	bacterial	lipopeptides
		fungal	Chitin (Fuchs *et al.* 2018), Zymosan
TLR2 with 6	surface	bacterial	Diacetylated lipopeptides
		synthetic	Pam2CSK4
TLR3	endosome	viral	Double-stranded RNA
		synthetic	Poly (I:C)
TLR4 with MD2	surface	bacterial	Lipopolysaccharide (LPS)
	endosome	bacterial	Lipopolysaccharide (LPS)
TLR5	surface	bacterial	flagellin
TLR7	endosome	viral	Single-stranded RNA
		synthetic	R848, Imiquimod (IMQ)
TLR8	endosome	viral	Single-stranded RNA
		synthetic	R848, TL8-506
TLR9	surface or endosome	bacterial viral	CpG, hypomethylated DNA motifs Herpesvirus DNA
TLR10 (human)	surface	?	?
TLR11 (mouse)	surface	bacterial	Uropathogenic bacteria, profilin-like molecules
TLR11 with TLR12 (mouse)	surface	parasitic	*Toxoplasma gondii* profilin protein
TLR13 (mouse)	endosome	bacterial	Ribosomal RNA

*** adapted from O'Neill et al. (O'Neill *et al.* 2013) and completed by literature mentioned in the text above and below and by synthetic TLR ligands which can be purchased by InvivoGen**

TLR1, 2, 4, 5, 6 are expressed on the cell surface and predominantly bind bacterial membrane structures, whereas TLR3, 7, 8 and 9 are found in endosomes where they bind nucleic acids (in more detail Table 1.1). TLR10 was recently described to be an anti-inflammatory (Oosting *et al.* 2014) and inhibitory regulator of innate immune signaling (Jiang *et al.* 2016). Although TLR10 has been shown to play a crucial role in inflammatory responses to *Listeria monocytogenesis* (Regan *et al.* 2013) its precise ligand still has to be identified. Commonly, TLR signaling requires dimerization of receptors, with the exceptions of TLR7, 8 and 9 that exist in preformed dimers in the endosome (Gay *et al.* 2014). Some TLRs also form hetero-dimers such as TLR2 which complexes with either TLR1 or TLR6, while the mouse TLR11 dimerizes with TLR12 (Gay *et al.* 2014).

Upon MAMP binding, the so-called Toll–IL-1-receptor (TIR) domains of the receptor molecule engage TIR domain-containing adaptor proteins, such as MyD88 (myeloid differentiation primary response protein 88) (except for TLR3). Binding of MyD88 activates a kinase cascade involving IRAKs (IL-1R associated kinases), activation of NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) and production of pro-inflammatory cytokines (O'Neill *et al.* 2013). Later, another adaptor protein was discovered and named TRIF (TIR domain-containing adaptor protein inducing IFNβ). TRIF was shown to mediate signaling of TLR3 and

MyD88-independent TLR4 signaling (with the help of the TRIF-related adaptor molecule (TRAM)), induced by internalized TLR4. Additionally, another TIR adaptor protein called MAL (MyD88-adaptor like protein) was described for TLR2 and TLR4 signaling, bridging MyD88 and TLR2/4. Furthermore, for successful LPS recognition, TLR4 is in need of a co-receptor, namely myeloid differentiation factor 2 (MD2) which binds LPS to induce signaling. Therefore, TLR4 signaling is so far considered the most complex signaling mechanism in the family of TLRs, either inducing surface receptor dependent activation of NF-κB (via MyD88-MAL) or cytoplasmic sensing and induction of anti-viral interferon response (via TRIF-TRAM) (see Figure 1.1) (O'Neill *et al.* 2013).

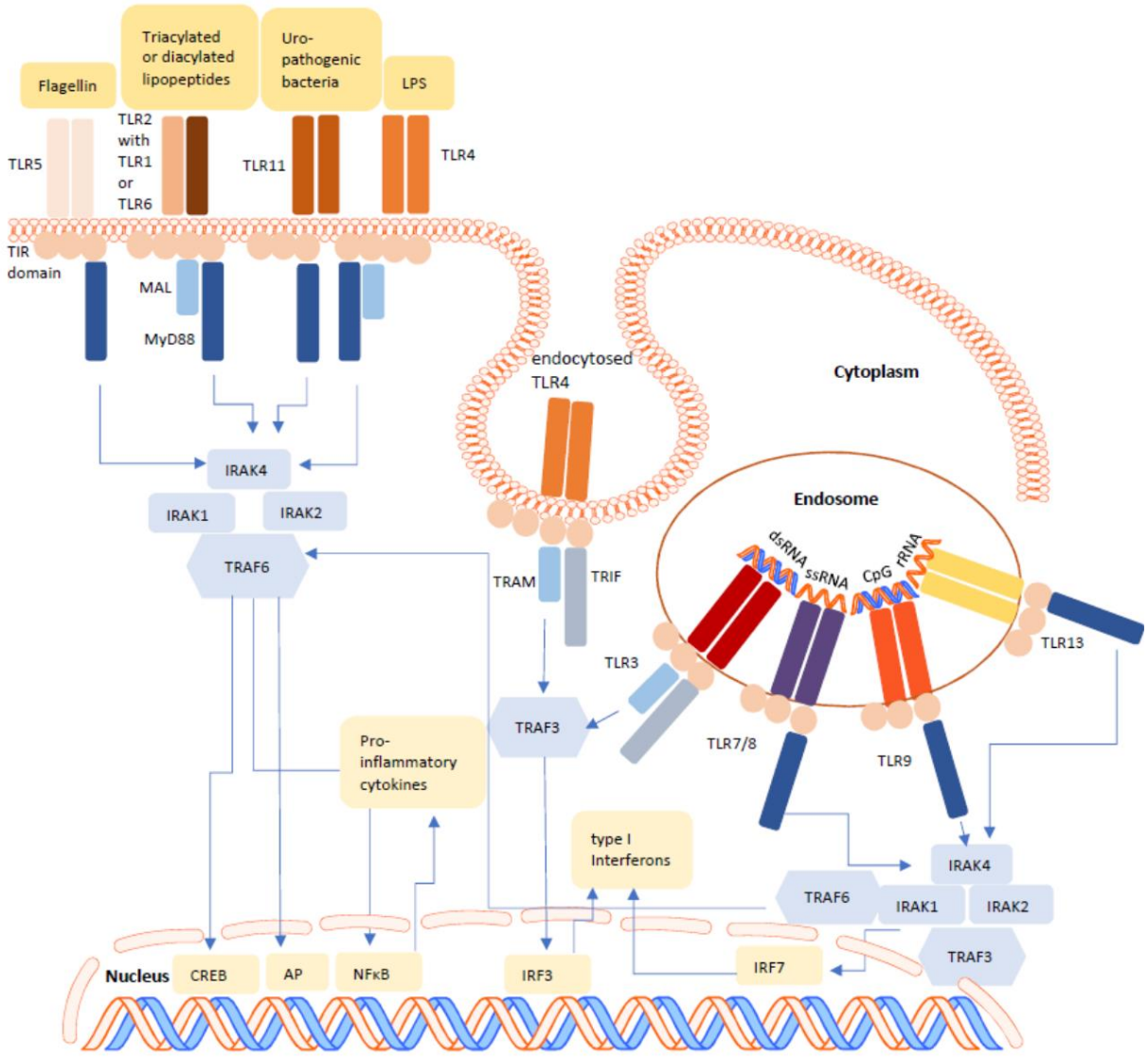


Figure 1.1: TLR signaling pathways

Heterodimers of TLR2-1 or TLR2-6, TLR5 and TLR11 bind their ligands on the cell surface, whereas, TLR3, TLR7, TLR8, TLR9 and TLR13 recognize nucleic acids in endosomal compartments. TLR4 is localized either on the cell surface or in endosomes. TLR signaling is initiated by ligand binding, following receptor dimerization. Thereafter, the Toll–IL-1-receptor (TIR) domains of TLRs bind TIR domain-containing adaptor proteins (MyD88 and MyD88-adaptor-like protein (MAL), or TRIF and TRAM). When TLR4 translocates to the endosome, signaling is switched from MyD88 to TRIF. TLR downstream signaling involves interaction of IRAKs, with TRAFs (TNF receptor-associated factors) and leads to kinase activation and transcription of NF-κB and Interferon-regulated factors (IRFs). Consequently, pro-inflammatory cytokines are released, or in case of endosomal TLRs type I interferons (IFNs). Adapted from O' Neill 2013 (O'Neill *et al.* 2013).
dsRNA, double-stranded RNA; LPS, lipopolysaccharide; rRNA, ribosomal RNA; ssRNA, single-stranded RNA

1.2.2. TLRs and their contribution to autoinflammation

TLRs are expressed on a variety of innate immune cells and their signaling is important for activation of adaptive immunity by e.g. induction of pro-inflammatory cytokines or enhancing expression of costimulatory molecules for APCs (Li *et al.* 2009). TLR signaling is considered to be crucial to link innate and adaptive immunity. However, continuous activation of TLRs or dysregulated TLR signaling likely contributes to the development of autoimmune diseases (Fischer and Ehlers 2008).

TLR signaling has therefore also gained interest in numerous autoimmune diseases in part as potentially druggable targets and new treatment options. Especially self-DNA recognition by endosomal TLRs has been extensively studied. For instance, in the autoinflammatory disease rheumatoid arthritis which leads to destruction of the joints, endosomal TLR ligands e.g. RNA are found in synovial fluids of the patients (Brentano *et al.* 2005), but also endogenous TLR4 ligands like heat-shock proteins (HSPs) (Roelofs *et al.* 2006) which can both potentially lead to inflammation. In systemic lupus erythematosus (SLE), autoantibodies against DNA or RNA are observed and clearance of apoptotic cells is impaired. This promotes the formation of immune complexes (ICs) of self-DNA fragments and their respective autoantibodies. The ICs are in turn recognized by TLR9 and thus induce pro-inflammatory immune response, leading to activation of DCs, T and B cells and culminate in an autoinflammation (Barrat *et al.* 2005) (Barrat and Coffman 2008). Small inhibitory oligonucleotides (iODNs) that block TLR9 were suggested as potential therapeutic intervention for SLE patients. Some of these iODNs are currently tested in clinical studies (Wu *et al.* 2015). In line with this, it was shown that neutrophils releasing self-DNA by neutrophil extracellular traps (NETs), could activate B cells in SLE patients. This DNA derived from NETting neutrophils, together with an antimicrobial peptide, entered B cells and induced activation by TLR9 signaling. This stimulation in turn led to autoantibody production against those NET structures (anti-neutrophil antibodies). A genetically modified B cell line that was deficient for TLR9 showed diminished immune stimulation and antibody production in response to DNA-ICs, proving that TLR9 is essential for sensing of self-derived DNA and subsequent inflammation in SLE (Gestermann *et al.* 2018).

For psoriasis, an autoinflammatory disease of the skin (see paragraph 1.5.), it was found that self-derived DNA and RNA can activate plasmacytoid DCs (pDCs) to produce type I interferon. IFN production depended on TLR9 and TLR7 respectively (Lande *et al.* 2007) (Ganguly *et al.* 2009) and NET contents released by neutrophils induced immune activation in pDCs (Lande *et al.* 2011). The presumed autoantibody in psoriasis is still unknown, but it is conceivable that a similar mechanism as found in SLE underlies the inflammatory mechanisms in the skin with endosomal TLRs as central mediators.

1.3. Neutrophilic granulocytes (PMNs)

Neutrophilic granulocytes, also commonly referred to as polymorphonuclear leukocytes (PMNs), are the most abundant granulocytes in the human blood, representing 60-70% of white blood cells in healthy human adults (Imhof and Dunon 1995). PMNs are usually considered the first cells to be recruited to the sites of inflammation and are very important for clearance of infections. PMNs are short lived, however one study showed PMN viability of up to 5.4 days in the human body (Galli *et al.* 2011) (Pillay *et al.* 2010). These findings are controversial because the method which was used by Pillay et al., also labeled PMNs in the bone marrow resulting in false viability rates of blood PMNs (Tofts *et al.* 2011). Nevertheless, PMNs show prolonged survival when activated by the presence of pathogens *in vivo* to assure complete clearance of the infection and adequate recruitment of other immune cells to the sites of inflammation (reviewed in (Kolaczkowska and Kubes 2013)). Also, the balance between PMN release and retention is tightly regulated by chemokines. Phagocytosis of apoptotic neutrophils by macrophages is an important mechanism regulated by the liver X receptors (LXR). Its dysregulation is often linked to autoimmune diseases (reviewed in (Kruger *et al.* 2015)).

Moreover, PMNs are packed with different kinds of granules which are divided in four subclasses: primary or azurophilic granules, secondary or specific granules, tertiary or gelatinase granules and secretory granules. All of them are filled with different antimicrobial peptides or bactericidal components (Lacy 2006) (Selders *et al.* 2017). Furthermore, PMNs are able to display a variety of different responses to combat pathogens such as phagocytosis, degranulation, ROS formation, release of pro-inflammatory cytokines or chemokines to attract other immune cells or even trapping and killing of pathogens by releasing NETs (Kruger *et al.* 2015).

1.3.1. PMNs and their role in inflammation

To effectively combat microorganisms in the periphery, leukocytes, including PMNs, need to rapidly translocate from the circulation into peripheral tissues. The leukocyte recruitment cascade is usually divided in five steps: tethering, rolling, adhesion, crawling and transmigration. When a pathogen enters the body, endothelial surface integrin expression changes due to stimulation by tissue resident leukocytes interacting with the inflammatory

mediator or due to direct activation of the endothelium by the pathogen. This causes PMNs in circulation to slow down and transient bonds are formed between PMNs and the endothelium (tethering and rolling). Then, integrins on the surface of PMNs change from low-affinity to high-affinity binding state, inducing attachment to the endothelial wall (adhesion) (Kolaczkowska and Kubes 2013). PMNs then actively crawl to cell-cell junctions, where they transmigrate through the endothelial barrier into the tissue. This mechanism is tightly regulated by interactions between the endothelium expression of ICAM1 (Intercellular Adhesion Molecule 1) and the neutrophil expression of MAC1 (macrophage adhesion ligand-1) (Phillipson *et al.* 2006). To leave the blood vessel, PMNs cross the endothelium and subsequently the basement membrane. This process requires tight regulation of integrins and cell-junction proteins. Also, endothelial cells rearrange their cytoskeleton to loosen the attachment to the extracellular matrix which finally enables transmigration of PMNs (Kolaczkowska and Kubes 2013). How PMNs overcome the tight barrier of the basement membrane is not fully understood but it is speculated that PMNs selectively transmigrate in regions where the expression of extracellular matrix proteins is low (Wang *et al.* 2006).

The major mechanisms of PMNs to fight pathogens are degranulation, ROS formation, NETosis and phagocytosis. PMNs are very potent phagocytes. After ligation with an opsonizing receptor, PMNs engulf foreign particles or pathogens. Phagocytosis is a complex mechanism involving diverse signaling cascades and cytoskeletal rearrangements that mediate target-engulfment within minutes. After engulfment, primary and secondary granules fuse with phagosomes and release their antimicrobial contents to kill the engulfed microbes (Kruger *et al.* 2015). When specific granules fuse with the phagosome, the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is activated and produces free superoxide radicals, i.e. reactive oxygen species (ROS) that have antimicrobial activity and are harmful to pathogens (Selders *et al.* 2017).

The mechanism regulating degranulation is not completely understood. Increase of intracellular Ca^{2+} which is largely mediated by ionophores, is of central importance (Lacy 2006). Interestingly, PMNs can release so-called Neutrophil extracellular traps (NETs) in response to bacteria, fungi or other stimuli like cholesterol crystals (Brinkmann *et al.* 2004) (Warnatsch *et al.* 2015). Their function will be discussed in more detail in the following section (1.3.3.).

Although PMNs are highly potent antimicrobial immune cells another important function is signaling to and recruitment of cells to sites of inflammation, by releasing pro-inflammatory cytokines and chemokines. Therefore, neutrophils and DCs are often found in close proximity at sites of inflammation. Furthermore, PMNs were shown to attract Th17 cells (Pelletier *et al.* 2010) and $CD8^+$ cytotoxic T cells (Lim *et al.* 2015) and they were recently found to even present antigens to $CD4^+$ T cells in an APC-like fashion (Vono *et al.* 2017).

1.3.2. Expression and roles of TLRs in PMNs

Activation of neutrophils can induce cytokine release, degranulation, i.e. release of pre-stored antimicrobial peptides and proteins, release of reactive oxygen species (ROS), active phagocytosis or NETosis (see also 1.3.3.) (Kruger *et al.* 2015). These are all important mechanisms for adequate immune response against pathogens. Some of those mechanisms are strongly dependent on TLR signaling.

PMNs also readily and functionally express TLRs on their surface and in endosomes. Hayashi et al. found that stimulation of TLRs with their specific ligands expectedly induced IL-8 release from PMNs of healthy donors – except for TLR3 ligands. Hence, they found that PMNs do not express TLR3 mRNA (Hayashi *et al.* 2003). Another study also demonstrated the lack of TLR7 expression in PMNs (Janke *et al.* 2009), leaving TLR8 and TLR9 as the only remaining endosomal TLRs in neutrophils. Importantly, Berger et al. confirmed the lack of TLR3 and TLR7 expression but showed cytosolic RNA receptors e.g. RIG-I to be functionally expressed in neutrophils (Berger *et al.* 2012) which might compensate for missing TLR3 and TLR7. Interestingly, Lindau et al. showed TLR9 (a DNA sensing endosomal TLR) to be functional on the surface of human and mouse PMNs. Of note, stimulation of neutrophils with the TLR9 ligand CpG enhanced surface expression of TLR9. The authors claimed that expression of TLR9 on the cell surface might be relevant in case TLR9 ligands are not able to enter the endosome to still ensure immune response to bacterial DNA (Lindau *et al.* 2013).

TLR stimulation in neutrophils was shown to efficiently induce release of pro-inflammatory cytokines, whereas it is not clear whether release of ROS strongly depends on TLR signaling. Zymosan is a protein-carbohydrate complex derived from yeast cell wall (Dillon *et al.* 2006) which serves as a ligand for TLR2 and Dectin-1. Zymosan was shown to induce ROS release from human PMNs (Gantner *et al.* 2003). Therefore, ROS formation can depend on TLR2 and/or Dectin-1 signaling. Further investigations showed, that zymosan depleted of TLR2 ligands was found to robustly induce ROS formation, meaning that ROS release largely depends on Dectin-1 (No *et al.* 2000). Our work showed that indeed cytokine release from neutrophils was highly dependent on TLR2 ligand binding and subsequent signaling, whereas Dectin-1 induced robust ROS release (Fuchs *et al.* 2018). Also, further TLR ligands e.g. LPS (TLR4), CpG (TLR9), R848 (TLR7/8), Pam3CSK4 (TLR2/1) or Pam2CSK4 (TLR2/6) (unpublished data) did not result in ROS release by PMNs. These results indicate, that TLR stimulation is not a robust ROS inducer but rather activates release of pro-inflammatory cytokines or NETosis (see 1.3.3. for more detail).

1.3.3. Neutrophil extracellular traps (NETs)

Neutrophil extracellular traps (NETs) have been described as long, fiber-like decondensed chromatin structures, loaded with cytosolic and granular proteins, e.g. antimicrobial peptides (Papayannopoulos 2018). Brinkmann et al. first discovered these structures and found NETs to trap and kill bacteria (Brinkmann *et al.* 2004). A couple of years later, the cell lytic form of

NET release was called “NETosis” (see Figure 1.2), because of its distinct differences to other forms of cell deaths like apoptosis or necrosis (Steinberg and Grinstein 2007) (Fuchs *et al.* 2007). Even though PMNs may have committed to eventual death, they still perform multiple tasks such as crawling or phagocytosis even after having lost the majority of their nuclear DNA (Yipp and Kubes 2013).

The major purpose of NETs is thus to eliminate pathogens. NETs not only trap bacteria but also contain antimicrobial peptides with activity towards a variety of different pathogenic species like bacteria, viruses or fungi. Also, other NET contents like neutrophil elastase (NE) were shown to inactivate virulence factors of pathogens (Brinkmann and Zychlinsky 2012). The exact mechanism allowing PMNs to distinguish when to release NETs or to induce alternative host protective mechanisms is far from being understood. A major factor influencing the choice of PMN response appears to be the size of a microbe: hyphae and huge bulks of bacteria that are too large to be phagocytosed are hence rather trapped by NETs, while smaller pathogens usually are internalized and digested. This selective induction of NETosis prevents overshooting immune reactions and necrotic tissue damage (Papayannopoulos 2018). Interestingly, also tiny bacteria that escape phagosomes, such as *Listeria,* or otherwise interfere with phagocytosis are also trapped and killed by NETosis. Additionally, bacterial toxins were found to kill PMNs and induce NETosis - whether bacterial toxins are required for NET formation remains unclear (reviewed in (Papayannopoulos 2018)).

Over the last couple of years, it became apparent that NETs are also released without cell lysis e.g. by *S.aureus* (Pilsczek *et al.* 2010) or by TLR4-activated platelets which bind to and subsequently mediate NET release by PMNs (Clark *et al.* 2007). TLR-mediated NET release is a fast process induced 30-60 minutes after stimulation compared to NETosis induced via Phorbol myristate acetate (PMA) (peaking at 4 hours after stimulation, complete cell lysis is induced). Here, the plasma membrane integrity is maintained and DNA is packed into small vesicles which fuse with the plasma membrane to release DNA (Pilsczek *et al.* 2010) (Jorch and Kubes 2017). In NETosis which is induced by PMA (originally observed by Brinkman et al. (Brinkmann *et al.* 2004)), NADPH oxidase induces ROS formation and subsequently stimulates protein-arginine deaminase 4 (PAD4) that converts arginine to citrulline on histones, thus inducing decondensation of chromatin in the nucleus (Jorch and Kubes 2017). Furthermore, histone citrullination that promotes chromatin decondensation was initially shown to be mediated by Neutrophil elastase (NE) (Papayannopoulos *et al.* 2010) which then is released during NET formation. However, histone H3 citrullination was independent of NE activity in NET release induced by fungi (Branzk *et al.* 2014). Also, in a deep vein thrombosis model, NE-deficient mice still showed robust NET formation (Martinod *et al.* 2016). Therefore, it might not be sufficient to investigate NE release *in vitro* to prove NET formation and usually microscopic analysis, preferably live cell imaging, has been considered a necessary means to illustrate the formation of *bona fide* NETs.

A remaining open question is also the source of DNA found in NETs. Yousefi and Simon showed that DNA in NETs is not exclusively nuclear chromatin but can also originate from mitochondria (Yousefi and Simon 2016). Interestingly, mitochondrial NETs and ROS induction were associated with autoinflammation in SLE. There, mitochondrial NETs were sensed by STING inducing type I IFN in a mouse model (Lood *et al.* 2016). But, whether nuclear or mitochondrial DNA release via NETs follows distinct pathways or is induced by distinct stimuli still remains to be clarified (Boeltz *et al.* 2019).

Hence, the mode of action for NET release; how to distinguish NETosis clearly from other forms of cell death and the source of DNA in NETs is still under current investigation (Jorch and Kubes 2017) (Boeltz *et al.* 2019) (Nestle *et al.* 2009).The three different types of NETs that have been described so far are (1) rapid TLR-induced nuclear DNA-NETs, (2) PMA-induced slow nuclear DNA-NETs and (3) mitochondrial DNA-NET release (Figure 1.2).

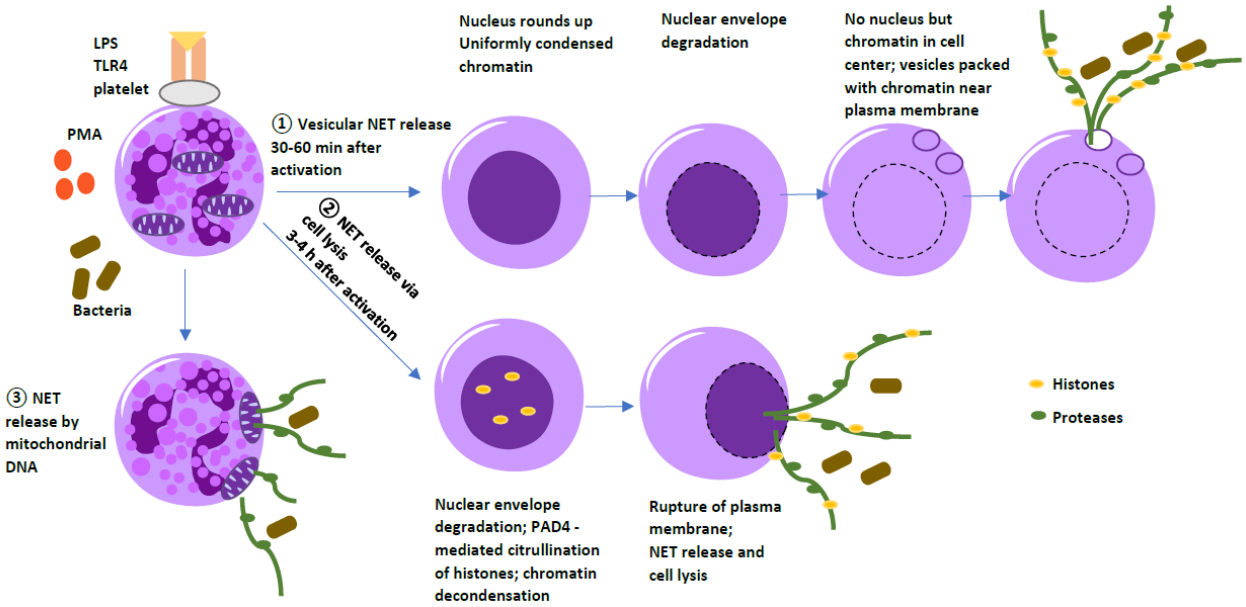


Figure 1.2: Different types of NETosis

Different mechanisms of NET release have been described. ① NETs can be released in response to initial TLR4-mediated activation of platelets and subsequent interaction with and activation of PMNs. The nucleus rounds up and uniformly condensed chromatin is found in the cell center. The nuclear envelope breaks down and DNA is packed into small vesicles. These vesicles eventually fuse with the plasma membrane and NETs are released to trap bacteria. This process is very fast and occurs approximately 30-60 min after stimulation of the cell. ② NETs are also released by cell lysis, where the nuclear membrane is degraded. Chromatin decondensation is mediated by PAD4-induced citrullination of histones. This mechanism is usually induced by PMA and occurs 3-4 h after stimulation. ③ NET release by mitochondria was also observed but not very much is known about its mode of action. Adapted from Phillipson & Kubes, 2011 (Phillipson and Kubes 2011).

1.3.4. NETs in disease and autoinflammation

NET formation is necessary for a fully functional and effective host defense while dysregulated NET induction can lead to severe health problems. Patients suffering from chronic granulomatous disease (CGD) are not able to mount efficient host response due to the lack of ROS induction. As ROS was shown to play a role in NET formation, PMNs from CGD patients also fail to induce NETosis, when infected with *Aspergillus* (a fungus which can form hyphae, thus being too large for phagocytosis). The patients' inability to clear the infection leads to severe and life-threatening inflammation (Brinkmann and Zychlinsky 2012). On the other hand, an overshooting NET formation can lead to severe tissue damage, as seen in sepsis (O'Brien *et al.* 2017). Usually in a healthy individual unwanted NETs are digested by DNases and thus removed. Interestingly, in patients with thrombosis less DNase I was found to clear NETs. In deep vein thrombosis, NET formation is induced due to activation of platelets and endothelium involving platelet-derived high mobility group protein B1 (HMGB1). (Papayannopoulos 2018). Furthermore, .in atherosclerosis, cholesterol crystals induce NETosis which in turn activate other immune cells, direct them to infiltrate the tissue and thus lead to a sterile (pathogen-free) inflammation (Warnatsch *et al.* 2015).

Autoimmunity in the context of NETs was first described for SLE. In SLE, B cells produce anti-neutrophil-cytoplasmic antibodies (ANCAs) and NETs were suspected as source for ANCA production (Yu and Su 2013). Meanwhile, Gestermann et al. showed that NET contents actively induce the production of anti-neutrophil antibodies in B cells from SLE patients (Gestermann *et al.* 2018).

In another auto-immune disease, rheumatoid arthritis (RA), patients suffer from inflamed joints. Interestingly, in an experimental mouse model for RA, mice deficient for PAD4 which are not able to induce NETosis, had less inflammation in their joints. Of note, the synovial fluid of RA patients contains large numbers of citrullinated proteins which even serve as diagnostic markers for RA (Branzk and Papayannopoulos 2013). In psoriasis (described in more detail in 1.5.) patients' NETs were found in blood and skin samples (Hu *et al.* 2016) and NET contents can stimulate pDCs to produce type I IFNs (Lande *et al.* 2011). These examples show that tight regulation of NET formation and degradation is crucial to maintain homeostasis and dysregulation has the potential to induce overshooting immune reactions and is associated with autoimmune diseases. Importantly, the possibility of RNA instead of DNA as a potential NET content and its contribution to the development of autoinflammation was so far not appreciated.

1.4. Platelets

Megakaryocytes found in the bone marrow are the origin of platelets, also called thrombocytes, that are absolutely crucial for blood coagulation. Platelets are small, anucleated cells and by far more abundant in human blood than leukocytes, counting over one trillion circulating cells in an adult human being. Besides the well-known and central role of platelets in coagulation, they are now also appreciated to be important players in immune responses (Habets *et al.* 2013). Platelets release inflammatory mediators (Golebiewska and Poole 2015) and attract other cells to the site of inflammation (Sreeramkumar *et al.* 2014) (Zuchtriegel *et al.* 2016). Also, there is a potential link between platelets or platelet-leukocyte aggregates and autoimmune diseases like rheumatoid arthritis, or skin diseases like atopic dermatitis or psoriasis (Tamagawa-Mineoka 2015).

1.4.1. The role of platelets in coagulation

The coagulation system is a well-known cascade of tightly regulated factors and generally two individual pathways are distinguished: the extrinsic, also known as tissue-factor pathway and the intrinsic or contact pathway. The tissue factor pathway is triggered by the cell surface protein “tissue factor (TF)”, whereas the contact pathway is regulated by activation of factor XII. Both pathways culminate in prothrombin cleavage into active thrombin which in turn converts fibrinogen to fibrin and additionally activates platelets (Smith *et al.* 2015). In Figure 1.3 an overview of all factors initiating blood coagulation is shown.

Platelets have so far mostly been appreciated for their role in blood coagulation. In a healthy individual, platelets usually circulate in close proximity to the blood vessel walls. In an intact endothelial barrier, platelet activation is inhibited by the secretion of nitric oxide (NO) or prostacyclin (PGI2) from the endothelium (Golebiewska and Poole 2015). Injury induces thrombus formation fast in a hierarchical manner. During the first phase (initiation) platelets are recruited to the site of injury by the von Willebrand factor (vWf) which binds to collagen fibers in the blood vessel wall. Usually, vWf circulates in an inactive form in the blood stream (secreted by endothelial cells) (Ruggeri 2007). Injury induces exposure of collagen fibers and exposed collagen fibers accumulate bound vWf. The vWf binding of surface antigen CD42b mediates recruitment of platelets. However, this weak binding of vWf to CD42b is not sufficient for robust platelet attachment and therefore subsequent conformational changes in surface integrins enhances attachment of platelets. Also, platelet activation is mediated by intrinsic activation pathways and e.g. generation of thrombin from the extravascular tissue (Tomaiuolo *et al.* 2017). After tight platelet adhesion and activation, the second phase (extension) follows, in which more platelets are recruited to form platelet-platelet aggregates. This process is mainly mediated by platelet binding to the plasma protein fibrinogen. This mechanism is induced by platelet dense granule contents e.g. adenosine-diphsophate (ADP) or by thromboxane A_2 released from already adherent platelets (Golebiewska and Poole 2015). In the third phase (stabilization) the thrombus plug is stabilized and protected from shear forces of the blood. Thrombin, besides activating platelets, also converts fibrinogen to

fibrin which forms strong fibers. Additionally, platelets release soluble agonists and interact with endothelial cells at the site of injury which functions as a positive feedback loop and further strengthens the thrombus plug (Tomaiuolo *et al.* 2017) (Golebiewska and Poole 2015).

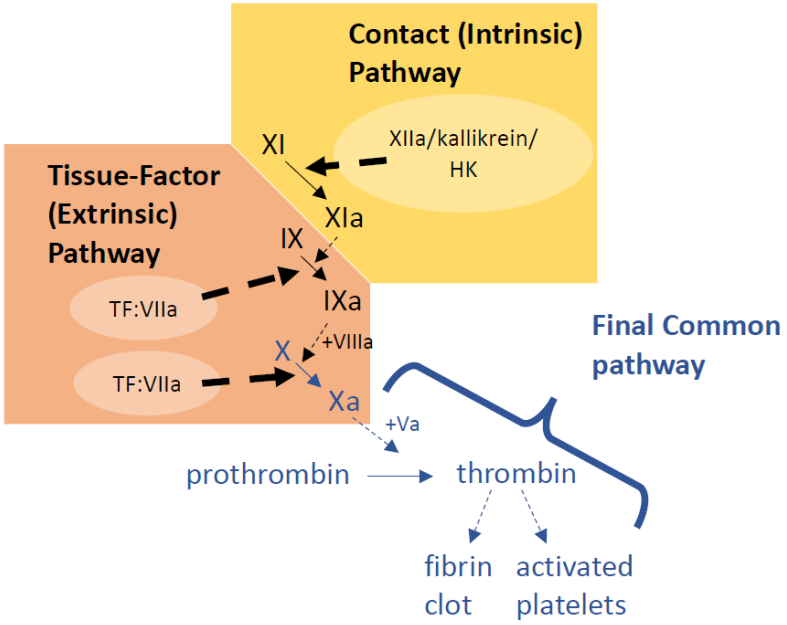


Figure 1.3: Blood coagulation cascade

Overview of the blood coagulation cascade. Two different pathways have to be distinguished: the Tissue Factor (TF) Pathway (extrinsic) or the Contact Pathway (intrinsic). The TF pathway is induced by cell-surface complex of TF and factor VIIa (TF:VIIa). The TF:VIIa complex proteolytically activates factor IX and factor X. The contact pathway is induced when factor XII, pre-kallikrein (PK) and HK (High-molecular-weight kininogen) form aggregates on a surface or polymer. These aggregates mediate activation of factor XII to factor XIIa and XIIa processes PK to kallikrein. Factor XIIa in turn activates factor XI to XIa which converts factors IX to factor IXa. Both pathways induce the production of active factor Xa which in turn induces the final step by generating thrombin leading to the transformation of fibrinogen to fibrin. Fibrin itself forms clots and mediates the activation of platelets. Adapted from Smith et al. 2015 (Smith *et al.* 2015).

1.4.2. Selected platelet-related diseases

There is a vast variety of different diseases associated with dysfunctional hemostasis classified as either thrombocytopathies or thrombocytopenias which can cause severe bleeding in the patients. The class of thrombocytopathies includes platelet-related diseases which have a defect in either adhesion, activation, secretion or aggregation of platelets (D'Andrea *et al.* 2009). Thrombocytopathies are usually very rare diseases. One of the most eminent defects is *Glazmann thrombastenia* which affects the megakaryocyte linage. Patients with *Glazmann thrombastenia* express functional deficient $\alpha_{IIb}\beta_3$ (CD41/CD61 complex) integrin which is important for binding of fibrinogen or vWf. The autosomal recessive inherited disease causes bleeding that ranges from mild to severe and the onset of the disease varies from early age to later in life (D'Andrea *et al.* 2009). Congenital thrombocytopenias were formerly considered as very rare but are now more frequently diagnosed by routine blood analysis. However, still

some cases are mistaken for an acquired thrombocytopenia e.g. idiopathic *thrombocytopenic purpura,* a low platelet count of an unknown cause (Kayal *et al.* 2014). Congenital thrombocytopenias are commonly divided into three groups: diseases with normal, increased or reduced platelet size. Patients with thrombocytopenia also suffer from rather mild to severe tendency to bleed (D'Andrea *et al.* 2009).

Better understood and characterized are the so-called *Haemophilias* like *Haemophilia A* and *B*. These X-chromosome-linked diseases almost exclusively affect men. Patients suffering from *Haemophilia A* usually have a deletion in Factor VIII gene that causes a mild to severe phenotype. *Haemophilia B* is characterized by deletion of Factor IX of the coagulation cascade. As the name of the disease already suggests: here patients also suffer from a higher tendency to bleed (Emilien *et al.* 2000).

Enhanced bleeding is not the only complication of platelet dysfunction: When platelet aggregation is increased, this can also cause thrombosis and other cardiovascular diseases (CVDs) like stroke or heart attack. Healthy platelets inhibit thrombus formation by releasing NO (Golebiewska and Poole 2015), this system is usually dysfunctional during thrombosis (Koupenova *et al.* 2017). Major risk factors for thrombosis or other CVDs are coagulation factor mutations (e.g. Factor V Leiden (Slusher 2010)), smoking, hypertension and high levels of cholesterol but interestingly autoimmune diseases e.g. Systemic Lupus Erythematosus have also been implicated (Koupenova *et al.* 2017).

As platelets are now considered to be not only important for blood coagulation but also emerging new players in immune activation, the question arises whether platelet dysfunction is also involved in autoimmunity. The role of platelets as immune cells and in autoimmunity is the topic of the following two paragraphs.

1.4.3. The role of platelets in immunity

Interestingly, former findings already indicate that platelets are able to direct leukocytes to sides of inflammation (Sreeramkumar *et al.* 2014) (Zuchtriegel *et al.* 2016). Leukocyte attraction was mainly induced by aggregate formation and platelet activation. Platelets also decorate bacteria, tagging them for PMN-mediated phagocytosis (Gaertner *et al.* 2017). Additionally, platelets can also induce neutrophil adhesion and recruitment of monocytes to the endothelium (Golebiewska and Poole 2015). Besides these rather passive mechanisms of platelet-induced immune reactions it is possible that they, although being anucleated, actively act as immune cells especially in innate immunity.

Indeed, in 2002, Tang et al. showed for example that thrombin-activated platelets are able to release seven different antimicrobial peptides, all of them being active against at least two pathogenic strains, mostly of bacterial and not of fungal origin (Tang *et al.* 2002). Additionally, platelets also express a variety of PRRs like TLRs (TLR1, 2, 4, 6, 8 and 9) (Morrell *et al.* 2014) and also NLRP3 and NOD2, both belonging to the same family of NOD-like receptors. Signaling

via these receptors activates anti-bacterial responses in platelets. TLR4 activation for example leads to the shedding of IL-1β containing microparticles from platelets and to interactions with other cells (Brown and McIntyre 2011). Hence, platelets also actively secrete pro-inflammatory cytokines such as IL-1β (Morrell *et al.* 2014) and are also important producers of the chemokine CXCL4 (PF4) which activates monocytes (Golebiewska and Poole 2015). However, whether platelets are also able to induce interferons as anti-viral response, has not been shown yet (Hottz *et al.* 2018).

Platelets not only affect innate immune cells (also see 1.4.5) or display innate immune cell functions but also influence adaptive immunity (Hottz *et al.* 2018). For example, platelet-derived CXCL4 binds to CXCR3 receptor which is highly expressed on activated Th_1 cells and induces T cell trafficking (Mueller *et al.* 2008). Additionally, platelets are the major producers of CD40L (stored in their α-granules) in the human body. CD40L released from platelets enhances T cells response to viral stimuli and is important for dendritic cell maturation and B cell isotype switch (Elzey *et al.* 2003) (Morrell *et al.* 2014).

1.4.4. Platelets and their role in autoimmunity

As outlined above, platelets accomplish functions in innate and adaptive immunity. Consequently, it is conceivable to assume that dysregulated platelet function is involved in development of autoimmune diseases. However, only little is known about platelets and their impact on autoinflammation. Still, there are indications that platelets affect the pathogenesis of numerous autoinflammatory diseases e.g. rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis, multiple sclerosis (MS), atopic dermatitis (AD), allergic contact dermatitis and possibly psoriasis (Habets *et al.* 2013) (Tamagawa-Mineoka 2015).

In RA for example, platelets are activated by fibrin accumulations in the joint and this in turn attracts PMNs to the sites of inflammation where they get trapped in the fibrin-platelet network and induce inflammation (Habets *et al.* 2013). SLE patients show enhanced platelet activation and increased serum levels of soluble CD40L which mainly originates from platelets (Desai-Mehta *et al.* 1996) and also systemic sclerosis patients show increased levels of platelet-derived molecules, like β-thromboglobulin (Postlethwaite and Chiang 2007). Moreover, increased platelet counts are also found in peripheral blood of MS patients (Sheremata *et al.* 2008) (Habets *et al.* 2013).

Still, platelet functions are only poorly studied in autoinflammatory skin diseases, especially in psoriasis. Nevertheless, there are indications from analysis in other skin-related diseases which indicate that platelets and platelet-PMN interactions might also be important in psoriasis (Tamagawa-Mineoka 2015) (Habets *et al.* 2013). For example, in AD, platelet-derived chemokines like CXCL4 are strongly upregulated in patients' blood (Tamagawa-Mineoka *et al.* 2008). Furthermore, in a murine mouse model for AD, increased PMN-platelet aggregate

formation is observed in the blood (Tamagawa-Mineoka 2015). Platelet-PMN interactions are discussed in further detail in the next paragraph.

1.4.5. PMN-platelet aggregation or interaction

More than four decades ago, it was shown that platelets and leukocytes form aggregates, especially when platelets are activated (Chanarat and Chiewsilp 1975) (Jungi *et al.* 1986) (de Bruijne-Admiraal *et al.* 1992). It is also well established that platelets are important mediators in innate immunity and in inflammatory diseases, e.g. in sepsis (Dewitte *et al.* 2017). Consequently, platelets are likely to interact with other cells such as PMNs which are initially recruited to sites of inflammation and induce further immune responses. Indeed, platelets do interact with PMNs: P-Selectin on platelets binds to P-selectin glycoprotein ligand 1 (PSGL-1) on PMNs (Lisman 2018). Interaction is also achieved via CD40L binding to CD40 (Zuchtriegel *et al.* 2016). Stable PMN-platelet aggregates commonly enclose activated platelets - either in circulation or when platelets bind to activated endothelium (Lisman 2018).

In the last couple of years, it has been increasingly appreciated that platelet activation might be the first response in innate immunity. In this scenario platelets guide leukocytes to the sites of inflammation (Ludwig *et al.* 2004) (Sreeramkumar *et al.* 2014) (Zuchtriegel *et al.* 2016). Ludwig et al. showed that the interactions of P-selectin and PSGL-1 on platelets and neutrophils respectively are responsible for rolling along skin micro vessels (Ludwig *et al.* 2004). Later, it was reported that PMNs, when recruited to injured tissue, scan the area for activated platelets and the interactions of P-selectin and PSGL-1 is indispensable for PMNs to extravasate or to form NETs (Sreeramkumar *et al.* 2014). However, Zuchtriegel et al. later postulated that platelets navigate leukocytes to extravasate and infiltrate tissues. In these studies platelets adhered at junctions in the endothelium and captured neutrophils by CD40-CD40L interactions. The recruitment accumulated cells at the site of inflammation and the cell-cell crosstalk induced a conformational change in integrins to subsequently mediate extravasation (Zuchtriegel *et al.* 2016).

Furthermore, PMN-platelet interactions have also been described in injury and thrombosis. In a transfusion associated mouse model of lung injury, platelets induced NETosis in PMNs which caused severe tissue damage and death (Caudrillier *et al.* 2012). Another important interplay between platelets and PMNs was observed in the pathogenesis of thrombosis. In various mouse models a key role of PMNs in the induction of thrombus formation was shown (Lisman 2018). Interestingly, human thrombi even contain NETs (Savchenko *et al.* 2014) and NET components are elevated in patients suffering from thrombosis (van Montfoort *et al.* 2013). This shows an important rationale between platelet and PMN activation in platelet-related complications *in vivo*.

It is also accepted that platelet-leukocyte interactions are not only important in platelet-related diseases, injury and immunity but also potentially cause autoinflammation. However, for inflammatory skin diseases, very little is known about PMN-platelet interactions. In a

mouse model of allergic contact dermatitis platelet-PMN aggregates are found adherent on vessel walls with PMNs which internalized platelets. The authors interpreted these findings by assuming that PMNs which initially attempted to clear platelet aggregates subsequently extravasated from blood vessels (Daito *et al.* 2014). Moreover, in a mouse model of atopic dermatitis platelet-leukocyte-complexes were increasingly found in blood, while in thrombocytopenic conditions, the chronic inflammation was reduced (Tamagawa-Mineoka *et al.* 2009). Importantly, in psoriasis, platelet aggregates were shown a long time ago (Berrettini *et al.* 1985) and aggregate formation is reduced after amelioration of skin inflammation. Nevertheless, the indicated contribution of platelet-PMN aggregates to the pathology of psoriasis awaits further confirmation in patient derived samples.

1.5. Psoriasis

1.5.1. General features of psoriasis

Psoriasis is an autoinflammatory disease of the skin with an incidence of up to 3% of adults in the Western population. Its major characteristics are epidermal hyperplasia due to increase in keratinocyte proliferation, angiogenesis accompanied with bleeding of the dermis and immune cell infiltration, mostly PMNs in the dermis (Griffiths and Barker 2007). The most common form of psoriasis (90% of cases), is the so-called *psoriasis vulgaris* or *plaque psoriasis* (histological picture of skin biopsy, see Figure 1.4). Another major characteristic of *plaque psoriasis* is the "Koebner phenomenon", where new lesions develop at sites of trauma or pressure e.g. on elbows, knees or the scalp (Griffiths and Barker 2007). In children, an acute form of psoriasis, called *psoriasis guttata*, can occur which is triggered by a streptococcal infection of the throat. Another, but quite rare form of psoriasis is the so-called *pustular psoriasis* which is characterized by small, sterile pustules on the skin (Griffiths and Barker 2007). The severity of psoriasis it scored by the Psoriasis-Area-Severity-Index (PASI) that includes parameters such as the affected area of the skin, the severity of inflammation and sometimes even the personal well-being of the patients. The PASI can reach values of up to 72 in very severe cases; a moderate to severe case is defined by a PASI higher than 10 (Fredriksson and Pettersson 1978) (Cabrera *et al.* 2015). Since 1971 the treatment of choice for most patients is the FDA (Food and Drug Administration)-approved systemic therapy with methotrexate, especially for patients that do not respond to topical treatments with steroids or phototherapy (Menter and Griffiths 2007). However, so called "biologicals", such as antibodies or small molecule inhibitors that block signaling of certain cytokines, e.g. TNF or IL-17, are currently tested in clinical studies or have already been approved as drugs for the treatment of psoriasis (Eberle *et al.* 2016).

Studies aimed to identify genetic predisposition for the development of psoriasis have shown limited success. Until now exclusively the presence of the HLA-Cw6 allele shows a good correlation with familial inherited early-onset psoriasis (Nair *et al.* 2006). Additionally, several

psoriasis-associated susceptibility loci (Tsoi *et al.* 2012) have been described. However, the main trigger for the development of psoriasis is still unknown.

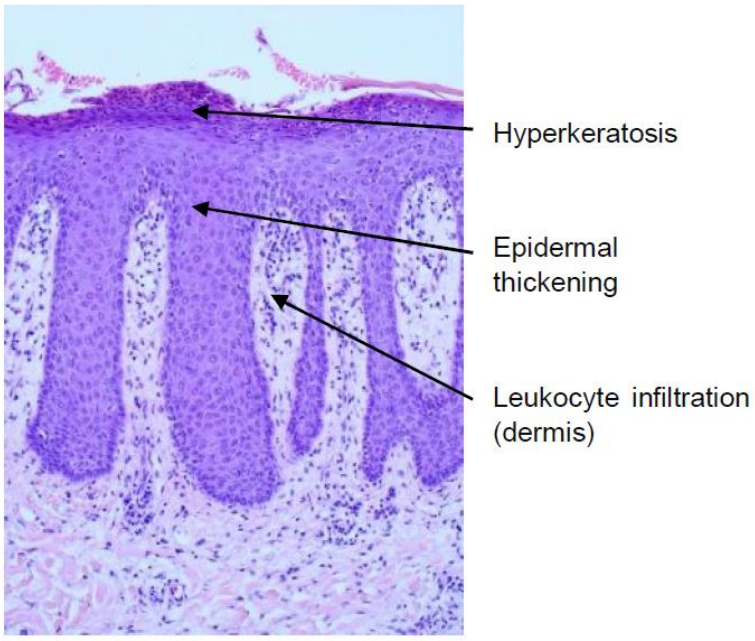


Figure 1.4: Skin biospy of lesional skin in psoriasis

Histological appearance of psoriatic lesions with hyperkeratosis, epidermal thickening and leukocyte infiltration of the dermis. (picture provided by Kamran Ghoreschi)

1.5.2. Innate immune responses (especially of PMNs) in psoriasis

Adaptive immune responses have been extensively studied in psoriasis. Based on these studies it is generally believed that psoriasis, certainly in its chronic state, is a T cell-driven disease. Thus, innate immune responses were largely neglected and are just beginning to gain interest in recent years.

Especially the work of Lande et al. initiated a shift in the paradigm of T-cell driven psoriasis by showing that complexes of self-derived DNA and the antimicrobial peptide LL37 which is overexpressed in psoriatic lesions, induce type I IFN release from pDCs via endosomal TLR signaling (Lande *et al.* 2007). In their work the authors explain the high type I IFN levels found in psoriatic lesions and how type I IFN in turn can activate other cells and induce T cell differentiation.

In addition to MAMPs, so-called Heat-shock-proteins (HSPs) and S100 proteins which have antibacterial properties, serve as endogenous ligands for TLRs. Finally, because HSPs and S100 proteins are frequently expressed in psoriatic skin, the binding of HSPs to TLRs in turn induces pro-inflammatory cytokine production in psoriasis (Tsan and Gao 2004) (Sweeney *et al.* 2011) (Holzinger *et al.* 2018). Importantly, keratinocytes can also initiate immune reactions of the skin and in psoriasis keratinocytes express higher levels of TLRs (TLR1 and TLR2) as compared to healthy skin. The enhanced expression of TLRs in psoriatic keratinocytes potentially also drives pro-inflammatory immune activation (Sweeney *et al.* 2011).

About the role of innate immune cells, different from DCs, only very little is known in psoriasis. Langerhans cells, i.e. dendritic cells that reside in the epidermis (Valladeau and Saeland 2005) are dysregulated in psoriasis (Sweeney *et al.* 2011). Macrophages, which are professional phagocytes, infiltrate psoriatic lesions and were proposed as the main producers of the pro-inflammatory cytokine TNF-α (Marble *et al.* 2007). The role of TNF-α in the pathogenesis of psoriasis is incompletely understood but TNF-α antagonists are used for the treatment of psoriasis and ameliorate the skin pathology (Yost and Gudjonsson 2009).

Although infiltration of PMNs into psoriatic lesions is a hallmark of psoriasis, the causative role of PMNs and their contribution to the pathogenesis of psoriasis is only poorly studied and therefore still incompletely defined (Schon *et al.* 2017). PMNs release various pro-inflammatory cytokines and chemokines which could drive proliferation and activation of keratinocytes but also attract further PMNs to the site of inflammation and activate them (Tecchio *et al.* 2014) (Terui *et al.* 2001). Furthermore, PMNs are the main producers of LL37 in the human body (Sorensen *et al.* 1997) which could explain excessive amounts of LL37 found in psoriatic skin. Interestingly, PMNs from psoriasis patients are more prone to activation and more sensitive to induction of NETosis in blood, also NETs are found in psoriatic lesions. The number of NETting neutrophils even correlates with disease severity (Hu *et al.* 2016). Additionally, signaling by TLRs expressed by PMNs (Prince *et al.* 2011) can induce very fast immune responses by release of pro-inflammatory cytokines or induction of NETosis.

The cytokine IL-17 is mainly produced by T helper 17 (Th17) cells and Th17 cells are considered as the major driver of inflammation in psoriasis (Di Cesare *et al.* 2009). An IL-17 specific antibody is approved for the treatment of psoriasis (Eberle *et al.* 2016) (Hueber *et al.* 2010). Due to the psoriasis associated role of IL-17 and the abundance of PMNs in psoriatic lesions it is speculated that PMNs are a potential source of IL-17 and could be targeted by the same treatment as T cells (Schon *et al.* 2017). This would link innate (PMNs) and adaptive (Th17 cells) immunity in psoriasis.

The central importance of PMNs in the patho-physiology of psoriasis is further highlighted by the fact that depletion of neutrophils in a psoriasis mouse model (Sumida *et al.* 2014) and in patients strongly ameliorates inflammation of the skin (Ikeda *et al.* 2013).

1.5.3. Adaptive immune responses in psoriasis

Research on pathogenic mechanisms in psoriasis generally is focused on T cells. Both, $CD4^+$ and $CD8^+$ T cells are found in the epidermis of psoriasis patients (Gaspari 2006). Also, T cells in psoriatic skin are mostly mature and activated memory T cells. However, the trigger for T cell homing to the skin has not yet been conclusively reported although it is known that LL37, which is overexpressed in psoriatic skin, acts as an MHC class II autoantigen that is recognized by T cells in psoriasis (Lande *et al.* 2014). There are also indications that molecular mimicry - a strong reaction to microbes involving immunodominant epitopes that cross-react with self-

antigens - can occur in psoriasis (Christen and von Herrath 2004). This mechanism could explain the onset of *psoriasis gutatta* (see above).

An important feature of memory T cells found in psoriatic skin is the expression of CLA (cutaneous lymphocyte associated antigen) and CCR10 (chemokine receptor type 10), both being almost exclusively detected in autoimmune diseases of the skin (Prinz 2003). Once they have infiltrated the skin, T-cells secrete cytokines (e.g. IFN-γ, TGF-β, IL-6 etc.) that induce proliferation of keratinocyte leading to the commonly observed skin thickening (Strange *et al.* 1993).

Intriguingly, it was only recently described that in psoriasis patients, polymorphisms are found in genes that are associated with Th17 immune signaling. Th17 cells are the major producers of IL-17 (Eberle *et al.* 2016) and IL-17A induces proliferation and impaired differentiation of keratinocytes. IL-17A induces weakening of the skin barrier and amplifies inflammation by promoting the release of pro-inflammatory cytokines and chemokines from keratinocytes. Additionally, IL-17A induces migration of leukocytes and in combination with TNF and IL-22, IL-17A upregulates the production of IL-1 family cytokines (Brembilla *et al.* 2018). Finally, genetically engineered mice that overexpress IL-17A in keratinocytes spontaneously develop psoriasis-like skin disease within a short period of time (Karbach *et al.* 2014). In line, anti-IL-17 antibodies significantly ameliorate skin pathology in psoriasis patients (Hueber *et al.* 2010) (Eberle *et al.* 2016). Hence, psoriasis is considered to be a Th17-IL-17 driven disease, although it is unclear how Th17 cells are triggered to produce IL-17 (Brembilla *et al.* 2018).

In contrast to numerous studies focusing on the role and function of T-cells in psoriasis, B-cells have only rarely been studied and, in line, autoantibodies have not been identified. Recently a correlation of B cell blood counts and severity of psoriasis was reported (Lu *et al.* 2016). The authors suggest that elevated B cell blood counts indicate enhanced activation of B cells in psoriasis patients. Also, the relative number of IL-10-producing regulatory B cells is decreased in psoriasis as compared to healthy individuals, indicating impaired B cell regulation in psoriasis patients (Hayashi *et al.* 2016).

1.5.4. Cardiovascular comorbidities in psoriasis

Psoriasis is often associated with additional autoinflammatory diseases like arthritis or even with cancer. In regard to cancer, however, it is not clear whether psoriasis or its treatment is the major cause (Griffiths and Barker 2007). The most noticeable comorbidity is the risk to develop cardiovascular diseases (CVDs) which was already described in 1973 (McDonald and Calabresi 1973). CVDs affect the heart or blood vessels and rising numbers of CVDs are an increasing problem worldwide. In 2016, the WHO (reference see Table 6.1 and 6.3.1.) published an alarming chart of leading causes of death, where the top 2 were ischemia heart disease and stroke, i.e. two severe CVDs. The treatment of CVDs, in order to prevent death, costs the US several hundred billion dollars a year (Jindal and Jindal 2018).

Besides the fact that CVDs are a general and worldwide problem of modern society, it was first suggested that psoriasis is an independent risk factor of CVD development by Gelfand et al. in 2006. The authors analyzed a general practitioner (GP) database from 1987 to 2002 and found an elevated risk of myocardial infarction for patients with severe psoriasis (Gelfand *et al.* 2006). Later, Ludwig et al. described psoriasis as an independent risk factor for CVDs (Ludwig *et al.* 2007) (Caiazzo *et al.* 2018). Mehta et al. postulated in 2011, that patients suffering from severe psoriasis not only have a higher risk to develop any CVD in general but that psoriasis is a risk factor experience major cardiac events (Mehta *et al.* 2011) (Boehncke 2018).

It has been suggested that hyperactive platelets are causing the enhanced risk of CVD in psoriasis. Psoriatic hyperactive platelets would cause excessive aggregation that in turn favors the development of CVDs. This proposed mechanism links cardiovascular disease and inflammatory skin disease psoriasis (Tamagawa-Mineoka 2015). The causal nature of psoriatic hyperactive platelets is in line with the observed increased amounts of platelet aggregates in blood samples from psoriasis patients. Furthermore, *in vitro* observed platelet aggregates were strongly reduced after improvement of the patients' skin condition (Berrettini *et al.* 1985). Only recently antagonistic antibodies directed towards IL-17A, a driver of inflammation in psoriasis, were reported to effectively ameliorate (Eberle *et al.* 2016) (Schon and Erpenbeck 2018) not only skin inflammation but also CVDs (Schuler *et al.* 2018). This shows that dysregulated platelet activation is strongly associated with psoriasis and likely causing CVDs of various kinds.

1.5.5. PMNs and platelets in psoriasis

Infiltration of PMNs in the skin is a central hallmark of psoriasis (Griffiths and Barker 2007). Besides, the impact of PMNs on the pathogenesis and inflammatory status in psoriasis is insufficiently understood. In recent years it became evident that PMNs might provide important inflammatory mediators such as ROS and cyto-or chemokines which induce the commonly observed hyperproliferation of keratinocytes and immunologic activation of endothelial and additional immune cells (Terui *et al.* 2001) (Tecchio *et al.* 2014). Furthermore, in autoinflammation, activation of immune cells can result from NET-provided self-antigens originating from PMNs (Lande *et al.* 2011). Interestingly, PMNs have been suggested as source of IL-17 which is a key mediator of psoriatic inflammation, and IL-17 release occurs via NETs (Lin *et al.* 2011). PMN-mediated release of NETs and IL-17 can therefore potentially link innate and adaptive immune responses in psoriasis.

Platelets are recognized as drivers of cardiovascular comorbidities in psoriasis. Also, the role of platelets as immune mediators/modulators is increasingly appreciated. Increased platelet mass index and mean platelet volume (Unal 2016) (Canpolat *et al.* 2010) as well as the identification of activated platelets in patient samples (Ludwig *et al.* 2004) corroborate their role in psoriasis. Finally, platelet activation reportedly correlates with diseases severity (Tamagawa-Mineoka *et al.* 2010) and platelets from psoriasis patients show a lower threshold

for activation and aggregate formation *in vitro* (Tamagawa-Mineoka 2015) which indicates a dysregulated platelet function in psoriasis patients. Interestingly, cardiovascular comorbidities are also linked to IL-17, as IL-17 is not only a major driver for psoriatic inflammation but also for cardiovascular diseases (Karbach *et al.* 2014) and by inhibiting IL-17 both inflammation and cardiovascular comorbidities can be ameliorated (Schuler *et al.* 2018).

Comparatively only scarce knowledge exists about platelet-PMN interplay in psoriasis and its impact on inflammation or cardiovascular comorbidities. Two studies from Turkey and Korea showed that neutrophil-to-lymphocyte and platelet-to lymphocyte ratios are elevated in psoriasis patients and correlate with disease severity (Polat *et al.* 2017) (Kim *et al.* 2016). However, an explanation of skin infiltration, platelets helping PMNs to extravasate and the impact of platelet-PMN aggregates on disease severity remains elusive.

1.6. Nucleic acid-LL37 complexes

LL37 belongs to the family of cathelicidins, i.e. peptides with antimicrobial activity. The active LL37 is a 37 amino acid long, amphipathic and positively charged peptide with two N-terminal leucine residues (Durr *et al.* 2006). The major producers of LL37 in the human system are PMNs which store LL37 in their specific granules (Sorensen *et al.* 1997). The active LL37 is generated from its precursor hCAP18 (human Cationic Antimicrobial Peptide 18 kDa) by proteolytic processing. The processing protease is amongst others the neutrophil-derived proteinase-3 (PR3). PR3 cleaves hCAP18 after the signal sequence and cathelin-domain to release the active C-terminal part LL37 (Sorensen *et al.* 2001). Thus, neutrophils provide both, the mature LL37 and the enzyme specifically processing the precursor. In healthy individuals LL37 is upregulated upon infection and displays efficient anti-microbial activities to most gram-positive and gram-negative bacteria but also to fungi (Turner *et al.* 1998).

Because of its positive charge, LL37 tends to form complexes with negatively charged molecules, such as DNA or RNA. DNA-LL37 complexes are delivered inside of cells by some form of endocytosis (Zhang *et al.* 2010) and endosomal TLR recognition of DNA or RNA induces robust immune activation (Lande *et al.* 2007) (Ganguly *et al.* 2009). Hence, LL37 especially protects fragile RNA from rapid degradation by RNases (Ganguly *et al.* 2009). Of note, complexes of human LL37 with nucleic acids are more potent in inducing immune activation compared to complexes of nucleic acids with the mouse ortholog CRAMP (cathelin related antimicrobial peptide) (Gallo *et al.* 1997) (Singh *et al.* 2013). Moreover, similar to LL37, β-defensins 2 and 3, which belong to a second class of antimicrobial peptides, naturally form complexes with DNA and fuel activation of immune cells (Tewary *et al.* 2013).

In addition to endosomal TLR binding, DNA or RNA in nucleic acid-LL37 complexes might also be recognized by other intracellular nucleic sensing receptors (e.g. RIG-I, or even inflammasomes (Loo and Gale 2011, Xiao 2015)) or LL37 itself can potentially bind to a receptor to induce immune stimulation. In keratinocytes, for example, IFN-β production is

induced by RNA-LL37 complexes that bind to cytosolic RNA receptors like RIG-I (Zhang *et al.* 2016). Confusingly, complexing of dsDNA by LL37 was reported to prevent stimulation of the dsDNA sensing AIM2 (absent in melanoma 2) inflammasome in keratinocytes, suggesting an anti-inflammatory effect of LL37 (Dombrowski *et al.* 2011).

Furthermore, LL37 was reported to act as a ligand for formyl-peptide receptor 2 (FPR2) (Zhang *et al.* 2009) which is also expressed on innate immune cells like e.g. PMNs. In line, the group of Richard Gallo postulated that LL37 enables self-RNA recognition via LL37-binding by scavenger receptors, inducing endocytosis of RNA and subsequent nucleic acid recognition of PRRs (Takahashi *et al.* 2018). These discrepant publications show that recognition and signaling of nucleic acid-LL37 complexes is incompletely understood and needs further investigation.

A major characteristic of psoriasis is the overexpression of LL37 in lesional skin. Complexes of nucleic acids with LL37 activate pDCs which induces strong IFN-α release (Lande *et al.* 2007) (Ganguly *et al.* 2009). These studies also report pDC infiltration of psoriatic lesions (Lande *et al.* 2007). In both studies, LL37 promoted uptake of DNA or RNA by cells and thus fueled recognition by endosomal TLRs, TLR7 for RNA and TLR9 for DNA in pDCs (Ganguly *et al.* 2009) (Lande *et al.* 2007). The authors claimed, that nucleic acid-LL37-mediated activation of pDCs might fuel an early inflammatory response in psoriasis which in turn can lead to T cell activation and chronification of the disease (the major findings are shown in Figure 1.5).

Furthermore, in autoinflammation of SLE, contents released by NETting neutrophils, i.e. DNA and LL37, induce robust activation not only of pDCs (Lande *et al.* 2011) but also of B cells and nucleic-acid-sensing of endosomal TLRs is indispensable for immune activation. These activated B-cells further produce autoantibodies against DNA and LL37 (Gestermann *et al.* 2018). However, autoantibodies have not been identified for psoriasis. Assumedly, a similar mechanism as seen for SLE applies to chronic inflammation in psoriasis. Intriguingly, LL37 itself also acts as an MHC class II autoantigen presented to T cells (in psoriasis) (Lande *et al.* 2014). This would connect innate immune responses (PMNs, pDCs) and adaptive immunity (T and B cells) providing a possible mechanism for fulminant and chronic inflammation in psoriasis.

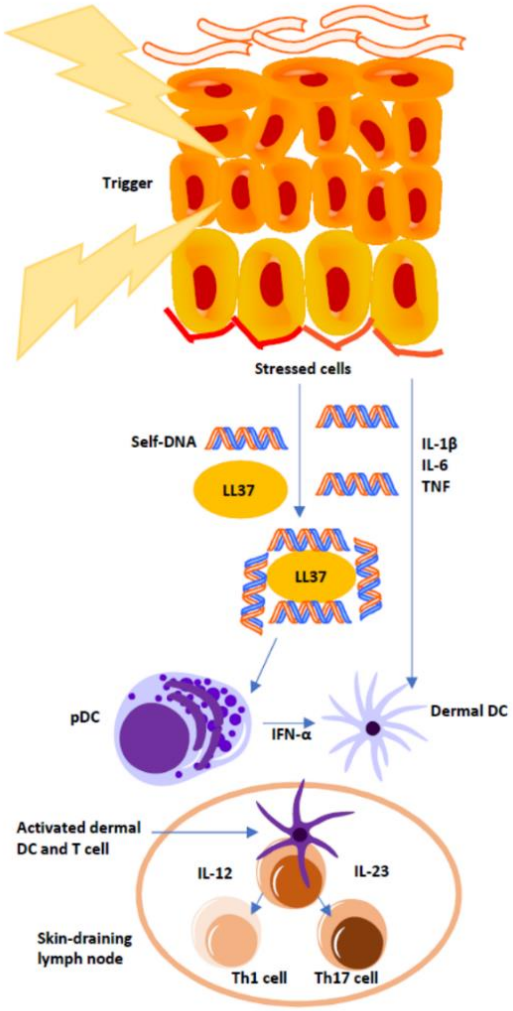


Figure 1.5: Complexes of self-DNA and LL37 induce activation of pDCs in psoriasis

Environmental triggers like stress or injury induce the formation of psoriatic lesions in individuals with a background of genetic susceptibility for psoriasis. In the beginning, stressed keratinocytes release self-DNA which form complexes with LL37, overexpressed in psoriatic skin. Plasmacytoid dendritic cells (pDCs) are activated to produce interferon-α (IFNα). Keratinocytes are also activated and release interleukin-1β (IL-1β), IL-6 and tumour necrosis factor (TNF). pDC-derived IFNα and the pro-inflammatory cytokines released from keratinocytes activates dermal DCs. These activated dermal DCs migrate to the skin-draining lymph nodes to present an antigen (of self or microbial origin) to naive T cells. This in turn promotes their differentiation into T helper 1 (Th1) and/or Th17 cells. (not shown: Th17 cells are activated and release IL-17 which leads to keratinocyte hyperproliferation and weakening of the skin barrier. Also, the production of pro-inflammatory cytokines is induced in other immune cells, leading to more inflammation and further immune infiltration of the skin.) Adapted from Nestle et al. (Nestle *et al.* 2009).

1.7. Hypothesis and aims of part I

Plasmacytoid dendritic cells (pDCs) and their ability to respond to complexes of nucleic-acids and the antimicrobial peptide LL37 were intensely studied. The group of Michel Gilliet could show that pDCs induced high levels of type I IFN when stimulated with DNA or RNA complexed to LL37 (Lande *et al.* 2007) (Ganguly *et al.* 2009) in the context of psoriasis. Furthermore, they showed that NET contents were immunostimulatory for pDCs, suggesting that nucleic acid-LL37 complexes are found in NETs (Lande *et al.* 2011).

However, PMN skin infiltration is a hallmark of psoriasis. PMNs not only outnumber pDCs in human blood and skin of psoriasis patients, they are furthermore the first cells recruited to the sites of inflammation and produce considerable amounts of pro-inflammatory cytokines like IL-8 or TNF. Therefore, it was assumed that PMNs play an important role in immune responses in psoriasis. Most importantly, it is still uncertain which cells might provide LL37 or nucleic acids, as both cannot be readily released by pDCs.

It was hypothesized that PMNs, which are major producers of LL37 in the human body and can also easily extrude nucleic acids by a term called NETosis, can provide the first triggers for inflammation in psoriasis.

The aim of this study was to investigate whether PMNs are able to induce cytokine release or NETosis in response to nucleic acid and LL37 complexes and if those NETs contain further nucleic acids and LL37. If this is the case, those *de novo* generated nucleic acid-LL37 complexes could potentially induce further immune reactions in PMNs which might fuel a self-propagating inflammatory loop possibly explaining early inflammatory events in psoriasis (see Figure 1.6).

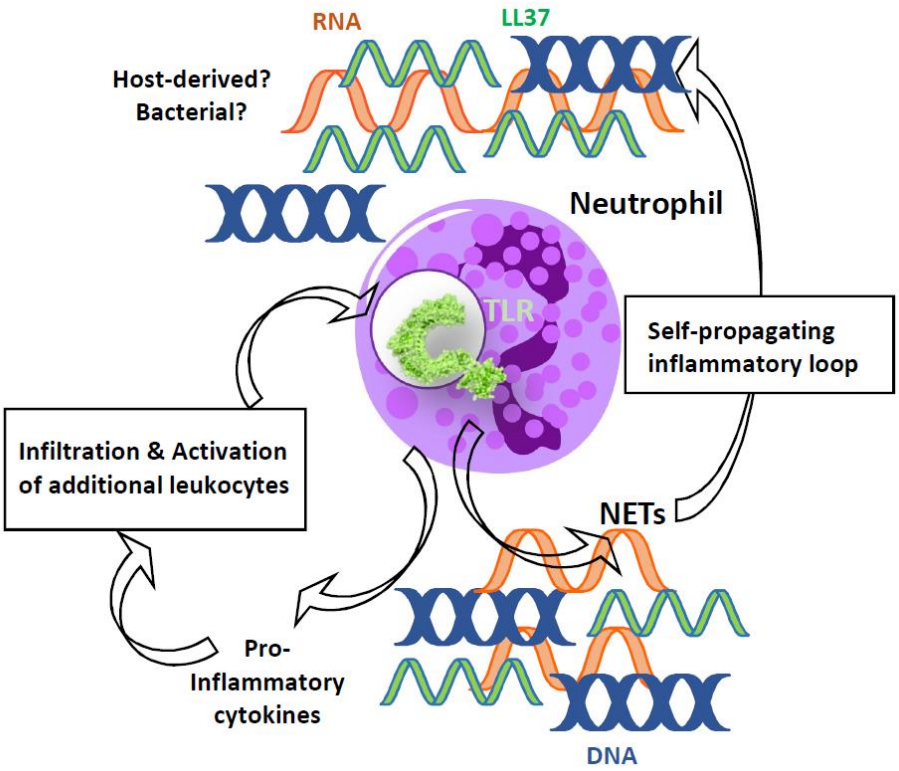


Figure 1.6: PMN activation might fuel a self-amplifying inflammatory loop in psoriasis

LL37 forms complexes and shuttles nucleic acids (DNA and RNA) into endosomal compartments of PMNs. Endosomal TLRs recognize these nucleic acids and induce cytokine- and NET release from PMNs. These NETs contain further nucleic acids and LL37 which in turn activate more PMNs via further endosomal TLR signaling. Pro-inflammatory cytokines released by PMNs upon stimulation with nucleic acid-LL37 complexes induce the infiltration and activation of additional leukocytes. This mechanism might explain an early inflammatory event of TLR-dependent activation of PMNs in psoriasis.

1.8. Hypothesis and aims of part II

Psoriasis is an autoinflammatory skin disease, where a variety of immune cells from the blood infiltrate the skin lesions. The trigger for immune cells to infiltrate the skin in psoriasis remains elusive. To identify surface antigens which are differentially expressed on immune cells of psoriasis patients and healthy controls, a surface antigen screen was performed. The major goal was to better understand skin homing of immune cells, especially PMNs, and to give a glimpse on the potential mechanism underlying skin infiltration in psoriasis (see Figure 1.7).

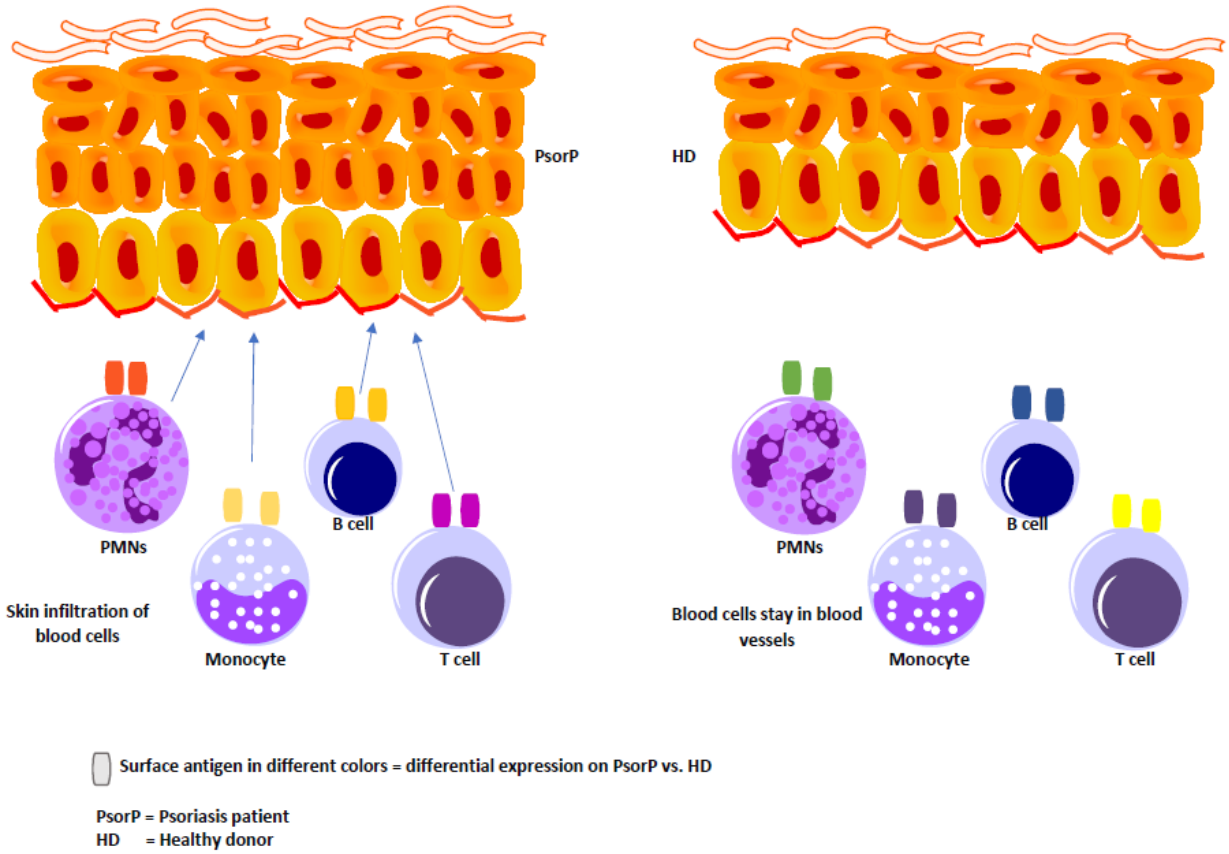


Figure 1.7: Differential expression of surface antigens might explain skin homing of blood cells in psoriasis

Blood leukocytes of psoriasis patients differentially express surface antigens compared to healthy controls. This might explain the tendency of immune cells to infiltrate psoriatic lesions.

2. Materials and Methods

2.1. Materials

2.2.1. Reagents and Chemicals

Table 2.1: Reagents and Chemicals

Reagent/Chemical	Company	Product no.
RPMI	Sigma-Aldrich	R8758
VLE-RPMI	Merck-Biochrom	FG1415
FCS	TH-Geyer	11682258
Pen./Strep.	Gibco	15140122
L-Glutamine	Gibco	25030081
Sodium pyruvate	Gibco	11360070
HEPES	Sigma-Aldrich	H0887
PBS	Thermo Fisher	14190-169
RNase/DNase free water	Thermo Fisher	10977049
Ampuwa	Fresenius Kabi	1833
Ficoll/Biocoll	Biochrom	ab211650
β- estradiol	Sigma-Aldrich	E2758
Ammonium chloride (NH_4Cl)	Roth	5470.1
Potassium bicarbonate ($KHCO_3$)	Fluka	60220
EDTA pH=8	ThermoFisher	15575020
Trisodium citrate dihydrate ($C_6H_5Na_3O_7 * 2\ H_2O$)	Carl Roth	4088.1
Citric acid ($C_6H_8O_7$)	Carl Roth	X863.2
Fixation buffer	BioLegend	420801
Cell staining buffer	BioLegend	420201
Precision Count beads™	BioLegend	424902
Pooled human serum	Transfusion medicine Tübingen	-
Saponin	Applichem,	A4518.0100
ProLong Diamond Antifade	ThermoFisher	P36965
RNase A	ThermoFisher	EN0531
Roti Histol	Carl Roth	6640.1
Immersion oil	OLYMPUS	81012

2.1.2. TLR ligands and inhibitors

Table 2.2: TLR ligands and inhibitors

Component	company	Product no.
LL37	InvivoGen	tlrl-l37
TL8-506	InvivoGen	tlrl-tl8506
LPS-EK (ultrapure)	InvivoGen	tlrl-peklps
R848 (Resiquimod)	InvivoGen	tlrl-r848-5
Chloroquine	InvivoGen	tlrl-chq
PMA	InvivoGen	tlrl-pma
HK S. pyogenes	Tatjana Eigenbrod	-
HK E.coli	Tatjana Eigenbrod	-

2.1.3. RNA/DNA and inhibitors

Table 2.3: Synthetic RNA/DNA and inhibitors

Component	Sequence	company
CpG2006	5'TsCsGsTsCsGsTsTsTsTsGsTsCsGsTsTsTsTsGsTsCsGsTsT3'	TIB MOLBIOL
ssRNA40 (unconjugated, conjugated with AF647 or AF488) *	5'GsCsCsCsGsUsCsUsGsUsUsGsUsGsUsGsAsCsUsC3'	IBA Lifescience
ssDNA60	5'AC$(AC)_{28}$AC3'	TIB MOLBIOL
IRS661	5'TsGsCsTsTsGsCsAsAsGsCsTsTsGsCsAsAsGsCsA3'	TIB MOLBIOL
IRS954	5'TsGsCsTsCsCsTsGsGsAsGsGsGsGsTsTsGsT3'	TIB MOLBIOL
* phosphorothioate backbone.		

2.1.4. Antibodies and recombinant proteins

Table 2.4: Antibodies and recombinant proteins

Item	fluorophore	species	isotype	dilution	company	Product no.
Isotype control	PE	mouse	IgG1 κ	1:100	eBioscience	12471442
Isotype control	FITC	mouse	IgM κ	1:80	BioLegend	401605
Isotype control	APC	mouse	IgG1 κ	1:100	BD Bioscience	550854
Isotype control	BV421	mouse	IgG1 κ	1:100	BioLegend	400157
Isotype control	AF488	mouse	IgG2a κ	1:200	BioLegend	400233
Isotype control	FITC	mouse	IgG2b κ	1:200	BioLegend	401206
Isotype control	AF488	mouse	IgG1 κ	1:100	BioLegend	400134
Isotype control	AF647	mouse	IgM κ	1:100	BioLegend	401618
Isotype control	PE-Cy7	mouse	IgG1 κ	1:500	BioLegend	400126
Anti-hCD15	PE	mouse	IgG1 κ	1:100	BioLegend	323006
Anti-hCD66b	FITC	mouse	IgG1 κ	1:80	BioLegend	305103
Anti-hCD62L	BV421	mouse	IgG1 κ	1:100	BioLegend	30482
Anti-hCD14	PE	mouse	IgG1 κ	1:100	ImmunoTools	21620144
Anti-hCD3	AF488	mouse	IgG2a κ	1:200	BioLegend	317310
Anti-hCD4	PE	mouse	IgG1 κ	1:200	BioLegend	300508
Anti-hCD11b	APC	mouse	IgG1 κ	1:100	BioLegend	301310
Anti-hCD19	BV421	mouse	IgG1 κ	1:500	BioLegend	302234
Anti-hCD8	APC	mouse	IgG1 κ	1:200	ImmunoTools	21810086
Anti-hHLA-DR	FITC	mouse	IgG2b κ	1:200	BioLegend	327006
Anti-hCD15	PE-Cy7	mouse	IgG1 κ	1:500	BioLegend	323030
Zombie yellow fixable dye	-	-	-	1:200	BioLegend	423103
Anti-hCD41	PE	mouse	IgG1 κ	1:60-200	BioLegend	303706
Anti-hCD61	PE	mouse	IgG1 κ	1:60-200	BioLegend	336406
Anti-hCD66b	AF647	mouse	IgM κ	1:100	BioLegend	305109
Anti-hCD62P	AF488	mouse	IgG1 κ	1:100	BioLegend	304916
anti-mLy6C	VioBlue	rat	IgG2a κ	1:100	Miltenyi	130-102-929
Anti-mCD45	APC-Vio770	rat	IgG2b κ	1:100	Miltenyi	130-118-687
Anti-mCD41	FITC	rat	IgG1 κ	1:100	Miltenyi	130-105-929
Anti-mLy6G	PE	rat	IgG1 κ	1:100	Miltenyi	130-102-895
Anti-mCD11b	APC	rat	IgG2b κ	1:100	Miltenyi	130-113-793

Propidium iodide	n/a	-	-	1:100	Miltenyi	130-093-233
TruStain fcX	-	-	-	1:25	BioLegend	422302
Recombinant hMIP-1β	-	-	-	1:3.3x10^6 or 1:0.6x10^6	ImmunoTools	11343223
Recombinant hIL-16	-	-	-	1:3.3x10^5 or 1:0.6x10^5	ImmunoTools	11340163
Recombinant hSDF-1α	-	-	-	1:1000	ImmunoTools	11343363
Recombinant hIL-3	-	-	-	1:1000	Peprotech	200-03
Recombinant hM-CSF	-	-	-	1:1000	Peprotech	300-25
SYTO RNAselect	n/a	-	-	1:10 000	ThermoFisher	S32703
Hoechst33342	n/a	-	-	1:10 000	Sigma-Aldrich	B2261
Anti-hLL37	unconjugated	rabbit	IgG	1:80-500	LSBio	LS-B6696-500
Anti-ψU	unconjugated	mouse	IgG1	1:200-500	MBL	MBL-D347-3
Anti-hNeutrophil Elastase (NE)	unconjugated	mouse	IgG1	1:100-500	Novus Biologicals	MAB91671-100
Anti-hCD41	unconjugated	rabbit	IgG	1:100	Abcam	ab63983
Anti-hCD42b	unconjugated	goat	IgG	1:100	Santa Cruz	sc-7070
Anti-mCD41	unconjugated	rat	IgG1	1:100	GeneTex	GTX-76011
Anti-h/mMPO	unconjugated	goat	IgG	1:200	R&D systems	AF3667
Anti-mCD42b	unconjugated	rat	IgG	4 µg/g or 2 µg/g	Emfret Analytics	R300
Isotype control	unconjugated	rat	IgG	4 µg/g or 2 µg/g	Emfret Analytics	R301
Anti-rabbit IgG	AF647	chicken	IgY	1:500	ThermoFisher	A-21443
Anti-mouse IgG	AF594	chicken	IgY	1:500	ThermoFisher	A-21201
Anti-mouse IgG	AF488	chicken	IgY	1:500	ThermoFisher	A-21200
Anti-mouse IgG	AF647	chicken	IgY	1:500	ThermoFisher	A-21463
Anti-rat IgG	AF488	chicken	IgY	1:500	ThermoFisher	A-21470
Anti-goat IgG	AF488	chicken	IgY	1:500	ThermoFisher	A-21467
Anti-goat IgG	AF594	chicken	IgY	1:500	ThermoFisher	A-21468

2.1.5. Special equipment and Softwares

Table 2.5: Special equipment and software

Plates/equipment	Company	Product no./Version
QIAcube	QIAGEN	9002840
Nikon Ti2 eclipse bright-field fluorescence microscope	Nikon	-
CO_2-O_2 controller	Okolab	-
Revolve	ECHO	FJSD2001
MACSQuant VYB	Miltenyi	130-096-116
FACS Canto II	BD Bioscience	-
ImageStream X MKII	Merck Millipore	-
Polycarbonate inserts, 24 well plates, 3 µm pores (for transwell experiments)	Corning	734-1570
½ Area flat bottom ELISA plates	Greiner	675061
U-bottom 96 well plates	Greiner	650101
V-bottom 96 well plates	Greiner	651101
µ-insert 4 well dish	Ibidi	80406
Poly-L-Lysine coated coverslips	Corning	734-1005
SuperFrost® Plus microscopy slides	VWR	631-9483
heparinized capillary tubes	ThermoFisher	22-362566
6 mm Acu punch	Acuderm	P650
Prism	GraphPad	V6-8
Excel	Windows Office	V2010 and V2019
FlowJo	FlowJo LLC	V10
FACSDiva	BD	V6
Fiji/ImageJ	-	win64
NIS Elements	Nikon	II
FCAP Array	BD Bioscience	V3
INSPIRE instrument controller software	Amnis	V2
IDEAS	Amnis	V4

2.1.6. Kits

Table 2.6: Kits used in this study

Kit	Company	Product no.
RNeasy Mini Kit	QIAGEN	74104
QIAamp DNA Blood Mini Kit	QIAGEN	51106
Human IL-8 ELISA MAX Deluxe Kit	BioLegend	431505
Human TNF-α ELISA MAX Deluxe Kit	BioLegend	430205
Human MIP-1β DuoSet ELISA Kit	R&D systems	DY271-05
Human IL-16 ELISA DuoSet ELISA Kit	R&D systems	DY316
Human LL37 ELISA Kit	HycultBiotec	HK321-02
Mouse TNF-α ELISA MAX Deluxe Kit	BioLegend	430902
Cytometric bead array Human Inflammatory Cytokine Kit	BD Bioscience	551811
Mouse Neutrophil Isolation Kit	Miltenyi Biotec	130-097-658
MACSxpress Whole Blood Neutrophil Isolation Kit	Miltenyi Biotec	130-104-434
NETosis Assay Kit	Cayman Chemical	601010
LEGENDScreen™Human PE Kit	BioLegend	700001

2.1.7. Buffers and media

Table 2.7: Buffers and media

Buffer/Media	Name
1.54 M NH_4Cl 100 mM $KHCO_3$ 1 mM EDTA; pH=8 dissolved in Ampuwa water pH adjusted to 7.3, sterile filtered (0.22 µm)	Erythrocyte lysis buffer (10x)
PBS 2% FCS (heat inactivated) 1 mM EDTA, pH=8	FACS buffer
PBS 0.05% Saponin	Permeabilization buffer
0.1 M $C_6H_5Na_3O_7 * 2\ H_2O$ pH adjusted to 6.0 with 0.1 M $C_6H_8O_7$	Citrate buffer
RPMI 10% FCS, heat inactivated; sterile filtered (0.22 µm)	Neutrophil culture medium (human and mouse)
RPMI 10% FCS 1% Pen./Strep. 1% L-Glutamine	PBMC culture medium
VLE-RPMI 10% FCS (heat inactivated) 1% Pen./Strep. 1% Sodium pyruvate 1% HEPES	BLaER1 culture medium
VLE-RPMI 10% FCS (heat inactivated) 1% Pen./Strep. 1% Sodium pyruvate 1% HEPES 10 ng/ml hIL3 10 ng/ml MCSF 150 nM β-estradiol	BLaER1 transdifferentation medium

2.2. Cell biology methods

2.2.1. Complex formation

For stimulation of human cells, 5.8 µM ssRNA40 (final concentration 34.4. µg/ml, sequence Table 2.3, AF647 or AF488 labeled where indicated) was mixed with 10 µg LL37 (final concentration 20 µg/ml, sequence Table 2.3, Atto-488 where indicated) and left at RT for 1 hour.

Where indicated, 1 µM ssDNA (final concentration 20 µg/ml, sequence see Table 2.3), genomic DNA (final concentration 20 µg/ml, isolated from whole blood using a QIAamp DNA Blood Mini Kit), total human mRNA (final concentration 20 µg/ml, isolated from HEK293T cells using the RNeasy kit on a QIAcube) or bacterial RNA (final concentration 10 µg/ml, from *S. aureus USA300 JE2* was isolated as described below) was also mixed together with 10 µg LL37 and left at RT for 1 hour.

For experiments with BM-PMNs, 5 µg bacterial RNA was complexed with 10 µg LL37 and used for four mice (final concentration: 10 µg/ml bacterial RNA and 20 µg/ml LL37). For the RNA-only or LL37-only conditions, the same amounts and volumes as for the complexes were used by adding sterile, endotoxin-free H_2O to the same final volume.

2.2.2. Isolation of bacterial RNA

Bacterial RNA isolation was performed by Natalya Korn from AG Wolz, Tübingen. For all experiments, RNA from *S. aureus USA300 JE2* was used. On the first day, the bacterial culture was inoculated in suitable medium (using antibiotics if needed). The next day, the culture was diluted (1:100) and the OD was measured with 600 nm absorbance. Then the culture was further diluted until OD=0.05, incubated while shaking at 37°C until reaching an OD of 0.5.

5 ml of the culture was harvested and spun down at 5 000 x g for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 1 ml Trizol (Invitrogen, 15596026) on ice and pipetted into freezing cups, containing zirconia silicon globules (Roth, N035.1). Then, the suspension was put on a shaker for 20 sec. at 6 500 rpm before freezing at –80°C.

The next day RNA isolation was performed. The samples were thawn at RT, 200 µl chloroform was added and mixed for 30-60 sec. After 3 min of incubation at RT, the samples were centrifuged at 12 000 x g for 15 min at 4°C. Thereafter, 500 µl isopropanol was pipetted into 1.5 ml RNase free Eppendorf tubes and the supernatant of the afore centrifuged samples (approximately 600 µl liquid phase) was added to the isopropanol. After mixing, and 10 min incubation at RT, the suspension was centrifuged at 12 000 x g for 30 min at 4°C. The supernatant was then removed by pipetting, using filter tips and the pellet was washed with 500 µl of 70% ethanol and centrifuged again at 7 500 x g for 5 min at 4°C. The supernatant was removed with filter tips, leaving the pellet to dry. In the end, 50 µl of sodium citrate (1 mM, pH=6.4 Ambion, AM7000) was added, incubated for 10 min at 55°C on a heating block (the

samples were thoroughly vortexed every 3-4 min) and frozen at -80°C. The RNA concentration was determined with a nanodrop (ng/µl) before using for experiments.

2.2.3. Study participants and sample acquisition

All blood and skin donors (psoriasis patients and healthy donors) provided their written informed consent before participation in the study. Approval for use of their biomaterials was obtained by the local ethics committees at the University Hospitals of Tübingen and Heidelberg, in agreement with the principles written down in the Declaration of Helsinki as well as respective laws and regulations. All blood or skin samples obtained from psoriasis patients, had a PASI score of ≥10 (except for 2 donors in Figure 4.3 and Figure 6.8, where the PASI score was ≥4.8), a median age of 41.8 years and no systemic therapy at the time of blood or skin sampling. The samples were obtained at the University Hospital Tübingen or in Heidelberg (both Department of Dermatology) and processed together with samples from at least one healthy donor (age- and sex-matched) which were recruited at the University of Tübingen, Department of Immunology. Skin sections were assessed from 12 patients with *psoriasis vulgaris* and 1 patient with *psoriasis guttata*. Platelet counts were determined in the course of clinical routines at the time of study blood sampling.

2.2.4. Isolation of bone-marrow derived PMNs (BM-PMNs)

Unc93b1$^{3d/3d}$ (Tabeta et al. 2006) or *Tlr13*$^{-/-}$ (Li and Chen 2012), kindly provided by Tatjana Eigenbrod from Heidelberg (both C57BL/6 background) and WT C57BL/6 mice (own breeding) between 8 and 20 weeks of age were used for this study. The mice were maintained following local institutional guidelines for animal experiments and hygiene monitoring. They were sacrificed using CO_2, followed by cervical dislocation.

Bone-marrow-derived (BM)-PMNs were isolated by magnetic separation (MACS separation) following the manufacturer's instructions (see Table 2.6). For (BM)-PMNs isolation, bone marrow of femur and tibia from all four legs was used to obtain a higher cell number. The cells were resuspended in neutrophil culture medium (see Table 2.7) and seeded by using 3 x 10^6 cells/ml (in 24 well plate, 250 µl per well and 96 well plate, 125 µl per well, the same concentration of cells for all experiments). After resting for 30 min, PMN stimulation was performed for 5 hours at 37 °C and 5% CO_2. Thereafter, supernatants were harvested and used for ELISA. For microscopy, the cells were seeded on Poly-L-Lysine coated coverslips (see Table 2.5, in a 24-well plate) stimulated for 16 hours and stained subsequently.

2.2.5. Human PMN isolation and stimulation

Whole blood (EDTA-anticoagulated) from healthy donors or psoriasis patients was diluted in PBS (1:2) and carefully loaded on 20 ml Ficoll (1.077 g/ml, using a 50 ml falcon). Then, density separation centrifugation was performed for 25 min at 509x g and 21°C (without brake). Thereafter, all layers were discarded (if the PBMCs were not used from the same donor) except for the erythrocyte-granulocyte pellet. Then, erythrocyte lysis (using 1x erythrocyte

lysis buffer, see Table 2.7) was performed for 20 min at 4°C on a roller shaker (the falcon tube was filled until 50 ml with erythrocyte lysis buffer). After centrifugation for 10 min (without brake), another erythrocyte lysis step was performed for 10 min at 4°C (the falcon tube was filled until 25 ml). After another 5 min of centrifugation (without brake), the remaining cell pellet was carefully resuspended in neutrophil culture medium (see Table 2.7). 1.6 x 10^6 cells/ml were seeded (24 well plate, 500 µl per well or 96 well plate, 125 µl per well). After resting for 30 min at 37°C, 5% CO_2, the cells were pre-treated with inhibitors (where indicated) for 30 min and subsequently stimulated with the indicated agonists for 4 hours (for ELISA) or for 30 min to 3 hours (for FACS analysis or microscopy).

2.2.6. Generation of NET contents

PMNs from healthy donors were isolated as described above. They were seeded in 10 cm dishes at a cell density of 5 x 10^6 cells/ml at 37°C and 5% CO_2. After resting for 30 min, the cells were stimulated with RNA-LL37 complex, LL37 alone, PMA (600 nM) or left unstimulated for 4 hours. Supernatants were removed carefully and the adherent cells/NETs were carefully washed three time with PBS. Then the cells and NETs were scraped off the bottom of the dish and frozen in neutrophil culture medium at -80°C.

2.2.7. Human PBMC isolation

Whole blood (EDTA anticoagulant) was diluted in PBS. After density gradient separation using Ficoll (described above), the PBMC layer was carefully transferred into a new reaction tube and diluted in PBS (1:1). The cell suspension was spun down at 645 x g for 8 min. Then, erythrocyte lysis was performed using 1x erythrocyte lysis buffer (see Table 2.7) for 5 min at RT. The cells were then washed twice more in PBS and spun down with decreasing speed (448 x g and 241 x g) for 8 min each. Then the cells were resuspended in PBMC culture medium (see Table 2.7) and seeded at cell density 1.6 x 10^6 cells/ml.

2.2.8. BLaER1 cells culture, transdifferentiation and stimulation

BLaER1 cells (WT and TLR8$^{-/-}$, kindly provided by Tim Vierbuchen and Holger Heine from Borstel, Germany (Vierbuchen *et al.* 2017)) were cultured in BLaER1 culture medium (see Table 2.7) at 37°C and 5% CO_2. After reaching a cell concentration not higher than 2 x 10^6 cells/ml, the cells were seeded in a 6 well plates (0.5 x 10^6 cells/ml, 2 ml per well) and transdifferentiated in BLaER1 transdifferentiation medium (see Table 2.7) for 6 days including 2 medium changes (on day 2 and 5). The cells become adherent and show a “star-shaped” morphology. On day 7 the adherent cells were detached by pipetting and re-seeded (96 well plate, 4 x 10^5 cells/ml, 125 µl per well) using BLaER1 culture medium (see Table 2.7) and left to rest for 1 hour. Subsequently, they were stimulated with TLR ligands for 18 hours and the supernatants were harvested and collected for ELISA measurements. The transdifferentiation efficiency was verified by FACS analysis, using CD19, CD14 and CD11b as cell surface markers as previously described (CD14$^+$CD11b$^+$ and CD19$^-$ cells are considered to be “monocyte-

/macrophage-like" cells; when following this protocol a purity of ≥ 95% can be reached) (Gaidt *et al.* 2018).

2.2.9. Live cell imaging of human PMNs

Human neutrophils were isolated by magnetic separation using MACSxpress whole blood neutrophil isolation kit (see Table 2.6) and were seeded into a µ-insert 4 well dish (7 µl cell suspension per insert). After resting for 20 min at 37°C, 5% CO_2, 1 µl Hoechst33342 (final concentration 1 µg/ml) and 1 µl SYTO RNAselect Green fluorescent dye (final concentration 50 µM) were added and the cells were incubated for another 20 min at 37°C and 5% CO_2. Live cell imaging was performed using a Nikon Ti2 eclipse microscope (40x magnification, no immersion oil) including a CO_2-O_2 controller. After adjustment of microscope and live cell imaging chamber, the cells were stimulated with TLR ligands (1 µl per well, using 34.4 µg/ml ssRNA and 20 µg/ml LL37 for complex formation and 600 nM PMA as control) and measurements were started immediately after adding of stimuli. Time-lapse analysis was performed by taking pictures every 3 min for at least 2 hours. Image analysis was performed using NIS Elements and Fiji analysis software.

2.3. Immunochemical methods

2.3.1. Flow cytometry of PMNs (purity and pre-activation assessment)

After PMN isolation and stimulation, the purity and activation status of PMNs was determined by flow cytometry. 200 µl of the cell suspension was transferred into a 96 well plate (U-shape) and spun down for 5 min at 448 x g and 4°C (all centrifugation steps were performed using the same speed and time and are referred to as "washing"). Blocking of Fc-receptors (FcR) was performed using 50 µl pooled human serum diluted 1:10 in FACS buffer (see Table 2.7) for 15 min at 4°C. After washing, the samples were stained with 50 µl antibody, diluted in FACS buffer (antibodies and respective dilutions see Table 2.4) for 30 min at 4°C in the dark. After further washing, fixation buffer (see Table 2.1) was added to the cell pellets for 10 min at RT in the dark. After washing, the cells were permeabilized with 0.05% Saponin diluted in PBS (see Table 2.7) for 15 min at RT in the dark. After an additional washing step, the cell pellets were resuspended in 150 µl FACS buffer. Measurements were performed on a FACS Canto II and analysis was performed using FlowJoV10. $CD15^+CD66b^+$ and $CD14^-$ cells were considered as PMNs. Using the aforementioned isolation method, a purity of ≥ 95% can be reached. Pre-activation was measured by staining of CD62L. When activated, the cells lost CD62L expression on their surface.

2.3.2. FACS analysis and fluorescence microscopy of fixed PMNs

The cells were isolated as previously described (see above) and seeded in a 96 well plate at 1.6 x 10^6 cells/ml, 125 µl per well. Subsequently they were stimulated with RNA-LL37 complexes for 30 min and 1 hour using RNA-AF647/AF488 and/or unlabeled LL37 or LL37-

Atto488 (where indicated, if the cells were not stimulated, this step was not performed). FcR block, staining, fixation and permeabilization were performed as for Flow cytometry (see above). Nuclei were stained with Hoechst33342 (final concentration 1 µg/ml) for 5 min at RT in the dark. After a final washing step, the cell pellets were resuspended in 50-100 µl FACS buffer. 40 µl of the cell suspension was pipetted on a Poly-L-Lysine coated coverslip and the cells were left to attach for 1 hour in the dark. ProLong Diamond Antifade (see Table 2.1) was used to mount the coverslips on uncoated microscopy slides. The slides were left to dry overnight at RT in the dark and stored at 4°C prior to analysis. The measurements were conducted with a Nikon Ti2 eclipse (100x magnification, using immersion oil) and the analysis was performed using Fiji analysis software.

2.3.3. Fluorescence microscopy of fixed NETing neutrophils (human and mouse)

For NET analysis, PMNs (1.6 x 10^6 cells/ml for human cells and 3 x 10^6 cells/ml for mouse cells) were seeded in 24 well plates, containing Poly-L-Lysine coated coverslips and stimulated with RNA-LL37 complexes (for mouse cells bacterial RNA-LL37 complexes were used) or PMA (600 nM) for 3 hours. NETs were fixed and stained using the protocol from Brinkmann et al.(Brinkmann et al. 2010). Where indicated, 100 µg/ml RNase A (DNase, protease-free, see Table 2.1) was added after fixation and incubated overnight (approximately 12 hours) at 37°C. Blocking was performed using pooled human serum (1:10 in PBS) for 15 min at RT. After three washes with PBS (5 min each), LL37 and PMNs were visualized using an unconjugated rabbit anti-LL37 antibody or an unconjugated mouse anti-Neutrophil elastase (NE) antibody (for 1 hour at RT) (see Table 2.4) with subsequent staining with an AF647-conjugated anti-rabbit or an AF594-conjugated anti-mouse secondary antibody, for 30 min at RT in the dark respectively (see Table 2.4). After washing, RNA was stained using SYTO RNAselect Green fluorescent dye (see Table 2.4, final concentration 50 µM) for 20 min or an unconjugated anti-ΨU antibody (see Table 2.4) for 1 hour at RT in the dark and subsequently adding an AF594-conjugated anti-mouse secondary antibody for 30 min at RT also in the dark. In the end, nuclear DNA was stained using Hoechst33342 (see Table 2.4, final concentration 1 µg/ml) for 5 min at RT in the dark. For mouse cells, only SYTO RNAselect Green fluorescent dye and Hoechst33342 were used to visualize RNA and DNA.

The coverslips were finally mounted with ProLong Diamond Antifade mounting solution (see Table 2.1), were left to dry overnight at RT in the dark and were then stored at 4°C before analysis. Secondary antibodies alone did not yield any significant staining. The measurements were conducted with a Nikon Ti2 eclipse (100x magnification, using immersion oil) and the analysis was performed using Fiji analysis software.

2.3.4. Transwell experiments

Transwell inserts were loaded with 100 µl of PBMC suspension (0.8 x 10^6 cells/insert). In the lower chamber 500 µl media containing TLR stimuli (2 µg/ml R848, RNA, LL37, RNA-LL37 complex, see Table 2.2) or 500 µl media containing only MIP-1β (30 and 150 pg/ml), IL-16 (300

and 1500 pg/ml) or SDF-1α (positive control, 100 ng/ml) were added. After 4 hours, the cells in the lower compartment were harvested and FACS staining was performed as described above. The total number of migrated cells was acquired using counting beads (see Table 2.1) on a FACS Canto II. Analysis was performed using FlowJo analysis software.

2.3.5. ImageStream analysis

ImageStream analysis was used to analyze internalization of RNA-LL37 complexes using spot-counts and tracking single cells. The cells were first seeded in a 96 well plate at a cell density of 8 x 10^6 cells/ml, 125 µl neutrophil culture medium per well. Subsequently, they were stimulated for 1 hour with RNA-AF647 (see Table 2.3) and/or LL37-Atto488 (kindly provided by Hubert Kalbacher, University of Tübingen). FcR block and surface staining (here CD15 PE) was performed as described above. After fixation, the cells were permeabilized with permeabilization buffer (see Table 2.7) for 15 min at RT in the dark. After washing, nuclei were stained with Hoechst33342 (final concentration 1 µg/ml) for 5 min at RT in the dark. After a last washing step, the cells were resuspended in 50 µl FACS buffer (see Table 2.7) and transferred into a 1.5 ml Eppendorf tube. The measurement was performed with the help of Simone Pöschel. At least 10.000 cells were acquired for each sample with 40x magnification using an ImageStream X MKII with the INSPIRE instrument controller software. Data were analyzed using the IDEAS Image analysis software. All samples were gated on single cells in focus.

2.3.6 Luminex cytokine multiplex analysis

Luminex measurements and analysis were performed by Nicole Schneiderhan-Marra and Thomas Knorpp at the NMI in Reutlingen. All samples were stored at -70°C until testing. The samples were thawn at room temperature, vortexed, spun at 18 000 x g for 1 min to remove debris and the required sample volumes were removed for multiplex analysis according to the manufacturer's recommendations. The samples were successively incubated with the capture microspheres, a multiplexed cocktail of biotinylated, reporter antibodies, and a streptavidin-phycoerythrin (PE) solution. Analysis was performed on a Luminex 100/200 instrument and the resulting data were interpreted using proprietary data analysis software (Myriad RBM). Analyte concentrations were determined using 4 and 5 parameter-, weighted- and non-weighted curve fitting algorithms included in the data analysis package.

2.3.7. Cytometric bead array

A cytometric bead array was performed using the "Human inflammatory cytokine kit" (see Table 2.6) and following the manufacturer's instruction. 25 µl of samples and standards were added to 25 µl of the capturing bead mixture. Additionally, 25 µl of PE detection reagent was pipetted to all tubes and incubated for 3 hours at RT in the dark. Thereafter, 1 ml of wash buffer was added to each tube and centrifuged at 200 x g for 5 min. The supernatant was carefully removed and the pellets were resuspended in 300 µl wash buffer each.

Measurements were performed with the FACS Canto II. Analysis was performed with Soft Flow FCAP Array v3 analysis software.

2.3.8. ELISA

To ensure that the supernatants were cell free, cell culture plates were spun down for 5 min at 796 x g. Subsequently, the supernatants were harvested and stored at -80°C prior to use. In order to save antibodies, ½ Area plates were used (see Table 2.5, using duplicates or triplicates of each sample). The absorbance was measured with a standard plate reader at 450 nm. The assays were performed according to the manufacturer's instructions (all ELISA kits used in this study are listed in Table 2.6), using appropriate dilutions of the supernatants. For LL37 determination a kit from HycultBiotech (see Table 2.6) with pre-coated plates was used following the manufacturer's instructions.

2.3.9. Neutrophil elastase NETosis assay

Neutrophil extracellular trap formation was determined using the colorimetric NETosis Assay Kit (see Table 2.6) based on the enzymatic activity of NET-associated neutrophil elastase. PMNs from various healthy donors were isolated as described above and stimulated with RNA-LL37 complex, or PMA (100 nM, provided by the kit) and a calcium ionophore (A-23187, 25 µM, provided by the kit) as positive controls for 1 to 3 hours. The assay was performed following the manufacturer's instructions but also using ½ Area plates to save substrate. The absorbance was then measured at 405 nm using a standard plate reader.

2.3.10. Fluorescence microscopy of tissue samples (human and mouse)

Skin samples from psoriasis patients with a PASI ≥ 10 and without systemic therapy at the time of skin sampling, healthy skin samples and mouse ears were paraffin-embedded according to standard procedures (Canene-Adams 2013). The paraffin blocks were cut in slices (thickness = 3 µm) and mounted on SuperFrost® Plus microscopy slides (this was done by Lukas Freund at the Dermatology Department in Heidelberg or by Sybille Kohler at the Dermatology Department Tübingen).

Thereafter, the tissue samples were deparaffinized (2 times for 10 min) with Roti Histol solution (see Table 2.1) and rehydrated using decreasing concentrations of ethanol (100% two changes, 95%, 80% and 70% every step 5 min). After rinsing in ddH_2O, antigen retrieval was performed by boiling for 10-20 min in citrate buffer (see Table 2.7). The skin tissue was then washed 3 times for 5 min with PBS. Blocking was performed using pooled human serum (1:10 in PBS) for 30 min at RT. The primary antibody (antibody list in Table 2.4) was added either overnight at 4°C or for 1 hour at RT. After 3 washes, the samples were incubated with secondary antibody for 30 min at RT in the dark. After another 3 washes, SYTO RNAselect Green fluorescent dye (final concentration 50 µM) was added for 40 min at RT in the dark. Thereafter, the samples were washed again and DNA was stained with Hoechst33342 (final concentration 1 µg/ml) for 5 min. Then 3 last washes were performed before using ProLong

Diamond Antifade and uncoated coverslips for mounting. The samples were left to dry overnight at RT in the dark before being used for microscopy or stored at 4°C. The specimens were analyzed on a Nikon Ti2 eclipse microscope (10x-60x magnification, using immersion oil for 40x and 60x magnification) and the analysis was performed using Fiji analysis software. Autofluorescence in multiple channels typical for the stratum corneum was labeled "AF". Blood vessels were labeled with "BV".

2.3.11. Cell surface antigen expression screening in whole blood samples

A cell surface antigen screening was performed using the LEGENDScreen™ (Table 2.6). Whole blood (EDTA anticoagulated) was drawn from five psoriasis patients (PASI≥10, no systemic therapy at the time of blood drawing) and five sex- and age-matched healthy controls. Erythrocyte lysis was performed for 5 min at 4°C on a roller shaker using 1x erythrocyte lysis buffer (see Table 2.7). After a short spin of 5 min at 509 x g without brake, FcR block was performed using pooled human serum (diluted 1:10 in PBS) for 15 min at 4°C, also on a roller shaker. Thereafter, the cells were stained in 1 ml volume per tube with anti-CD3 (T lymphocytes), CD15 (PMNs) and CD19 (B lymphocytes), excluding dead cells using Zombie Yellow (respective antibodies, fluorophores and dilutions see Table 2.4) for 30 min at 4°C on a roller shaker. Subsequently, each tube was filled with 12 ml PBS and the stained cells were aliquoted into 4 x 96 well plates (V-bottom), each well containing 5 µl of PE-labeled antibody directed against one of 332 surface antigens, and 10 isotype controls all labeled in PE (all PE-conjugated Abs were provided by the kit). Further steps were performed using manufacturer's instructions, except that one kit was divided for the measurement of 4 donors. FACS measurements were performed using a MACSQuant analyzer (AG Schindler, Tübingen) and subsequently FlowJo V10 was used to analyze the data. T cells, PMNs and B cells were gated according to the Abs in the master mix. The gating strategy is depicted in Figure 6.5. Monocytes were gated by size and granularity and not additionally labeled with CD14. However, in the well containing anti-CD14-PE antibody, all gated events were CD14-positive.

2.3.12. FACS analysis of whole blood samples

200 µl of the cell suspension prepared as in section 2.3.11. was transferred into a 96 well U-bottom plate and spun down for 5 min at 448 x g at 4°C. FcR block was performed using pooled human serum diluted 1:10 in FACS buffer for 15 min at 4°C. After washing, the samples were stained for 30 min at 4°C in the dark (antibodies and respective dilutions are listed in Table 2.4). Thereafter, fixation buffer was added to the cell pellets and incubated for 10 min at RT in the dark. After an additional washing step, the cell pellets were resuspended in 100 µl FACS buffer. Measurements were performed on a MACSQuant analyzer. Analysis was performed using FlowJo V10.

2.3.13. Fluorescence microscopy of fixed whole blood cells

Short erythrocyte lysis was performed as described above in (2.3.11.). 200 µl of cell suspension was aliquoted per well (96 well plate, U-bottom). FcR block, staining, fixation and

permeabilization were performed as for flow cytometry (described above in 2.3.1. and 2.3.12., antibodies and respective dilutions are listed in Table 2.4). Nuclear DNA was stained using Hoechst33342 (final concentration 1 µg/ml) as described above. Cell pellets were resuspended in 50-100 µl FACS buffer. 40 µl of the cell suspension was pipetted on a Poly-L-Lysine coated coverslip (see Table 2.5) and the cells were left to attach for 1 hour in the dark. ProLong Diamond Antifade was used to mount the coverslips on uncoated microscopy slides. The slides were left to dry overnight at RT in the dark and were then stored at 4°C before microscopy. Measurements were conducted with a Nikon Ti2 eclipse (100x magnification, using immersion oil) and the analysis was performed using Fiji analysis software.

2.3.14 FACS and microscopy settings

Table 2.8: FACS Canto II Settings

Color/dye	Laser	Wavelength (nm)
BV421	violet	450/50
FITC/AF488	blue	530/30
PE	blue	585/42
APC/AF647	red	660/20

Table 2.9: MACSQuant FACS Settings

Color/dye	Laser	Wavelength (nm)
BV421	violet	450/50
Zombie Yellow	violet	525/50
AF488	blue	525/50
PE	yellow	586/15
PE-Cy7	yellow	750/LP

Table 2.10: Nikon Ti2 eclipse Settings

Color/dye	Filter	Wavelength (nm)
Hoechst 33342	QuadDAPI	390
SYTO RNAselect Green fluorescent, AF488, FITC	QuadFITC	475
PE	QuadCy3	549
AF549	QuadmCherry	575
AF647	QuadCy5	632

2.4. *In vivo* mouse model

2.4.1. Mice

In vivo mouse experiments were performed by Nate Archer (group of Lloyd Miller in Baltimore, USA). In brief, C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were bred and maintained under specific pathogen-free conditions at an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility at Johns Hopkins University. They were handled according to procedures described in

the Guide for the Care and Use of Laboratory Animals as well as Johns Hopkins University's policies and procedures as set forth in the Johns Hopkins University Animal Care and Use Training Manual, and all animal experiments were approved by the Johns Hopkins University Animal Care and Use Committee. Sex- and age-matched 6-8-week old mice were used for each experiment.

2.4.2. Platelet depletion protocol

For platelet depletion in mice, 4 µg/g of anti-CD42b (see Table 2.4) or rat IgG isotype control (see Table 2.4) diluted in sterile PBS was injected intravenously (i.v.) one day before, and 2 µg/g intraperitoneally (i.p.) 3 days after the first imiquimod treatment.

2.4.3. Imiquimod model of psoriatic skin inflammation

Mice were anesthetized (2% isoflurane) and 62.5 mg of 5% imiquimod (Imiquimod creme, Tora Pharmaceuticals) was applied topically with a sterile cotton swab to the ventral and dorsal sides of the mouse ear. This was done daily for a total of 5 treatments. Prior to imiquimod application, ear thickness was measured with a manual caliper. A day before and at the end of imiquimod treatment, blood was collected retro-orbitally with heparinized capillaries for FACS analysis. In addition, ear thickness was terminally analyzed taking a 6mm punch for immunohistochemistry (IHC, in this case H&E staining) and immunofluorescence (IF).

2.4.4. Histology and epidermal thickness measurements

6-mm punch biopsy specimens were placed in 10% formalin and paraffin-embedded using standardized procedures. Skin cross-sections (thickness = 4 µm) were cut and mounted on SuperFrost® Plus microscopy slides and left to dry overnight. The staining with hematoxylin-eosin (H&E) was performed by the Johns Hopkins Reference Histology Laboratory according to clinical specimen guidelines, or utilized for immunofluorescent staining (as described above). To measure epidermal thickness, at least 10 measurements per mouse were averaged from images taken at 20x magnification (Revolve microscopy from ECHO, Table 2.5) using ImageJ/Fiji software.

2.4.5. Flow cytometry

For FACS analysis, retro-orbital blood samples were collected on day 0 and day 5 of IMQ-treatment, diluted in 300 µl TBS (Tris-buffered saline) containing 5 U/ml Heparin and subsequently further diluted with 500 µl PBS. FcR-block was performed using TruStain fcX (see Table 2.4) for 10 min at RT. Subsequent staining was performed using anti-Ly6C, anti-CD45, anti-CD41, anti-Ly6G, anti-CD11b, propidium iodide (all antibodies with respective dilutions are listed in Table 2.4) for 30 min at 4°C. FACS measurements were performed using a MACSQuant and subsequently analyzed with MACSQuantify software. Cell types were defined by flow cytometry according to the following gating strategies: platelets were identified from the $CD41^+$ population from live cells, and myeloid cells were gated on the $CD11b^+$ population

from live cells. PMNs and monocytes were identified as $Ly6G^{hi}Ly6C^{lo}$ and $Ly6G^{lo}Ly6C^{hi}$ cells, respectively. Platelet-PMN and platelet-monocyte aggregates were identified first by gating on the $CD41^{+}$ $CD11b^{+}$ population and then on $Ly6G^{hi}Ly6C^{lo}$ and $Ly6G^{lo}Ly6C^{hi}$ cells, respectively.

2.5. Statistical analysis

2.5.1. General statistics

Experimental data was analyzed using Excel 2010 or 2019 (Microsoft) and/or GraphPad Prism 6, 7 or 8. Microscopy data were analyzed with NIS Elements from Nikon and with ImageJ/Fiji. Flow cytometry data were evaluated with FlowJo V10. Extreme values, outliers were identified using the ROUT method at high (0.5%) strictness. For each experiment, normal distribution was evaluated using the D'Agostino-Pearson or Shapiro-Wilk test to choose either a parametric (ANOVA, Student's t-test) or non-parametric (e.g. Friedman, Mann-Whitney U or Wilcoxon) test for validation of significance. p-values (α=0.05) were then calculated and correction for multiple comparison was performed in Prism, which is always indicated in the Figure legends. Values < 0.05 were considered as statistically significant and indicated by * even if the calculated p-values were greatly lower than 0.05. Multiple comparisons were performed, typically comparing all values to the unstimulated control unless indicated otherwise. Wherever this was done, p-values were adjusted for multiple testing.

2.5.2. Differential expression analysis of surface marker screening data

Statistical analysis of the LEGENDScreen™ raw MFI (mean fluorescence intensity) data was performed by Marius Codrea and Simon Heumos at QBIC in Tübingen.
The goal was to identify surface antigens which were significantly different between patients and healthy donors within specific cell populations (on a surface antigen/protein expression level). This relationship was formulated as MFI ~ health_status + cell_type, where the mean fluorescence intensity (MFI) depended on the two main factors: health status and cell type. All cell populations were measured simultaneously in one FACS screen per subject (patient or healthy donor) and the resulting intrinsic (within-subject) variance (across all populations) was calculated by extending MFI ~ health_status + cell_type + subject/cell_type. In this notation, cell_type was "nested" within "subject". Analysis were performed in R [version 3.4.4, with linear mixed models using the R package nlme (version 3.1-131.1)] (Pinheiro 2000, Bates 2015). The fitted models were subject to post-hoc analysis with Tukey's "Honest Significant Difference" test to compute adjusted pair-wise differences among the cell types (Bretz 2011). The lsmeans (version 2.27-61) (Lenth 2016) R package implementation was used in order to compute the adjustments. lsmeans' pairs were used to calculate all pair-wise contrasts of patients versus healthy donors by the given cell_type. The p-values and the fold changes of all surface antigens were extracted from all contrasts of all different cell_type levels. This then provides multiple-comparison adjusted p-values and fold changes between patients versus healthy donors for each cell population. For the generation of the principle component

analysis (PCA) plots, the R package ggplot2 (version 2.2.1) was used (Herster et al., 2019, *in revision*).

3. Results part I

3. Neutrophil extracellular trap-associated RNA (naRNA) and LL37 complexes enable self-amplifying inflammation in psoriasis

3.1. LL37 induces uptake of RNA and PMN activation by endosomal TLRs

The group of Michel Gilliet (Lande *et al.* 2007) (Ganguly *et al.* 2009) previously showed that pDCs internalized DNA or RNA, when complexed to LL37, which in turn induced strong type I interferon release. In order to investigate whether PMNs are also able to be activated by nucleic acids complexed to LL37, experiments with PMNs isolated from healthy donors were performed first.

3.1.1 PMNs only respond to RNA-LL37 and not to DNA-LL37 complexes

It was previously shown that human PMNs respond to RNA and DNA with cytokine release after stimulation for over 12 hours. However, the cytokine levels were for both much lower compared to stimulation with the commercially available TLR7/8 agonist, R848 (Janke *et al.* 2009) (Lindau *et al.* 2013). In this study, these previous findings were re-evaluated but using only short time period (e.g. 4 hours) for stimulation, taking into account that isolated PMNs are short lived in culture and to avoid off-target effects by apoptotic cells (after 4 hours in culture, a cell viability of at least 95% was observed, see Figure 6.1C).

PMNs were purified as described in chapter 2.2.4. in “Materials and Methods”. As they are very fragile and can potentially be activated during the process of isolation, purity and pre-activation status were always assessed by FACS prior to performing an experiment. For purity, cells which were $CD15^{+}CD66b^{+}$ were considered as PMNs; for pre-activation, CD62L shedding was used as a sensitive and early activation marker (Simon *et al.* 1995). Here, the purity was always >95% and cells were not pre-activated if handled very gently (see Figure 6.1A and B). PMNs from healthy donors were found to readily respond to stimulation with known TLR ligands e.g. R848 (TLR 7/8 agonist), LPS (TLR4 agonist) and CpG (TLR9 agonist) with cytokine release and CD62L shedding (R848 did not induce CD62L shedding) (see Figure 3.1A). However, in contrast to pDCs (Lande *et al.* 2007), in PMNs neither ssDNA (sequence see Table 2.3) nor human genomic DNA induced detectable IL-8 release or CD62L shedding, regardless of DNA being in complex with LL37 or not (Figure 3.1B). However, when using ssRNA40 (from now on further referred to as “RNA”, sequence see Table 2.3), this induced a robust release of IL-8 and moderate CD62L shedding but only in complex with LL37 (Figure 3.1C). These results indicate that neither RNA nor DNA alone are able to induce immune activation in primary PMNs. Only for RNA, but not for DNA, LL37 promotes cell activation.

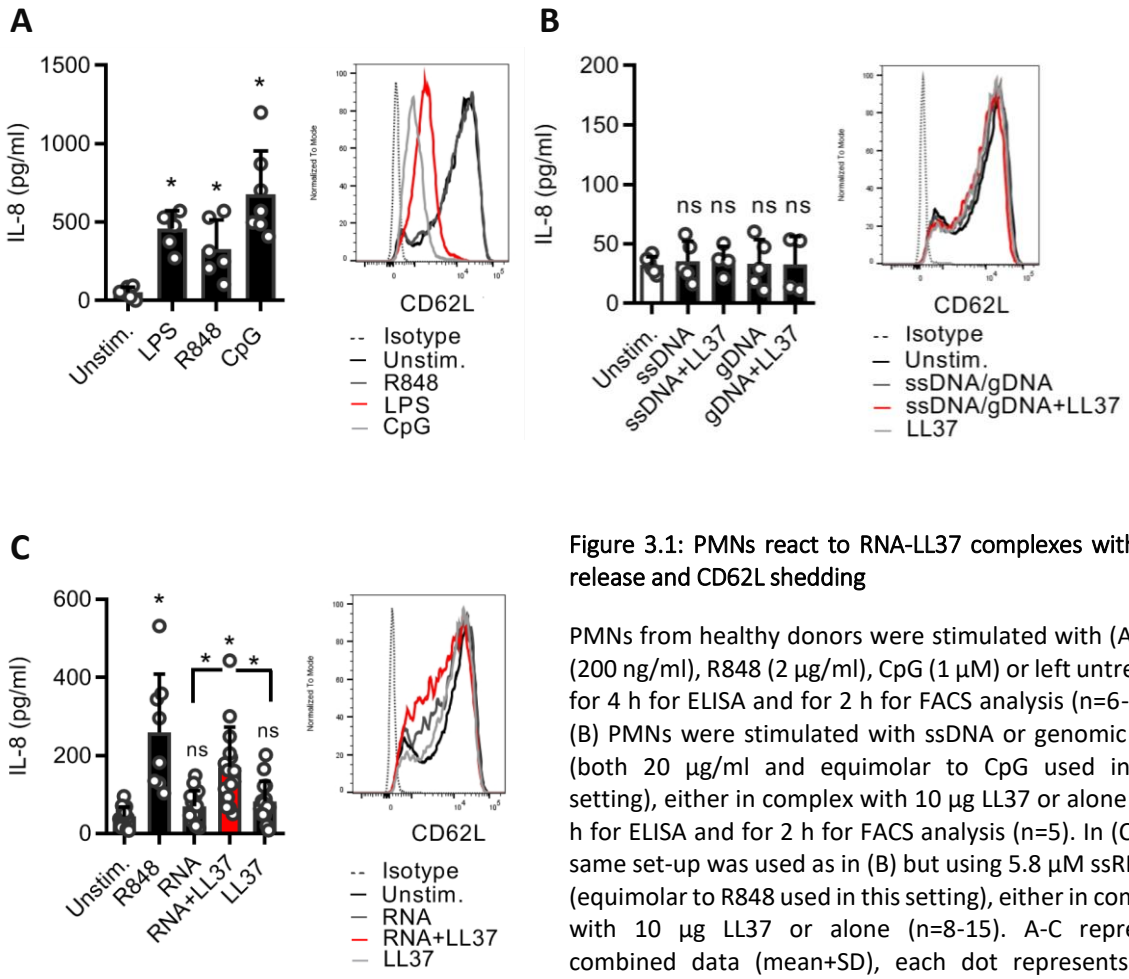


Figure 3.1: PMNs react to RNA-LL37 complexes with IL-8 release and CD62L shedding

PMNs from healthy donors were stimulated with (A) LPS (200 ng/ml), R848 (2 µg/ml), CpG (1 µM) or left untreated for 4 h for ELISA and for 2 h for FACS analysis (n=6-7). In (B) PMNs were stimulated with ssDNA or genomic DNA (both 20 µg/ml and equimolar to CpG used in this setting), either in complex with 10 µg LL37 or alone for 4 h for ELISA and for 2 h for FACS analysis (n=5). In (C) the same set-up was used as in (B) but using 5.8 µM ssRNA40 (equimolar to R848 used in this setting), either in complex with 10 µg LL37 or alone (n=8-15). A-C represent combined data (mean+SD), each dot represents one donor. * $p<0.05$ according to one-way ANOVA with Dunnett's correction compared to unstimulated unless otherwise indicated(A-C).

3.1.2. LL37 promotes RNA uptake in human PMNs

To further investigate if LL37 helps to promote RNA uptake, AlexaFluor (AF) 647-labeled RNA was purchased from IBA Lifescience. In FACS analysis more than 20% of PMNs were AF647 positive for RNA-LL37 complex-treated PMNs compared to under 5% for RNA alone (Figure 3.2A) upon stimulation for 1 hour. ImageStream bright-field cytometry confirmed that RNA did not only bind to the surface of the cell but was internalized and therefore found in intracellular compartments (Figure 3.2B). Additionally, after labeling LL37 with Atto488 (kindly provided by Hubert Kalbacher, Tübingen), fluorescence microscopy not only showed that RNA was detectable in intracellular compartments but also co-localized with LL37 (Figure 3.2C). These findings show that LL37 is able to shuttle RNA into intracellular compartments (probably endosomes, where nucleic acid sensing TLRs reside, (Berger *et al.* 2012)) and that complex formation *in vitro* works, which is in good agreement with previous studies performed with pDCs (Ganguly *et al.* 2009).

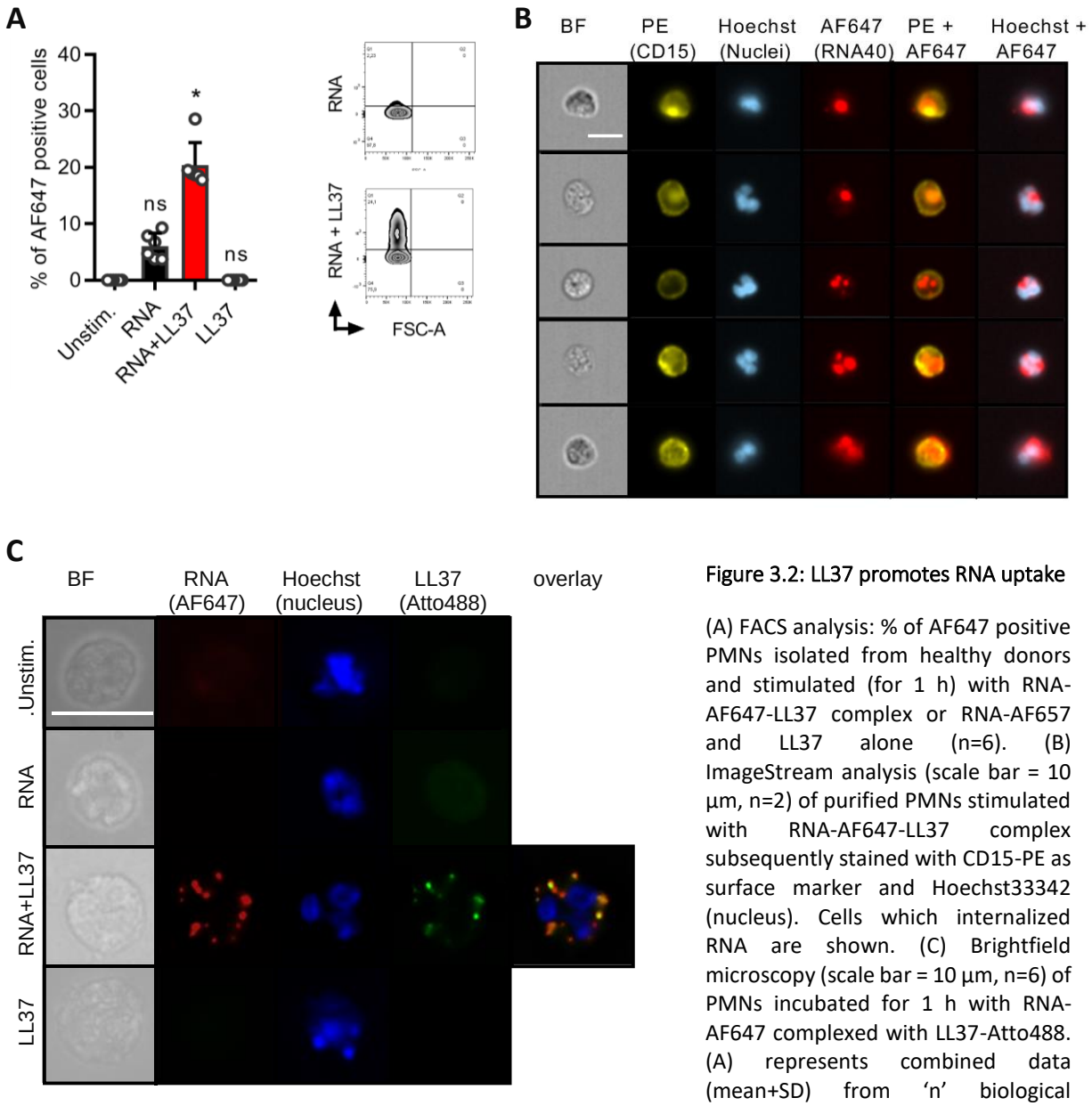


Figure 3.2: LL37 promotes RNA uptake

(A) FACS analysis: % of AF647 positive PMNs isolated from healthy donors and stimulated (for 1 h) with RNA-AF647-LL37 complex or RNA-AF657 and LL37 alone (n=6). (B) ImageStream analysis (scale bar = 10 µm, n=2) of purified PMNs stimulated with RNA-AF647-LL37 complex subsequently stained with CD15-PE as surface marker and Hoechst33342 (nucleus). Cells which internalized RNA are shown. (C) Brightfield microscopy (scale bar = 10 µm, n=6) of PMNs incubated for 1 h with RNA-AF647 complexed with LL37-Atto488. (A) represents combined data (mean+SD) from 'n' biological replicates (each dot represents one donor). In B and C representative samples of 'n' replicates or donors are shown. * $p<0.05$ according to Friedman test with Dunn's correction (A).

3.1.3. Synthetic, human and bacterial RNA form complexes with LL37 and induce cytokine release via endosomal TLRs in PMNs

Next, it was investigated whether RNA-LL37 uptake was specific for the synthetic RNA (sequence see Table 2.3) which was used for pervious experiments or whether the RNA sequence had no impact on complex formation with and uptake by LL37. Therefore, total human mRNA isolated from HEK293T cells and bacterial RNA, isolated from *S. aureus* as

previously described by Eigenbrod et al. (Eigenbrod *et al.* 2015) was used. As expected, human (Figure 3.3A) and, interestingly, bacterial RNA (from now on further referred to as bRNA) (Figure 3.3B) induced cytokine release more potently when being complexed with LL37. However, it has to be acknowledged that human PMNs in general reacted better to synthetic compared to bacterial RNA.

In order to test whether this observed cytokine release induced by RNA-LL37 complexes was dependent on endosomal TLRs, Chloroquine (CQ) was used to block endosomal TLR signaling (Kuznik *et al.* 2011). CQ inhibits endosomal acidification which is crucial for endosomal TLR activation. And indeed, CQ was able to inhibit IL-8 release by PMNs induced through CpG (a known TLR9 agonist, positive control) and RNA-LL37 complexes (Figure 3.3C and D). This was not due to a cytotoxic effect (Figure 6.1D). As chloroquine also does not affect cytosolic RNA sensing e.g. by RIG-I (Matsukura *et al.* 2007), it was assumed that RNA-LL37 signaling occurs due to endosomal TLR sensing and is independent of the RNA sequence.

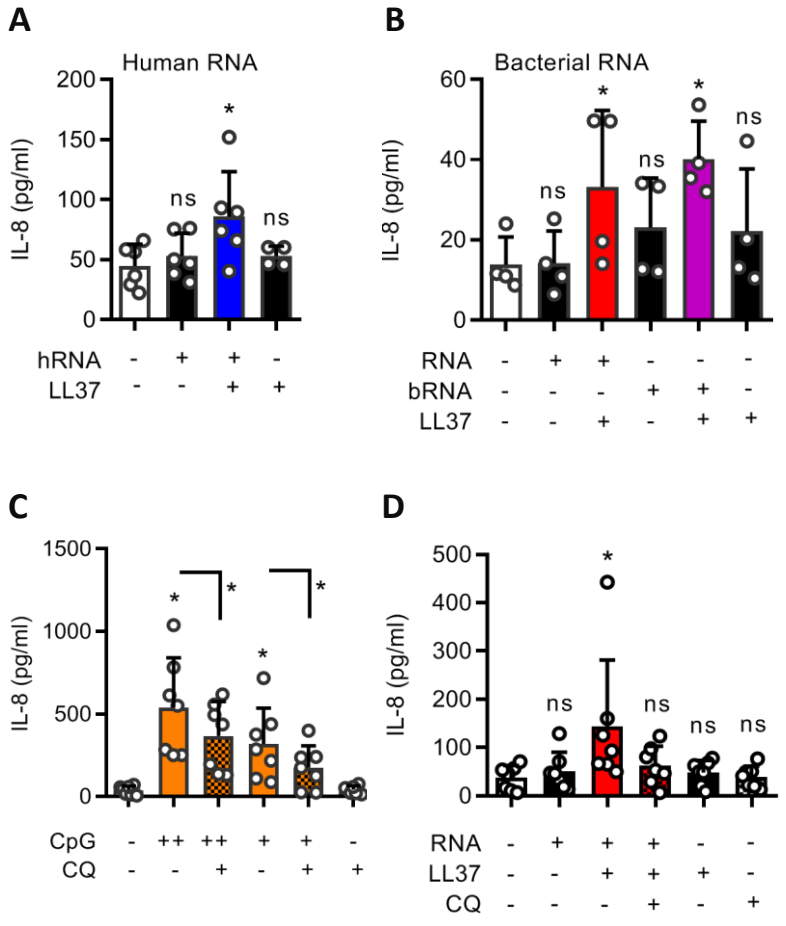


Figure 3.3: RNA-LL37 complexes induce cytokine release by PMNs via endosomal TLRs and this is independent of RNA sequence

(A) PMNs were stimulated with complexes of total human mRNA isolated from HEK293T cells and LL37 for 4 h (n=4-6). (B) Bacterial RNA (isolated from *S. aureus*) was complexed with LL37 and PMNs were stimulated with the complex for 4 h (n=4). (C) PMNs were pre-incubated with 10 µM CQ (30 min) and subsequently stimulated with CpG (1 µM and 500 nM) for 4 h (n=7). (D) The same setup as in C but stimulation with RNA-LL37 complex instead of CpG (n=7). A-D represent combined data (mean+SD) from 'n' biological replicates (each dot represents one donor). * $p<0.05$ according to one-way ANOVA with Dunnett's correction for A and D with Sidak correction for C and Friedman test with Dunn's correction for B.

3.2. PMNs release cytokines and chemokines in response to RNA-LL37 complexes which in turn induce migration of other immune cells

3.2.1. RNA-LL37 complexes induce the release of multiple cytokines and chemokines by human PMNs

Next, it was investigated whether PMNs only release IL-8 in response to RNA-LL37 complexes or also other pro-inflammatory cytokines and chemokines. Therefore, supernatants from two healthy donors were screened in Luminex analysis (the measurements were kindly performed by Nicole Schneiderhan-Marra and Thomas Knorpp at the NMI in Reutlingen). The analysis was performed as previously described (Brockmann *et al.* 2016). As a result, it was observed that not only IL-8 was released by PMNs in response to RNA-LL37 complexes but also other pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β (Figure 6.2A). Interestingly, the screen also revealed that PMNs released more IL-16 and MIP-1β in response to RNA-LL37 complexes (Figure 6.2B). IL-16, previously known as lymphocyte chemoattractant factor or LCF, was described as a chemoattractant for $CD4^+$ lymphocytes a long time ago (Center and Cruikshank 1982). However, not much is known about IL-16 release by PMNs. There are indications that its release might be related to cell death (Roth *et al.* 2015) which can be excluded here (Figure 6.1C), at least for the unstimulated samples. MIP-1β or better known as CCL4 (C-C Motive Chemokine Ligand 4) is a strong chemoattractant for a variety of immune cells (Menten *et al.* 2002). For PMNs, there is only one study showing that migrating neutrophils secreted MIP-1β which induced the migration of dendritic cells in turn (Chiba *et al.* 2004). Next, the results of the Luminex screen were re-evaluated with supernatants from more donors using Cytometric bead array (CBA) or ELISA. As expected, it was confirmed that PMNs release more TNF-α, IL-6 and IL-1β in response to RNA-LL37 complexes (Figure 3.4A-C). Furthermore, IL-16 release was already induced by stimulation with LL37 only (Figure 3.4D), whereas the induction of MIP-1β was strongly dependent on RNA-LL37 stimulation (Figure 3.4E). These data show that PMNs are able to release not only IL-8 but also other cytokines and chemokines in response to RNA-LL37 which consequently could potentially attract other cells to the site of inflammation.

Although the amounts of cytokines released from RNA-LL37-stimulated PMNs in *in vitro* experiments were comparatively low, they might still have a great impact on immune responses. Furthermore, high concentrations of cytokines might well be elicited due to fast cytokine release (within 4 hours of stimulation) and the high number of PMNs in human blood or psoriatic skin lesions.

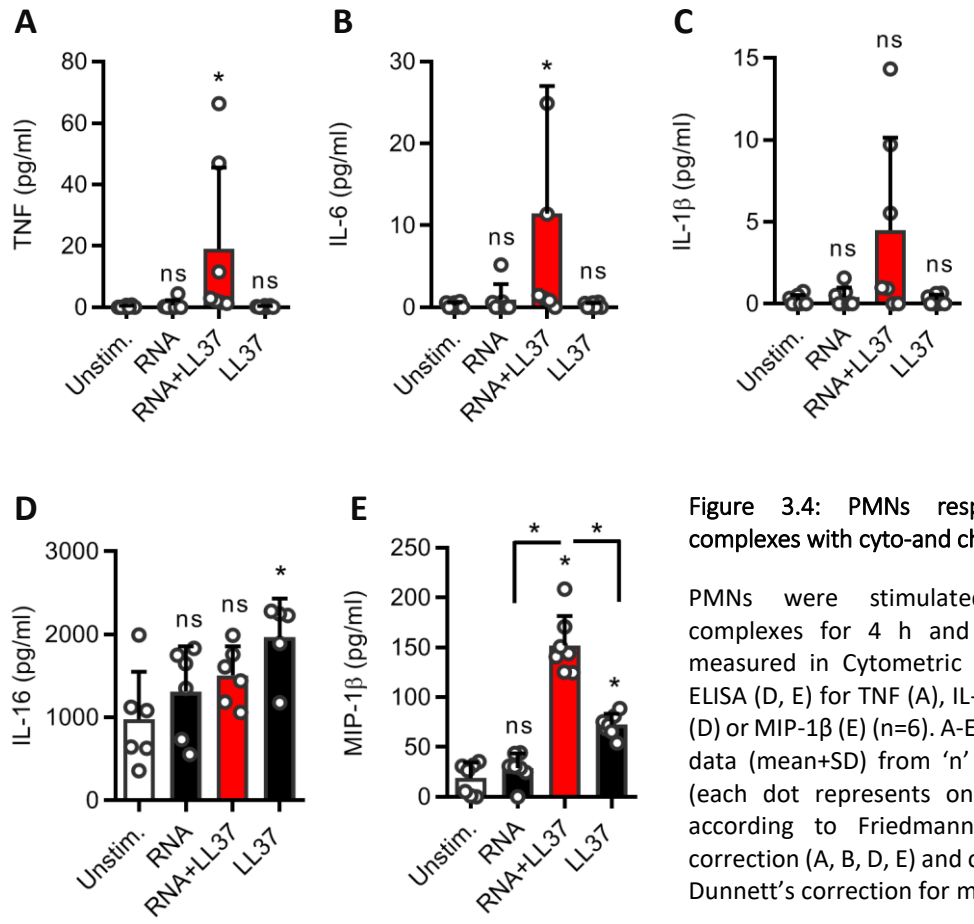


Figure 3.4: PMNs respond to RNA-LL37 complexes with cyto-and chemokine release

PMNs were stimulated with RNA-LL37 complexes for 4 h and supernatants were measured in Cytometric bead array (A-C) or ELISA (D, E) for TNF (A), IL-6 (B), IL-1β (C), IL-16 (D) or MIP-1β (E) (n=6). A-E represent combined data (mean+SD) from 'n' biological replicates (each dot represents one donor). * $p<0.05$ according to Friedmann test with Dunn's correction (A, B, D, E) and one-way ANOVA with Dunnett's correction for multiple testing in (C).

3.2.2. Low concentrations of MIP-1β and IL-16 induce the migration of other immune cells

As previously described, PMNs respond to RNA-LL37 stimulation with the release of a variety of pro-inflammatory cytokines and chemokines. To study whether these cyto-/chemokines (in low concentrations, as released by PMNs) are able to induce cell migration, transwell migration assays were performed. It was especially expected that IL-16 and MIP-1β, as known chemoattractants for a variety of cells (Center and Cruikshank 1982) (Menten *et al.* 2002), would induce cell migration. In the lower compartments, IL-16 (300 pg/ml and 1500 pg/ml) and MIP-1β (30 pg/ml and 150 pg/ml) or SDF-1α (stromal-cell derived factor 1 α, 100 ng/ml, used as migration control) were pipetted in RPMI medium. In the upper compartment PBMCs from the healthy donors were inserted. After 4 hours, the migrated cells in the lower wells were harvested, stained with antibodies against respective surface antigens and measured by flow cytometry. To assess the total number of migrated cells, counting beads were used.

With SDF-1α as known strong chemoattractant for T and B lymphocytes (Bleul *et al.* 1996), it was confirmed that the transwell experiment itself worked (Figure 6.3A-C). As shown in Figure 3.5 A-C, $CD3^+CD4^+$ helper T cells, $CD3^+CD8^+$ cytotoxic T cells and $CD14^+HLA\text{-}DR^+$ monocytes migrated towards the cytokines, even more prominently towards the lower concentrations

used in this experiment. Due to donor-to-donor variations, only a non-significant effect could be observed. Unexpectedly, when adding RNA-LL37 complexes and other TLR stimuli to medium in the lower compartment (intended as a negative control), $CD3^+CD4^+$ lymphocyte migration towards the RNA-LL37 complexes was observed (Figure 3.5D). Of course, it remains to be established whether this effect only applies to T cells within PBMCs, i.e. an indirect effect, or if RNA-LL37 works directly on $CD4^+$ T cells, a possibility that could be easily checked using isolated $CD4^+$ T cells in the future. These current data show that IL-16 and MIP-1β are able to attract a vast variety of immune cells and that even RNA-LL37 serves as a chemoattractant for $CD4^+$ T cells.

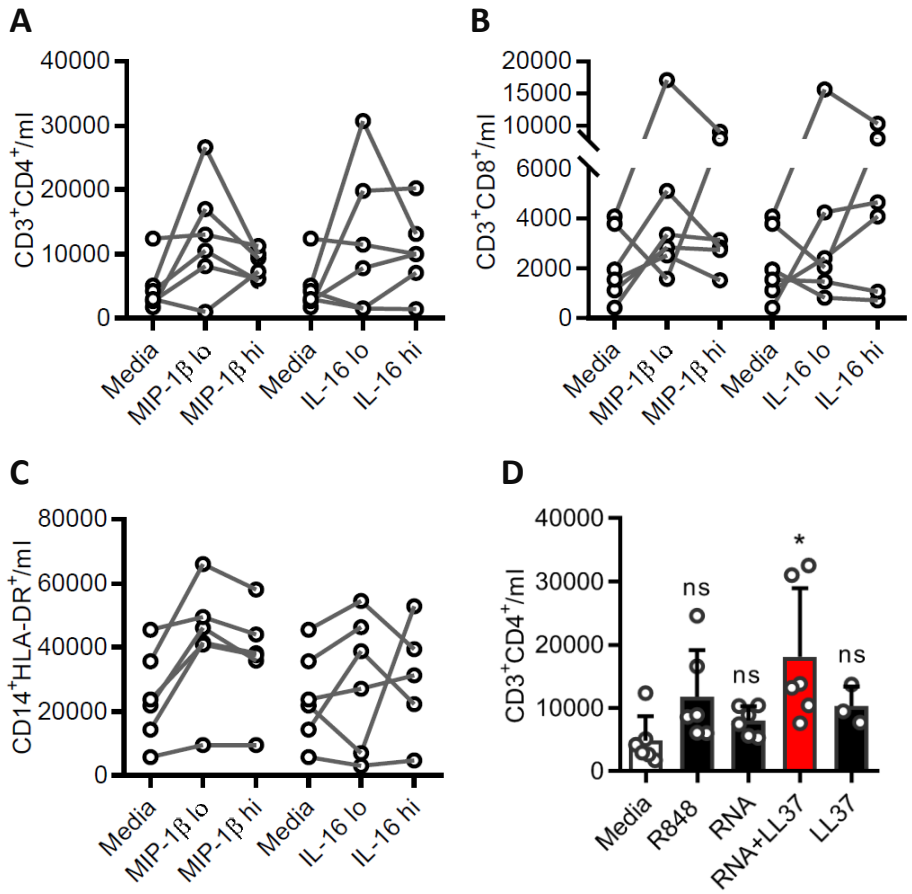
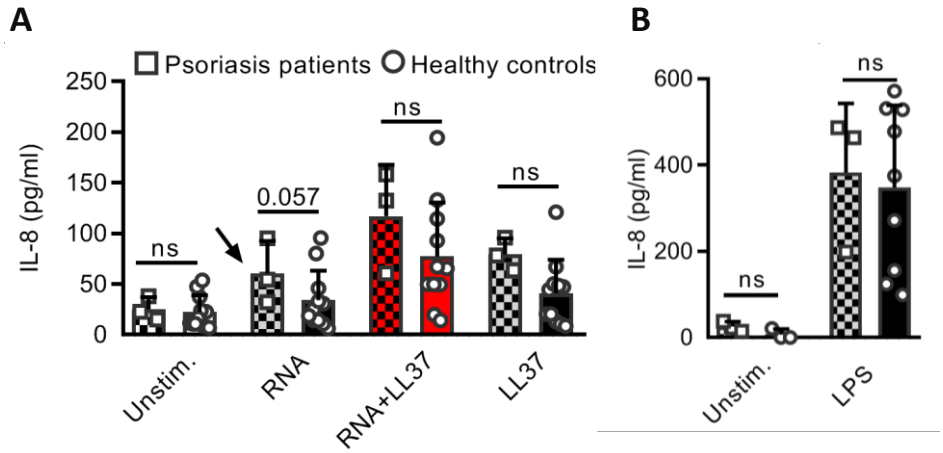


Figure 3.5: Immune cells migrate towards cytokines and chemokines released by PMNs in response to RNA-LL37 complexes

Total cell counts quantified by flow cytometry after transwell migration assay (after 4 h). Migrating $CD3^+CD4^+$ T cells (A), $CD3^+CD8^+$ T cells (B) and $CD14^+HLA\text{-}DR^+$ monocytes (C) towards either MIP-1β (30 and 150 pg/ml) or IL-16 (300 and 1500 pg/ml) are shown (n=6-7, $p>0.05$ for treatments vs. media). Total migrating $CD4^+$ T cell counts (D) towards R848 (2 µg/ml), RNA, RNA-LL37 complexes or LL37 are shown (n=3-7). A-D represent combined data (mean+SD) from 'n' biological replicates (each dot represents one donor). * $p<0.05$ according to Friedmann test with Dunn's correction (A-C) or one-way ANOVA with Dunnett's correction for multiple testing (D).

3.2.3. PMNs from psoriasis patients secrete more cytokines in response to RNA-LL37 complexes

Activation of pDCs by RNA-LL37 complexes has been reported, however, differences in response of pDCs from healthy individuals and psoriasis patients were not investigated (Ganguly *et al.* 2009). To elucidate whether PMNs of psoriasis patients and healthy individuals differentially react to RNA-LL37 complexes, PMNs from peripheral blood (from psoriasis patients and healthy controls) were isolated and subsequently stimulated with RNA-LL37 complexes. At the same time, at least one sex-and age-matched control was analyzed. As shown in Figure 3.6 A and B, PMNs from psoriasis patients released more IL-8 when stimulated with the complex, whereas other TLR agonists like LPS or R848 (not shown) did not display differences in cytokine levels between patients and healthy controls. This was even more obvious for MIP-1β (Figure 3.6C and D). There, psoriasis PMNs released significantly more MIP-1β only in response to the RNA-LL37 complex compared to healthy controls. Interestingly, it was observed that the IL-8 baseline for RNA alone was higher for psoriasis patients (relatively to the unstimulated control), whereas the levels of IL-8 for the healthy controls were not considerably different from the unstimulated sample. As previously shown, RNA uptake was very dependent on LL37 complex formation (Figure 3.1C). PMNs are the major producers of LL37 in the human body, therefore it was speculated whether psoriasis PMNs might constitutively secrete more LL37. If this was the case, readily released LL37 could in turn complex with added RNA and start the stimulation of other PMNs independently of exogenously added LL37. For this reason, LL37 levels were measured in supernatants of unstimulated PMNs from psoriasis patients and healthy controls and indeed higher LL37 baseline levels were found for patients compared to healthy donors (Figure 3.6E). These results indicate that psoriasis PMNs are more prone to RNA-LL37 stimulation, potentially due to a higher constitutive secretion of PMN-derived LL37.



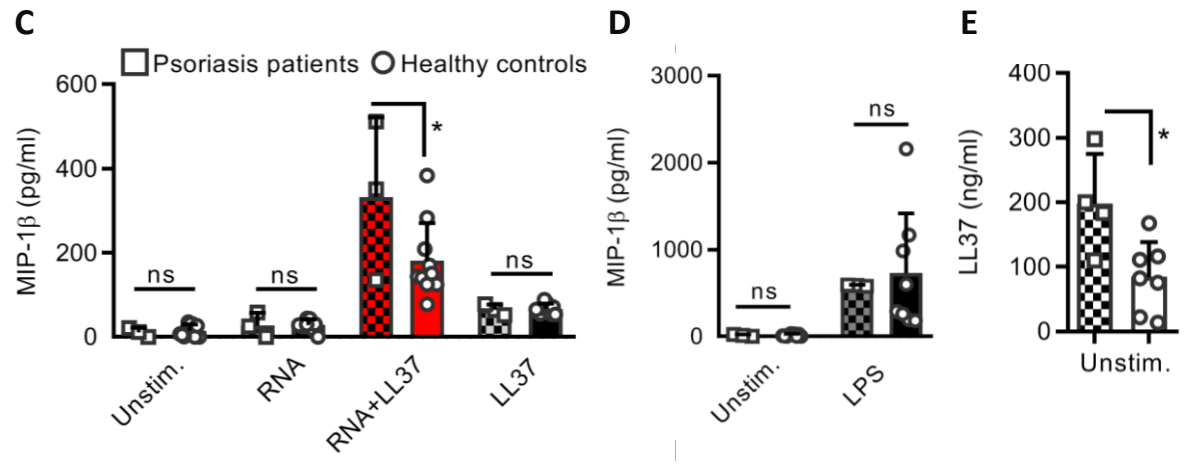


Figure 3.6: Psoriasis PMNs are more prone to RNA-LL37 stimulation

In (A) ELISA of IL-8 secreted from psoriasis PMNs or PMNs from sex-and age-matched healthy donors after 4 h of stimulation with RNA-LL37 complexes or (B) with 200 ng/ml LPS is shown. (C) the same setting as in (A) but measuring MIP-1β by ELISA. (D) the same set-up as (B) but measuring MIP-1β. (E) LL37 ELISA of unstimulated PMNs from psoriasis patients or from sex-and age-matched healthy donors (for all n=3-4 patients, chequered bars, n=7-10 healthy donors). A-E represent combined data (mean+SD) from 'n' biological replicates (each dot represents one donor). * $p<0.05$ according to one-way ANOVA with Dunnett's correction for multiple testing (A, C, E) or Kruskall-Wallis test with Dunn's correction (B, D).

3.3. RNA-LL37 complexes trigger the release of RNA-and LL37-containing NETs

This study so far showed that RNA and LL37 complexes induced pro-inflammatory cytokine release which could potentially contribute to inflammation in psoriatic lesions. Also, neutrophil-mediated chemokine release could trigger the infiltration of other immune cells to sites of inflammation. This response to RNA-LL37 complexes would be self-limiting, unless RNA-LL37 would trigger the release of further RNA and LL37 e.g. by NETosis. It was previously shown that Neutrophil extracellular traps (NETs) contain nucleic acids (DNA) (Brinkmann *et al.* 2010) and that this DNA, when complexed to LL37 (Lande *et al.* 2011), can activate pDCs. As shown here, PMNs were not able to induce immune reaction in response to DNA-LL37. Therefore, it was investigated whether RNA could be a new, unknown NET content involved in NET-mediated propagation via the DNA-unresponsive PMNs. Further studies were performed to reveal whether RNA-LL37 complexes were able to induce NETosis and whether these NETs can in turn contain LL37 and more importantly RNA.

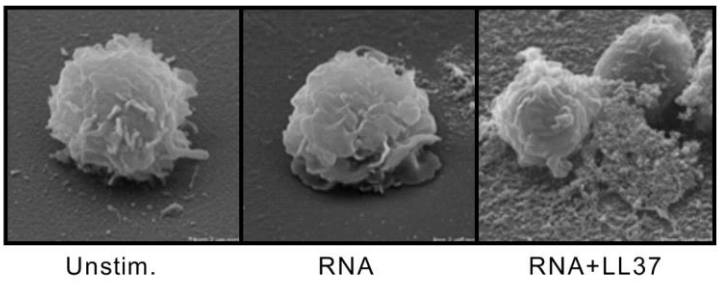
3.3.1 RNA-LL37 complexes induce NETosis

The first hint that RNA-LL37 complexes were able to induce NET formation were obtained from EM (electron microscopy) pictures taken by Jürgen Berger at the MPI (Tübingen). Here, fiber-like structures were observed in close proximity to PMNs which had been stimulated with RNA-LL37 complexes (Figure 3.7A). Therefore, NET formation was analyzed by detection of Neutrophil elastase (NE) release from PMNs (supernatants) using an enzymatic reaction ("NETosis kit") (Yizengaw *et al.* 2016). Interestingly, Neutrophil elastase was not only released in response to RNA-LL37 but also to LL37 alone (Figure 3.7B) (PMA was used as a positive

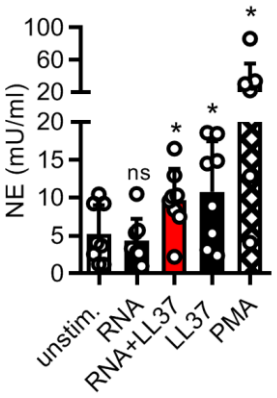
control). Since it is under debate whether NE is really necessary for the induction of NETosis (Martinod *et al.* 2016), further investigations of NET formation were performed by fluorescence microscopy. Hence, DNA, RNA and LL37 were labeled and NET release by PMNs in response to RNA-LL37 complexes was evaluated. To show RNA in NETs, a dye called SYTO RNAselect which was previously described to be RNA-specific (Li *et al.* 2006), was used.

Importantly, RNA-LL37 complexes induced NETosis in human PMNs which contained not only DNA (blue) and LL37 (red) but also considerable amounts of RNA (green) (Figure 3.7C). This was quite exclusive for stimulation with RNA-LL37 complex (and PMA as positive control), because RNA or LL37 alone did not result in NET release (RNA alone is not shown). Next, the behavior of PMNs to stimulation with RNA-LL37 in live-cell-imaging, in order to exclude staining artefacts in fixed cells, was investigated. The cells were therefore labeled with Hoechst (nucleus) and SYTO RNAselect (RNA) which revealed that the complex first accumulated on the cell membrane, then entered the cell and was finally released with further endogenous RNA and DNA from the rupturing cell. This was in general a very fast process (Figure 3.7D, quantified in E). In summary, these data demonstrate, that RNA-LL37 complexes induce NETosis and that these NETs in turn contain DNA, RNA and LL37.

A



B



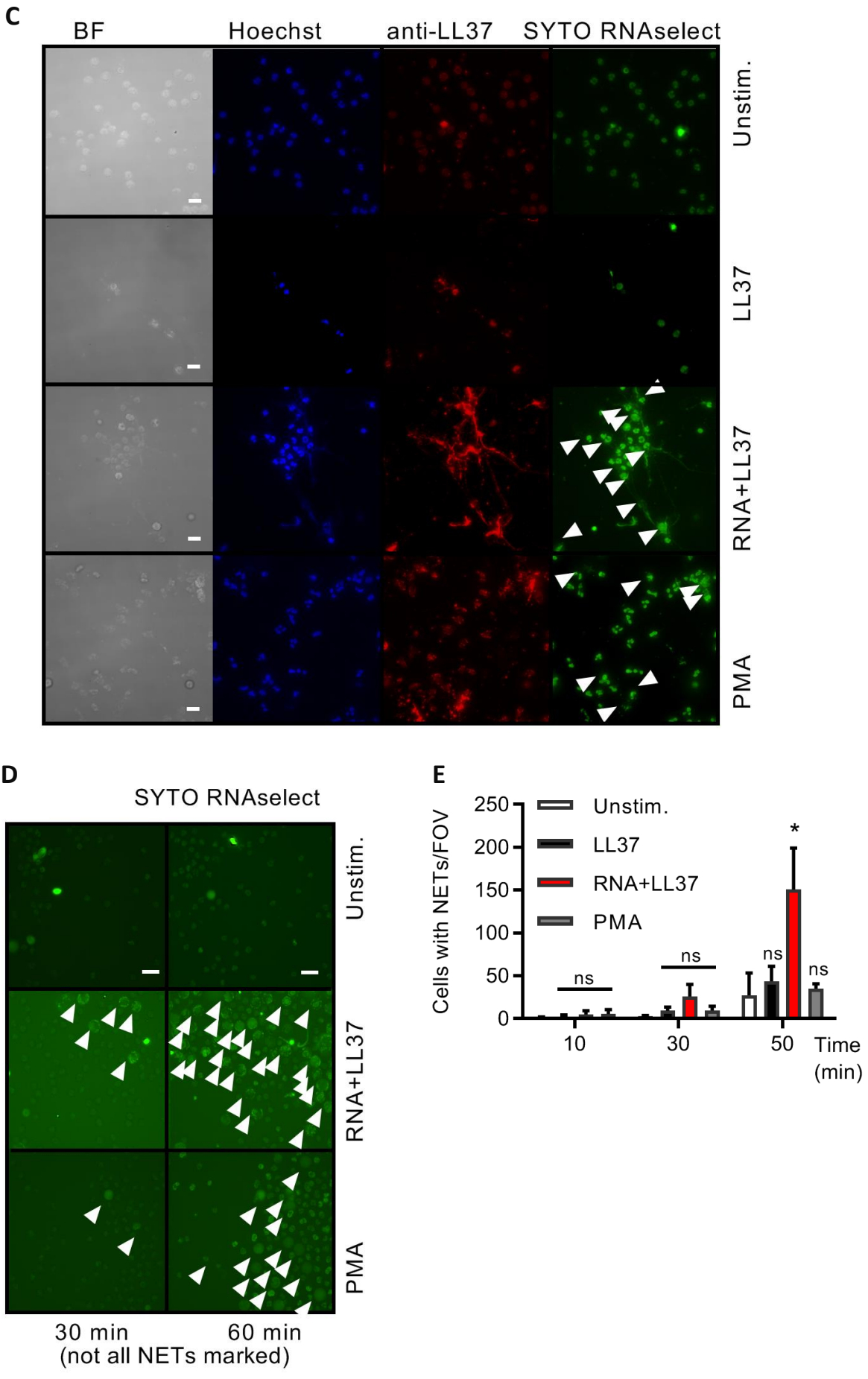

C
BF
Hoechst
anti-LL37
SYTO RNAselect
Unstim.
LL37
RNA+LL37
PMA
D
SYTO RNAselect
Unstim.
RNA+LL37
PMA
30 min
60 min
(not all NETs marked)
E
Cells with NETs/FOV
250
200
150
100
50
0
Unstim.
LL37
RNA+LL37
PMA
ns
ns
ns
*
ns
10
30
50
Time
(min)

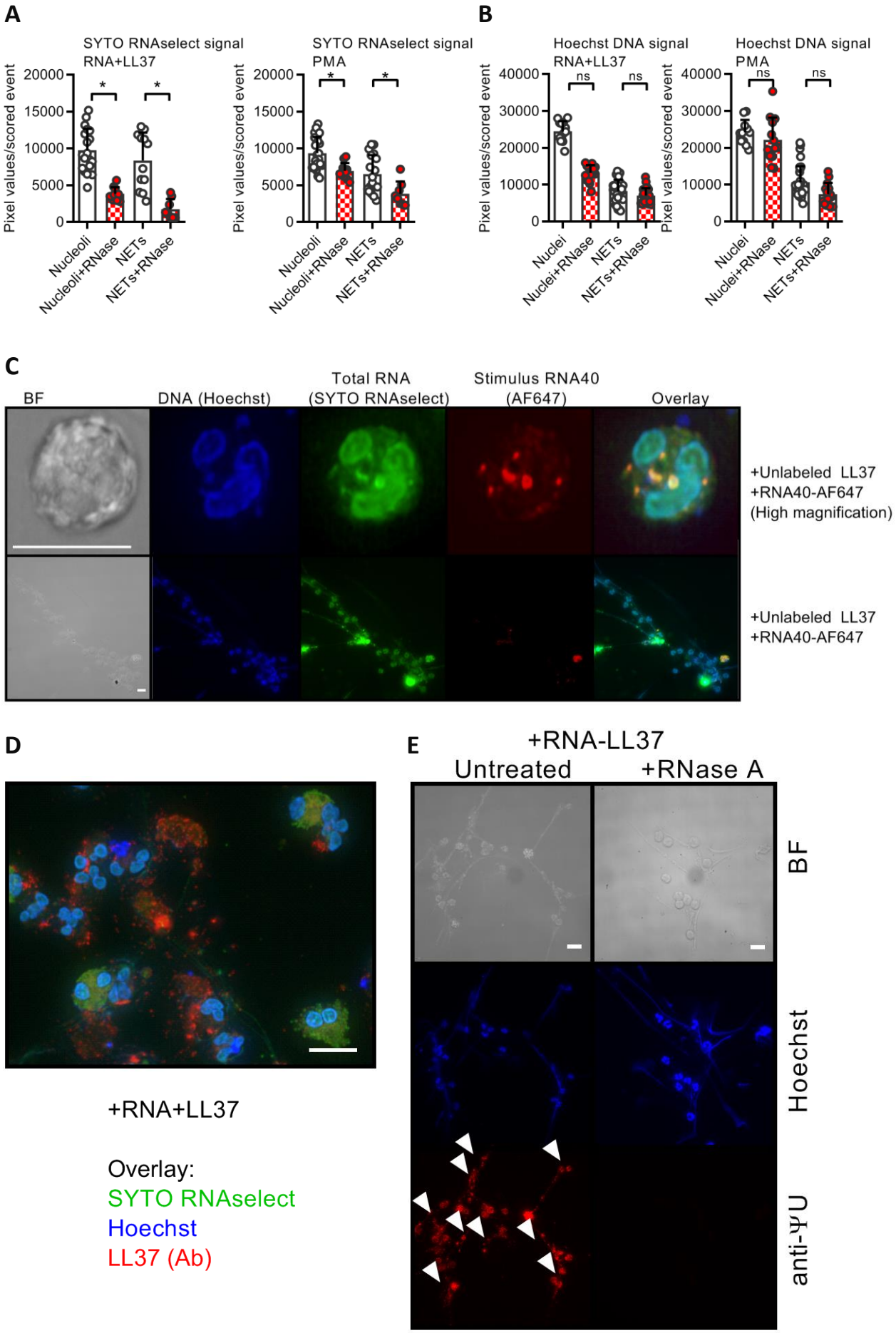
Figure 3.7: PMNs respond to stimulation with RNA-LL37 complex with NET formation

(A) EM pictures from PMNs stimulated with RNA-LL37 for 3 h (n=1), taken by Jürgen Berger at the MPI, Tübingen. (B) Neutrophil elastase (NE) released from PMNs stimulated for 3 h. 100 nM PMA was used as a positive control (n=8, each dot represents one donor). (C) Fluorescence microscopy of PMNs, stimulated with RNA-LL37 complexes or 600 nM PMA for 3 h. After fixation, the cells were subsequently stained with Hoechst33342 (nucleus, depicted in blue), anti-LL37 (unconjugated, rabbit, secondary antibody A647 labelled, depicted in red) and SYTO RNAselect (RNA, depicted in green) (n=6, scale bar = 10 µm). (D) Live-cell imaging of PMNs, stained with Hoechst33342 (nucleus) and SYTO RNAselect (RNA) and subsequently stimulated as indicated (n=4, scale bar = 20 µm). Pictures of 30 min and 60 min after stimulation are shown. (E) Quantification of live-cell-imaging. B and E represent combined data (mean+SD) from 'n' biological replicates. In A, C and D representative samples of 'n' replicates or donors are shown. Arrowheads indicate released RNA-containing NETs (C and D). * $p<0.05$ according to one-way ANOVA (B), two-way ANOVA (E) with Dunnett's correction.

3.3.2. RNA-LL37 complexes induce release of NET-associated-RNA (naRNA)

This study previously showed that RNA-LL37 induced NETosis and that those NETs in turn contained RNA and LL37. To prove the specificity of SYTO RNAselect, RNase A was used (Sharma *et al.* 1993) for RNA digest which yielded in significantly reduced RNA signal (SYTO RNAselect), leaving the DNA signal (Hoechst) unaffected (Figure 3.8A and B). Fluorescence microscopy using AF647 labeled RNA complexed with unlabeled LL37 as stimulus and subsequent staining with SYTO RNAselect revealed that the dye not just stained the synthetic RNA which was added to the cells but rather showed the total RNA in the cells or released in NETs (Figure 3.8C). Additionally, PMNs at the verge of NETosis or degranulation were observed, where the SYTO RNAselect dye was accumulating in granule-like vesicles (Figure 3.8D). It was assumed that RNA is potentially stored in granules of PMNs, as it was previously reported for eosinophils (Behzad *et al.* 2010).

Although the specificity of SYTO RNAselect was already proven by Li et al. (Li *et al.* 2006) and also in this study with previous experiments (Figure 3.8A-C), further confirmation was accomplished using an antibody against pseudoruidine (Ψ-U). Ψ-U is a frequent nucleotide modification exclusively found in RNA (Zhao and He 2015) and would thus also allow to distinguish stimulant RNA (which is synthetic and hence devoid of Ψ-U) and cellular RNA. When inducing NETosis by RNA-LL37 (but also with PMA as a control), Ψ-U-positive RNA in extracellular, fiber-like structures was clearly observed. When using RNase A, this RNA signal could be completely erased, whereas the DNA signal stayed unaffected (Figure 3.8E). Finally, when PMNs were stimulated with AF488-labeled synthetic RNA complexed with unlabeled LL37 and subsequently stained with Ψ-U-antibody (AF594-labeled secondary antibody), ssRNA40-AF488 could be depicted in defined cytosolic compartments, whereas Ψ-U was found spread over the whole cell or over the NET fibers rather than being accumulated in puncta (Figure 3.8F). These findings confirm the abundance of RNA in NETs which might serve as a new immunogenic stimulus. Since RNA has never before been reported as a component of NETs, the term "NET-associated-RNA" or short "naRNA" is proposed for future reference.


A
SYTO RNAselect signal
RNA+LL37
Pixel values/scored event
20000
15000
10000
5000
0
*
*
Nucleoli
Nucleoli+RNase
NETs
NETs+RNase
SYTO RNAselect signal
PMA
*
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B
Hoechst DNA signal
RNA+LL37
40000
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Nuclei
Nuclei+RNase
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PMA
C
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DNA (Hoechst)
Total RNA
(SYTO RNAselect)
Stimulus RNA40
(AF647)
Overlay
+Unlabeled LL37
+RNA40-AF647
(High magnification)
+Unlabeled LL37
+RNA40-AF647
D
+RNA+LL37
Overlay:
SYTO RNAselect
Hoechst
LL37 (Ab)
E
+RNA-LL37
Untreated
+RNase A
BF
Hoechst
anti-ΨU

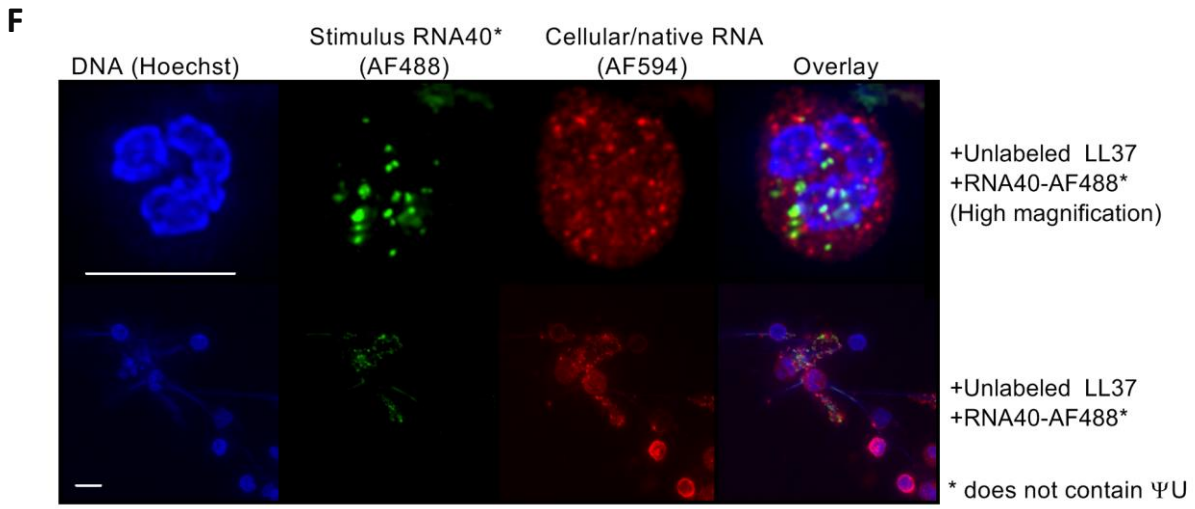


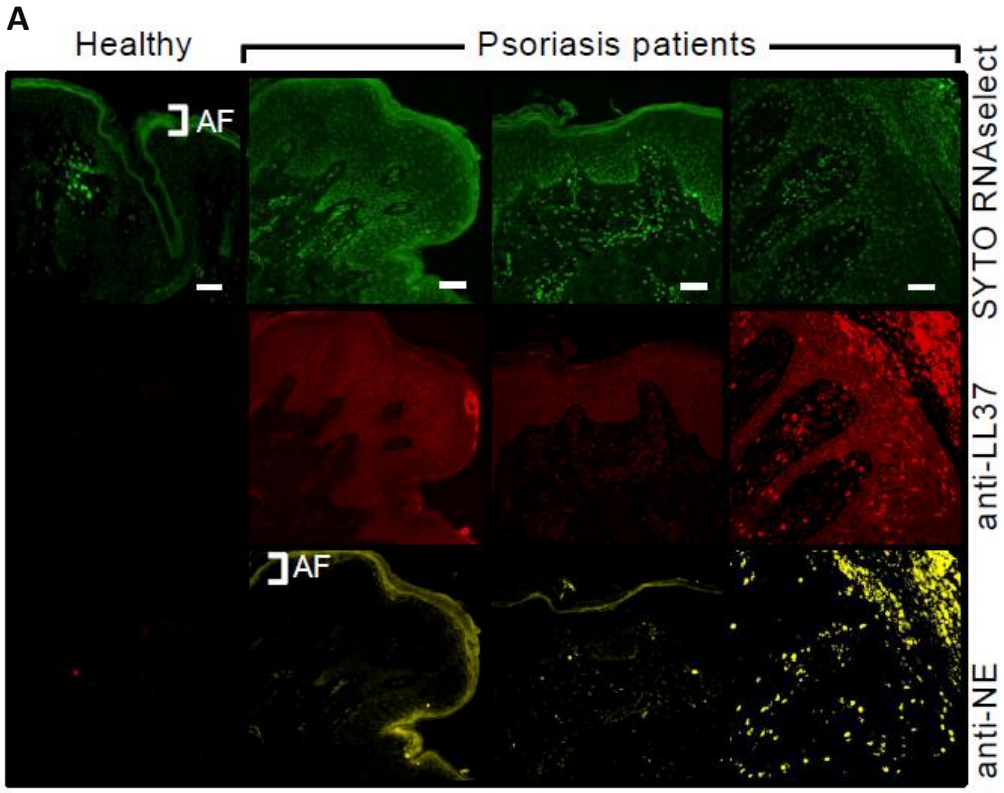
Figure 3.8: RNA-LL37 complexes induce the release of NET-associated-RNA (naRNA)

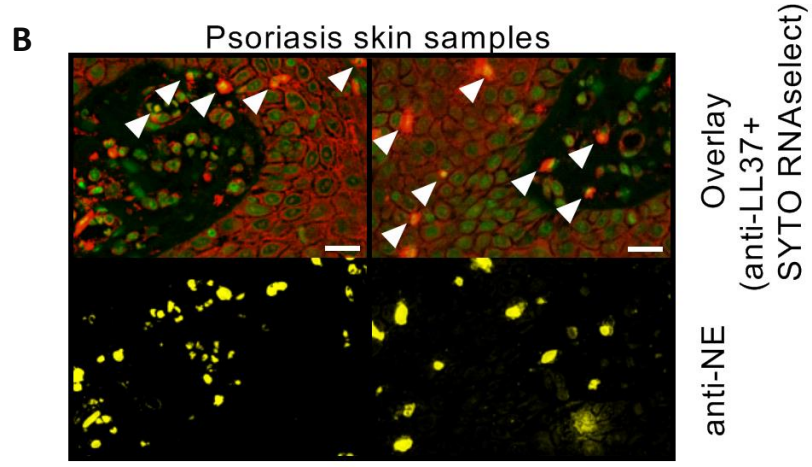
(A, B) Fixed Neutrophils, were treated with RNase A (100 µg/ml) or buffer control overnight at 37°C and subsequently stained with Hoechst33342 or SYTO RNAselect (n=4). Quantification of pixel values of nuclear or extracellular NET events scored by an independent and unbiased observer in the RNA (A) or DNA channels (B) is shown for one representative experiment. (C) Shows fixed PMNs, stimulated with RNA-AF647 (red) complexed to unstained LL37 for 1 h and subsequently stained with SYTO RNAselect (green) (n=2, scale bar = 10 µm). (D) Shows SYTO RNAselect (green) staining accumulation in granules, observed for every experiment (n = 10, scale bar = 10 µm). (E) PMNs were stimulated for 3 h with RNA-LL37 complexes (or PMA not shown here), treated with RNase A (100 µg/ml) or buffer control overnight at 37°C and subsequently stained with anti-Ψ-U (AF594 labeled secondary antibody, red) (n= 3, scale bar = 10 µm). (F) Stimulation as in (C) but using RNA-AF488 (green) and unlabeled LL37 for stimulation and subsequent staining with anti-ΨU (AF594 labeled secondary antibody, red) (n=2, scale bar = 10 µm). Arrowheads indicate RNA-containing NETs (E). In A-F one representative of 'n' replicates is shown. * $p<0.05$ according to one-way ANOVA with Holm-Sidak's correction (A) or Kruskal-Wallis test with Dunn's correction (B) to adjust for multiple testing.

3.3.3. RNA-LL37 complexes are found in psoriatic skin lesions

It was previously shown that increased levels of NETs were present in steady-state of blood-derived PMNs but also in skin of psoriasis patients (Hu *et al.* 2016). The presence of naRNA had obviously not been analyzed. It was therefore of interest to investigate whether more RNA and LL37 can be detected in psoriatic lesions, potentially released by NETting PMNs. As expected, high abundance of LL37, not only in the epidermis of the patient samples but also in the infiltrating PMNs (here additionally stained with anti-NE antibody), was observed. Interestingly, when using SYTO RNAselect, more RNA can be found throughout the whole epidermis of the patient skin (Figure 3.9A) compared to healthy controls but also due to the infiltrating cells which likewise contained RNA. When taking a closer look, RNA and LL37 appeared to be co-localized especially where PMNs infiltrated (Figure 3.9B). Also, SYTO RNAselect specificity for skin samples was confirmed using anti-ΨU: both signals clearly overlapped (Figure 3.9C). It can therefore be assumed that the RNA signal is attributable to naRNA. As RNA and LL37 are found in large quantities in psoriatic lesions, it was hypothesized

that RNA-LL37 complexes might have a physiological relevance in inflammation in psoriatic skin.





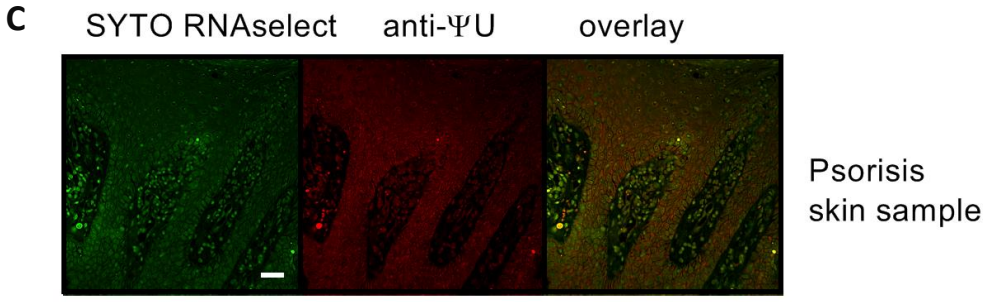


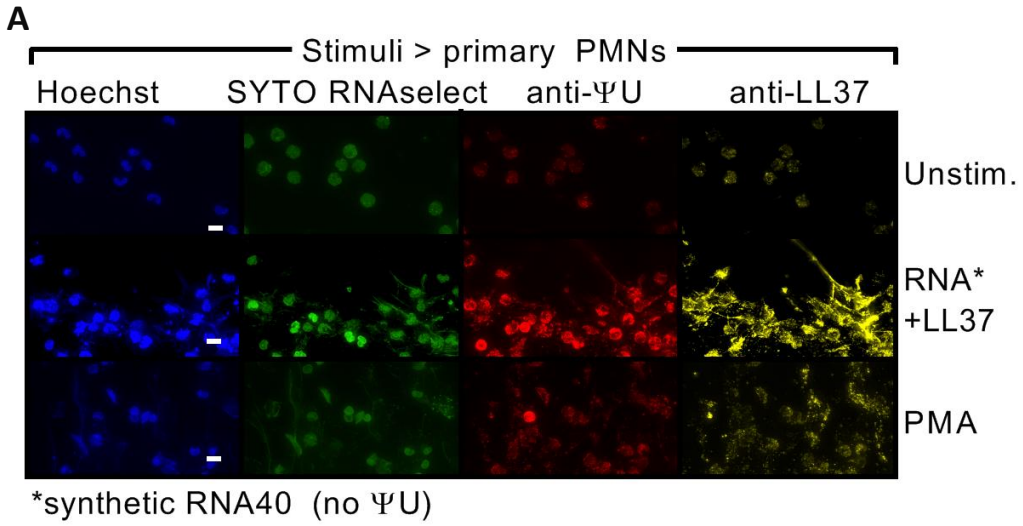
Figure 3.9: RNA-LL37 complexes are found in psoriatic skin lesions

(A,B) Skin sections from healthy skin and psoriatic lesions (n=12 patients and 3 healthy controls, scale bar = 20 µm), from patients with PASI score ≥ 10 and no systemic therapy at the time of skin biopsy collection, were stained with anti-LL37 (red), anti-NE (yellow) (with subsequent secondary antibodies anti-rabbit-AF647 and anti-mouse-AF594) and SYTO RNAselect (green). (B) Co-localization of RNA and LL37 is indicated by arrowheads. (C) SYTO RNAselect (green) staining strongly overlaps with anti-ΨU (red) (and subsequent anti-mouse-AF594) staining in psoriatic skin samples (n=12 patients, scale bar = 20 µm). AF = autofluorescence. In A representative samples of 'n' replicates or donors are shown. In B and C one representative of 'n' replicates is shown.

3.3.4. NET contents (naRNA-LL37 complexes) induce NETosis in further PMNs

Based on previous results, it was assumed that NETs induced by RNA-LL37 complexes (which contained naRNA and LL37) could trigger further NET formation in more PMNs. The repetitive release of naRNA and LL37 via NETosis, in turn could lead to a self-propagating inflammatory loop.

In fact, when using those NET-contents (from cells which nicely released NETs upon stimulation with RNA-LL37 complexes and PMA, as shown in Figure 3.10A) to stimulate naïve PMNs, those PMNs were prompt to "NET" (release) and NETs contained DNA, naRNA and LL37. In contrast, the "mock" NET contents from unstimulated PMNs were not able to induce any NET formation in naïve PMNs (Figure 3.10B, and quantified in Figure 3.10C). Taken together, this experiment showed that NETs released by RNA-LL37 has the ability to induce NET formation in naïve PMNs and their NETs contain further DNA, naRNA and LL37. Although it awaits confirmation *in vivo*, the self-propagating mechanism that could be envisaged, might be applicable to psoriatic skin as an early inflammatory stimulus (naRNA-LL37 complexes) and upstream event of pDC and T cell activation.



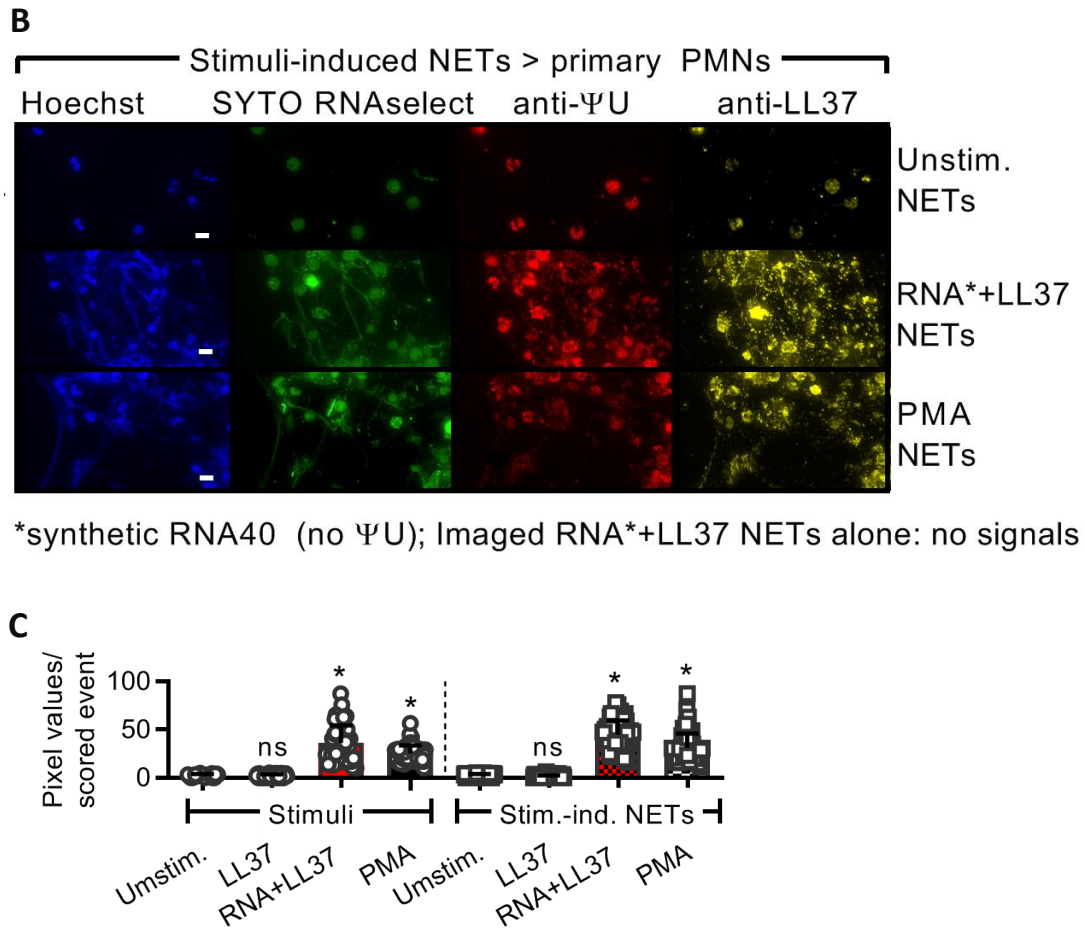


Figure 3.10: naRNA-LL37 complexes induce NETosis in further PMNs

(A) PMNs were stimulated with RNA-LL37 complex, LL37 alone or PMA (600 nM) for 4 h (n=4, scale bar = 10 µm). After fixation, cells were stained with anti-LL37 (yellow) (anti-rabbit-AF647 secondary antibody), anti-ΨU (red) (anti-mouse-AF594 secondary antibody), SYTO RNAselect (green) and Hoechst33342 (blue). These NET contents were harvested and (B) subsequently transferred to naïve PMNs (n=6, scale bar = 10 µm) for 4 h. Then, after fixation the cells were stained with anti-LL37 (yellow) (anti-rabbit-AF647 secondary antibody), anti-ΨU (red) (anti-mouse-AF594 secondary antibody), SYTO RNAselect (green) and Hoechst33342 (blue). (C) Quantification of (A) and (B) by showing pixel values per scored events. C represents combined data (mean+SD) from 'n' biological replicates. In A and B representative samples of 'n' replicates or donors are shown. * $p<0.05$ according to Kruskal-Wallis test with Dunn's correction (C).

3.4. RNA-LL37 complexes and NETs activate PMNs via TLR8 (human) and TLR13 (mouse)

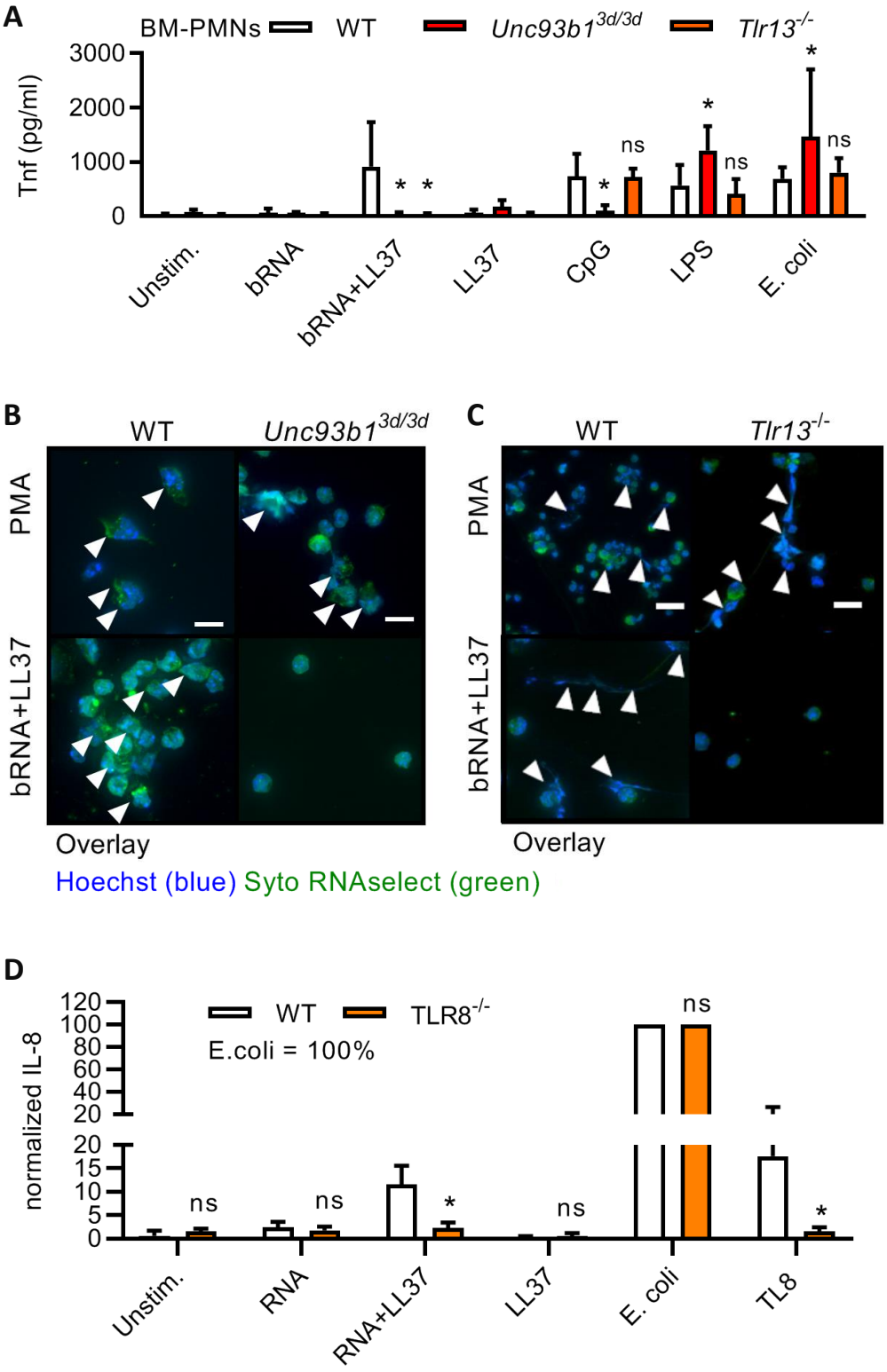
Previously it was observed that cytokine release from PMNs induced by RNA-LL37 complexes could be inhibited by blocking endosomal TLR signaling via chloroquine (Figure 3.3D). As CQ had no effect on cytosolic RNA sensors (Matsukura *et al.* 2007), the following experiments further focused on endosomal TLR signaling. Although it is now appreciated that PMNs survive a couple of days *in vivo* (Kruger *et al.* 2015), *in vitro* the cells are only very short-lived and are post-mitotic (Mayadas *et al.* 2014). Therefore, it is impossible to genetically modify them in culture e.g. via transfection or viral transduction. As a result, mouse BM-PMNs were used to study receptor-dependency. First, previous results (Figure 3.3D) were re-evaluated with BM-

PMNs from *Unc93b1*-deficient mice which lack functional endosomal TLR signaling because of a so-called "triple D" mutation (Tabeta *et al.* 2006) that suppresses the critical chaperone function of UNC93B1 for endosomal TLRs (Eigenbrod *et al.* 2012). For mouse BM-PMNs, bRNA together with (human) LL37 was used for stimulation, as this yielded the highest BM-PMN activation: bRNA-LL37 complexes were more potent in mouse PMNs to stimulate cytokine release compared to synthetic ssRNA40 (data not shown); and additionally, human LL37 was already shown to be more potent in binding DNA compared to the mouse ortholog CRAMP (Singh *et al.* 2013) (Gallo *et al.* 1997). The same effects were therefore assumed for RNA.

When stimulating BM-PMNs from *Unc93b1*-deficient mice, it was observed that cytokine release induced by bRNA-LL37 was completely dependent on endosomal TLR signaling. CpG, a known TLR9 agonist (Hemmi *et al.* 2000), served as control; cytokine release upon stimulation with CpG was also completely abolished in BM-PMNs from *Unc93b1*-deficient mice (Figure 3.11A). Of note, LPS or heat-killed E.coli (TLR4 ligands, (Poltorak *et al.* 1998)) were actually more potent in *Unc93b1*-deficient mice compared to WT mice.

As human PMNs were shown to functionally express endosomal TLRs but not TLR3 (Hayashi *et al.* 2003) and TLR7 (Janke *et al.* 2009) (Berger *et al.* 2012), it was assumed that TLR8 could be the RNA sensing receptor responsible for activation by RNA-LL37 complexes of human PMNs. Hence, testing of BM-PMNs from *Tlr13*-deficient mice was considered because of TLR13 being the murine equivalent for TLR8 in the humans (Li and Chen 2012) and TLR8 being non-functional in mice (Hemmi *et al.* 2002) (Heil *et al.* 2004). Indeed, when stimulating BM-PMNs isolated from *Tlr13*-deficient mice with bRNA-LL37 complexes cytokine release was abolished. Conversely, stimulation with LPS or heat-killed E.coli stayed unaffected (Figure 3.11A). After this very promising result, it was investigated next whether BM-PMNs from WT mice were able to induce NETosis in response to bRNA-LL37 stimulation and whether this was endosomal TLR- or even TLR13-dependent. When stimulating BM-PMNs from WT, *Unc93b1*-deficient or *Tlr13*-deficient mice with PMA, a chemical, TLR-independent NET-inducer (van der Linden *et al.* 2017) (Al-Khafaji *et al.* 2016), BM-PMNs from all mouse strains were able to induce NETosis. However, in response to bRNA-LL37 complexes only BM-PMNs from WT mice potently induced NET formation and BM-PMNs from *Unc93b1*- (Figure 3.11B) or *Tlr13*-deficient mice (Figure 3.11C) failed to do so.

Finally, to undoubtedly pinpoint TLR8 as receptor for RNA-LL37 complexes, a biological model system was used that closest resembles human innate immune responses. Therefore, since human PMNs cannot be engineered, BLaER1 cells (WT and TLR8$^{-/-}$) were used. BLaER1 cells are leukemic B cells that can be transdifferentiated to monocyte-/macrophage-like cells (Vierbuchen *et al.* 2017) and thus display a good model to study immune reactions in innate immune cells (Gaidt *et al.* 2018). With these cells it could be observed that cytokine release by RNA-LL37 complexes was completely dependent on TLR8 in the human system. As a control for TLR8 signaling TL8-506 (by Invivogen), a selective TLR8 agonist (Lu *et al.* 2012), was used (Figure 3.11D). These findings thus clearly show that RNA-LL37 complexes are dependent on TLR8 (human) and TLR13 (mouse) to induce robust cytokine and NET release in PMNs.


A
BM-PMNs
WT
Unc93b1 3d/3d
Tlr13 -/-
Tnf (pg/ml)
3000
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Unstim.
bRNA
bRNA+LL37
LL37
CpG
LPS
E. coli
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B
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Unc93b1 3d/3d
PMA
bRNA+LL37
Overlay
C
WT
Tlr13 -/-
PMA
bRNA+LL37
Overlay
Hoechst (blue) Syto RNAselect (green)
D
WT
TLR8 -/-
E.coli = 100%
normalized IL-8
120
100
80
60
40
20
15
10
5
0
Unstim.
RNA
RNA+LL37
LL37
E. coli
TL8

Figure 3.11: Cytokine and NET release induced by RNA-LL37 complexes is dependent on TLR8 (human) and TLR13 (mouse)

(A) BM-PMNs from WT, Unc93b$^{3d/3d}$ and Tlr13$^{-/-}$ mice were isolated and stimulated for 5 h with bRNA, LL37, bRNA-LL37 complexes, 1 µM CpG, 200 nM LPS and heat-killed E. coli MOI = 1 (provided by Tatjana Eigenbrod, Heidelberg) (n=4 Tlr13$^{-/-}$, n=5 Unc93b1$^{3d/3d}$, n=8 WT). (B) BM-PMNs were stimulated for 16 h as in (A), additionally using 600 nM PMA as a positive control and fixed. Subsequently fluorescence microscopy was performed, using Hoechst33342- (DNA, blue) and SYTO RNAselect- (RNA, green) stained cells (n=5 Unc93b1$^{3d/3d}$, n=8 WT; scale bar = 10 µm). (C) as in (B) but using WT and Tlr13$^{-/-}$ BM-PMNs (n=4 Tlr13$^{-/-}$, n=8 WT; scale bar = 20 µm). (D) *TLR8* CRISPR-edited BLaER1 cells were stimulated with RNA, LL37, RNA-LL37 complex, heat killed E. coli MOI = 1 and 100 ng/ml TL8-506 for 18 h and IL-8 ELISA was measured (n=7). The values were normalized to E. coli = 100%. Arrowheads indicate released NETs (B and C). A and D represent combined data (mean+SD) from 'n' biological replicates. In B and C one representative of 'n' replicates is shown (mean+SD of technical triplicates). * $p<0.05$ according to tone-way ANOVA with Sidak correction (A, D).

3.5. NET and cytokine release induced by RNA-LL37 complexes can be blocked by iODNs

After identifying TLR8 for being a receptor for RNA-LL37-induced immune reactions, it was further analyzed whether receptor binding could be potentially blocked with so-called inhibitory oligodeoxynucleotides (iODNs). iODNs were previously characterized in mice and are directed towards TLR7 (IRS661) or TLR7/9 (IRS954) to block ligand-mediated activation (Pawar *et al.* 2007). Both of the inhibitors used here were already proposed as treatment for another autoimmune disease, namely Systemic Lupus Erythematosus (SLE) (Barrat *et al.* 2005) (Barrat and Coffman 2008). Because of the similarities found in endosomal TLRs (Colak *et al.* 2014), it was assumed that the TLR7 antagonists might also block TLR8 signaling. Based on previous results obtained by a former member of the lab, David Eisel, PMNs from healthy donors were pre-incubated for 30 min with very low amounts of iODNs (IRS661 1 nM and IRS954 50 nM, not toxic for PMNs shown in Figure 6.4) and subsequently stimulated with RNA-LL37 complexes for 4 hours. And indeed, not only IL-8 and MIP-1β (Figure 3.12A, B) release from PMNs induced by RNA-LL37 complexes could be inhibited but this was also specific for TLR8 signaling, because LPS-induced cytokines were not affected (Figure 3.12C). Importantly, iODNs could also inhibit NETosis induced by RNA-LL37 complexes (Figure 3.12D and quantification in E), again at very low concentrations (IRS661 50 nM). These results are in agreement with previous findings on TLR8-dependent inhibition of NETosis induced by HIV infection (Saitoh *et al.* 2012). Based on these results, it can be concluded that iODNs might be useful to block TLR8-mediated binding of RNA-LL37 complexes and hence as a therapeutic intervention in psoriasis. Blocking of TLR8 signaling potentially inhibits cytokine and NET release induced by RNA-LL37 complexes and can potentially intervene with an early inflammatory event in psoriasis.

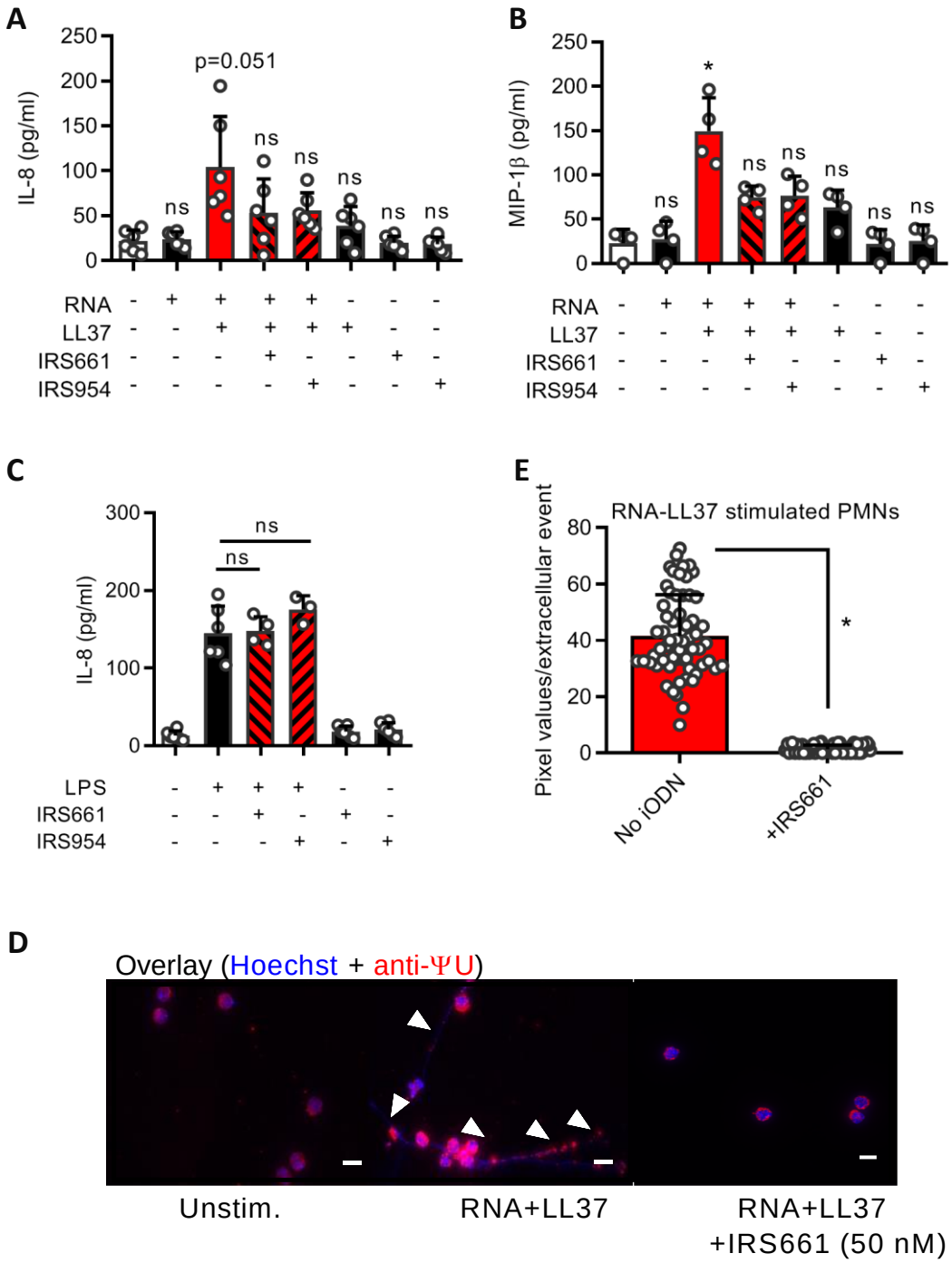


Figure 3.12: NET and cytokine release induced by RNA-LL37 complexes can be inhibited by iODNs

(A) PMNs were pre-treated with IRS661 (1 nM) or IRS954 (50 nM) for 30 min and then subsequently stimulated with RNA-LL37 complexes for 4 h (n=6). IL-8 release was measured by ELISA. (B) the same as (A) but measuring MIP-1β (G, n=4). (C) as (A) but using 200 ng/ml LPS as stimulus (n=4-6). (D) PMNs were pre-treated with IRS661 (50 nM) for 30 min and subsequently stimulated for 3 h with RNA-LL37 complexes. After fixation, fluorescence microscopy was performed with Hoechst33342- (nuleus, blue) and anti-ΨU- (RNA, red) (anti-mouse-AF594 secondary antibody) stained PMNs (n=3, scale bar = 10 µm). (E) Quantification of D by showing pixel values per counted extracellular events. A-C, and E represent combined data (mean+SD) from 'n' biological replicates (each dot represents one donor or value). Arrowheads indicate released NETs (D) * $p<0.05$ according to one-way ANOVA with Sidak correction (A and C) or Friedmann test with Dunn correction (B), and Mann-Whitney test (E).

4. Results part II

4. Platelet-PMN aggregates promote skin pathology in psoriasis

4.1. Circulating PMNs in psoriasis have a distinct platelet signature

In psoriasis, a variety of different immune cells infiltrate the skin. However, the major trigger for immune cells to infiltrate the skin in psoriasis remains elusive. In order to find surface antigens differentially expressed on cells from whole blood of psoriasis patients, which might contribute to skin homing, the so-called LEGENDScreen™ from BioLegend was performed. This kit contains 332 antibodies against different surface antigens and 10 corresponding isotype controls, all labeled with PE and are pre-aliquoted in individual wells in a total of 4 x 96-well plates (Figure 6.5A). Combined with the antibodies against CD15, CD3, CD19 and Zombie yellow (as a master mix added to each well), this further allowed to phenotype PMNs, T cells and B cells (excluding dead cells) respectively from different psoriasis patients and healthy donors for 332 unique surface antigens, in order to identify those differentially expressed in patients vs. controls. Whole blood samples from five patients with a PASI score of ≥ 10 and no systemic therapy at the time of blood sampling and five sex- and age-matched controls was analyzed.

4.1.1. Surface antigens are differentially expressed on blood cell populations of psoriasis patients

Whole blood from five patients and five healthy controls was used and, as aforementioned, additionally stained with CD15 (PMNs), CD3 (T cells), CD19 (B cells) and Zombie yellow (dead cells). Subsequently, the LEGENDscreen™ was performed. This was based on an already published approach investigating T cells (Graessel *et al.* 2015). The monocyte population was afterwards gated by size and granularity. However, CD14 positivity was confirmed by the antibody provided by the kit (PE-labeled antibody, kit content). In the following paragraph the term “monocytes” refers to cell, characterized by granularity and size. The general workflow and gating strategy of the experiment are shown in Figure 6.5A and B. Differences based on MFI (mean fluorescence intensity) raw data for each surface antigen and isotype control from each of the gated populations were calculated by Marius Codrea and Simon Heumos from QBIC (Quantitative Biology Center, Tübingen). Conceptually, they first analyzed significant differences in surface antigen expression between the two groups. Defined by $p<0.1$ (nominal by two-way ANOVA followed by Tukey’s multiple comparisons correction), differential expression of antigens for PMNs, T cells, B cells and monocytes (comparing psoriasis patients and healthy controls) were identified. In total, 30 antigens were identified to be significantly different on blood cells over several populations between psoriasis patients and healthy controls.

Figure 4.1A-E shows significantly different surface antigens for PMNs and in Table 4.1 and Figure 6.6 all significant differences in surface antigen expression with short explanation are listed or box plots are shown. Most affected were monocytes as a whole, with 12 surface antigens being significantly different between psoriasis patients and healthy controls.

With this experiment, it was confirmed that there are significant differences in surface antigen expression on circulating blood cells of psoriasis patients and healthy controls. PMNs as the cells of interest were analyzed in further detail but selected antigens will nevertheless be discussed below (see paragraph 6.2.1.).

Table 4.1: Significantly different surface antigen expression (psoriasis patients vs. healthy controls)

surface antigen	name*	cell type	up/down in patients	short explanation
CD20	B1 or Bp35	Monocytes	up	Plays a role in B cells differentiation into Plasma cells (Vale and Schroeder 2010)
CD21	Complement C3d receptor (C3dR), complement receptor 2 (CR2), Epstein-Barr virus receptor	Monocytes	up	Has been shown to interact with CD19 on B cells (Bradbury *et al.* 1992)
CD22	BL-CAM, Siglec-2, Lyb8	Monocytes	up	Belongs to SIGLEC family and is expressed on mature B cells (Crocker *et al.* 1998)
CD11c	Integrin αX subunit, CR4, p150, ITGAX	B cells	down	Expressed on a variety of different blood cells, potential role in phagocytosis and antigen presentation (Sadhu *et al.* 2007) (Collin *et al.* 2013)
CD13	Aminopeptidase N, APN, gp150	Monocytes	up	Expressed on granulocytes, myeloid progenitors and involved in processing of cytokines (Proost *et al.* 2007)
CD56	Leu-19, NKH1	Monocytes	up	Expressed on NK and NK-T cells (Almehmadi *et al.* 2014)
CD41	gpIIb, CD41a	Monocytes	up	Forms complexes with CD61. CD41 is required for platelet adhesion and aggregation (Anderson *et al.* 1991) (Mateo *et al.* 1996)
CD61	Integrin β3, gpIIIa	Monocytes	up	See CD41
CD162	PSGL-1, p-selectin glycoprotein ligand-1	Monocytes	up	Binds to P-selectin (CD62P); rolling (Xu *et al.* 2007)
CD163	GHI/61, M130, RM3/1, p155,	Monocytes	up	Upregulation happens in macrophages when it

	Hemoglobin/Haptoglobin Complex Receptor, macrophage-associated antigen			switches to pro-inflammation (Etzerodt and Moestrup 2013)
CD154	CD40L, gp39, TRAP, T-BAM, TNFSF5	Monocytes	up	Expressed on activated T cells and induces class switch in B cells (Lederman *et al.* 1994)
CD89	FcαR	Monocytes	up	Expressed on neutrophils, and monocytes/macrophages (Morton and Brandtzaeg 2001)
CD124	IL-4 receptor α subunit	B cells	up	Mostly responsible for Th2 responses (Gilmour and Lavender 2008)
CD209	Dendritic Cell-Specific Intercellular adhesion molecule 3 (ICAM-3)-Grabbing Nonintegrin	PMNs	down	Is a member of the C-type lectin family and can bind and transmit HIV (Geijtenbeek *et al.* 2000)
CD143	ACE, kininase II, dipeptidyl dipeptidase 1, peptidase P, carboxycathepsin	PMNs	down	angiotensin I and bradykinin, acting as a blood pressure regulator and is expressed on endothelial cells (Iwai *et al.* 1987)
CD215	IL-15RA, IL-15Rα, IL-15 Receptor α subunit	B cells	down	Binds IL-15 in high affinity (Giri *et al.* 1994)
CX3CR1	V28, GPR13, Chemokine C-X3-C receptor 1	T cells	down	Mediates both leukocyte adhesion and migration (Imai *et al.* 1997)
Delta Opioid receptor	OPRD, DOR, hDOR	B cells	down	Mainly expressed in the brain (Erbs *et al.* 2015)
CD261	TRAIL-R1, Apo-2, CD261, TNFRSF10A	B cells	up	Member of TNFR superfamily induces apoptosis (Dufour *et al.* 2017)
CD235ab	Glycophorin A/B, GPA/GPB	PMNs	up	expressed on red blood cell membrane, and erythroid precursors (Bruce *et al.* 1994)
CD272	BTLA, B and T lymphocyte attenuator	Monocytes	up	Co-inhibitory receptor comparable to e.g. PD-1 Induction of peripheral tolerance in vivo (Liu *et al.* 2009)
CD337	NKp30, NCR3, Activating NK receptor NKp30, natural cytotoxicity triggering receptor 3	PMNs	down	Expressed on resting and activated NK cells (Warren *et al.* 2005)
CD244	2B4, NAIL, SLAMF4	T cells	down	Ligation of CD244 induces enhanced NK cytotoxicity

				(Stark and Watzl 2006)
CD267	TNFRSF13B, CD267, Transmembrane Activator and CAML Interactor (TACI)	B cells	down	Important for humoral immunity, negative regulator of B cells (Sakurai *et al.* 2007)
FcεRIα	high affinity IgE receptor	Monocytes	up	Expressed on variety of inflammatory cells Plays a key role in allergy (Fiebiger *et al.* 1996)
CD268	TNFRSF13C, BAFF-R, BAFFR, BR3, BAFF Receptor	Monocytes	up	Co-stimulation of B cells See also CD267 (Sakurai *et al.* 2007)
IgD	Ig delta chain C region	Monocytes	up	Expressed in naïve B cells (Noviski *et al.* 2018)
Siglec-8	Sialic acid-binding Ig-like lectin 8 (Siglec-8), Siglec8L, Sialoadhesin family member 2 (SAF2)	Monocytes	down	Expressed mainly on eosinophils (Bochner 2009)
TRA-1-60-R	Podocalyxin, TRA-1	PMNs	down	Expressed on human embryonic stem cells, downregulated when cell differentiates (Pera *et al.* 2000)
MSC (W5C5)	Mesenchymal Stem Cells (MSC), Sushi domain containing 2 (SUSD2)	B cells	up	Expressed by bone marrow mesenchymal stem cells (Sivasubramaniyan *et al.* 2013)

*** names were downloaded from the data sheet provided by the LEGENDscreen™ from BioLegend**

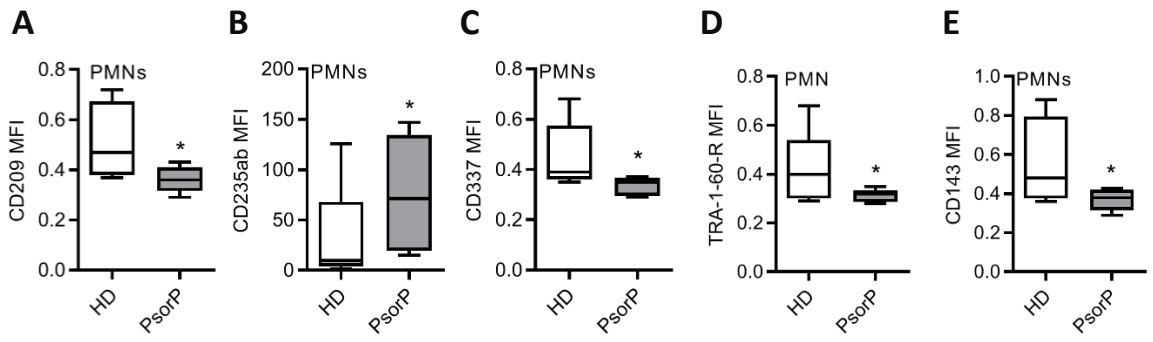


Figure 4.1: In PMNs from psoriasis patients, certain surface antigens are differentially expressed compared to PMNs from healthy controls

(A-E) Surface antigens (CD209, CD235ab, CD337, TRA-1-60-R and CD143 see Table 4.1 and Figure 6.6 for further information) with significant differences in MFIs between healthy donors (HDs) and psoriasis patients (PsorPs), n=5 each. A-E represent combined data (mean+SD) from 'n' biological replicates. Statistical analysis was performed by Marius Codrea and Simon Heumos from QBIC. * p<0.1 nominal by two-way ANOVA followed by Tukey's multiple comparisons correction.

4.1.2. Circulating PMNs from psoriasis patients display a distinct platelet signature

Although differences in individual surface antigen expression were clearly detected, psoriatic and healthy cell populations supposedly differ more significantly in combined expression of specific surface antigens. In order to adequately distinguish patients from healthy donors by combinations of surface antigens (later referred to as “signature”) a global principle component analysis (PCA) was performed (further information is provided in the section “Materials and Methods”). Surprisingly, especially for PMNs, the psoriasis patients strongly clustered together (Figure 4.2), indicating that PMNs of psoriasis patients can strictly be defined and are different from PMNs from healthy controls. For the other cell types, the variance was much larger and therefore the clusters were not so prominent (Figure 6.7A-C).

Mapping of those antigens, which mostly contribute to the separation of the groups, showed that for PMNs mainly five different antigens, namely CD6, CD11c, CD41, CD61 and CD235ab were responsible for the distinct “signature” of psoriasis PMNs in circulation. For monocytes, there was a much larger group of surface antigens contributing to the separation of groups. Also, B and T cells from patients or healthy controls only clustered weakly (Figure 6.7A-C).

Interestingly, CD6 has previously been shown to be important for T cell activation (Carrasco *et al.* 2017) and binds to the ligands CD166 (Bowen *et al.* 1995) and CD318 (Enyindah-Asonye *et al.* 2017) on epithelial cells like keratinocytes. CD11c is a known antigen found on a variety of blood cells in humans, e.g. classical DCs, monocytes but also to a lower extend on PMNs and B cells (Boltjes and van Wijk 2014) and was considered to be important for phagocytosis and for DC antigen presentation (Sadhu *et al.* 2007) (Collin *et al.* 2013). CD235ab, however, was is expressed on terminally differentiated erythrocytes (Bruce *et al.* 1994) (some of these antigens are further discussed in section 5.2.1.). When taking a closer look on the aforementioned antigens that contribute to the psoriasis PMN signature, it was appreciated that two antigens are known platelet antigens, in particular CD41 and CD61. CD41, also known as integrin α2b which is usually associated with CD61 (integrin β3) and is required for platelet adhesion and aggregation (Mateo *et al.* 1996) (Anderson *et al.* 1991).

Collectively, these data show that a group of five surface antigens can clearly discriminate between psoriasis patients and healthy controls, with platelet antigens as major representatives in the psoriasis PMN signature.

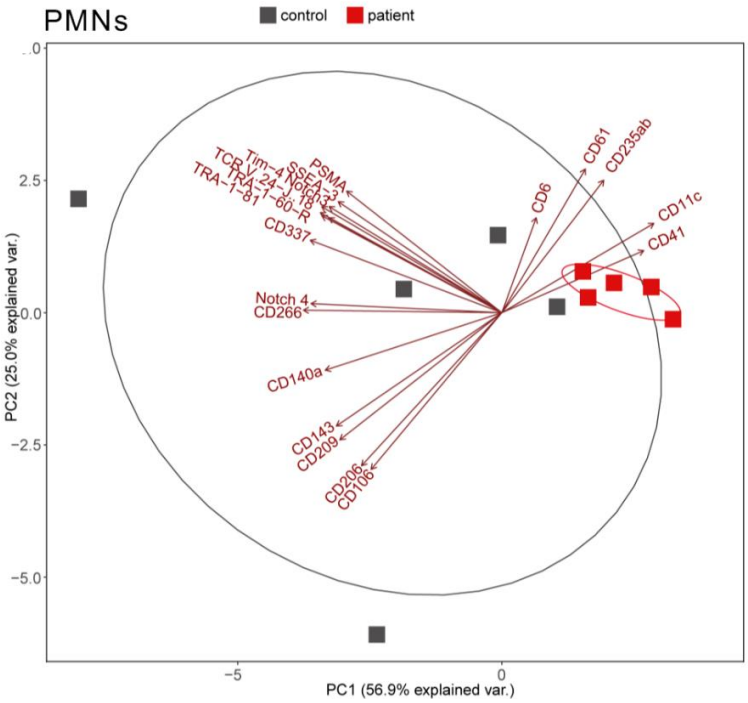


Figure 4.2: The PMN signature of psoriasis patients is defined by five surface antigens

Principal component analysis of PMN surface antigens for healthy donors (red) and psoriasis patients (grey). The top significant antigens (based on nominal p-value <0.1) which contribute to a separation of patients and healthy donors (n=5). Represents combined data (mean+SD) from 'n' biological replicates (each dot represents one donor).

4.2. Circulating PMNs directly interact with platelets in psoriasis

4.2.1. Platelet-PMN aggregates are found in blood from psoriasis patients

As shown before, PCA revealed that the signature of circulating psoriasis PMNs was mainly dependent on five surface antigens, namely CD6, CD11c, CD41, CD61 and CD235ab. As PMN infiltration of the skin is a hallmark of psoriasis, it was believed that one or more of those antigens could potentially explain PMN skin homing. First, the main focus was on CD41 and CD61 (as known platelet antigens) surface expression of psoriasis PMNs.

According to the literature, platelet (PLT)-leukocyte aggregate formation is often observed in an inflammatory background (Habets *et al.* 2013). They also seem to play a role in various experimental *in vivo* models for inflammatory skin diseases e.g. Atopic dermatitis (AD) (Tamagawa-Mineoka 2015). PMN-PLT aggregates are additionally frequently shown to form in whole blood which was also used for this study. Chanarat and Chiewsilp furthermore found that PMN-PLPT aggregates can be diminished by Ficoll density separation (Chanarat and Chiewsilp 1975). To investigate whether PMNs of psoriasis patients *de novo* expressed platelet surface antigens or whether platelets bound to PMNs, whole blood derived- and Ficoll-separated PMNs and PBMCs from additional psoriasis patients and healthy controls were analyzed. In Figure 4.3A, the initial MFIs from the LEGENDScreen™ are shown again. There, it was observed that CD41 and CD61 expression was higher on psoriasis PMNs compared to PMNs from healthy controls, but this did not reach statistical significance. When investigating

CD41 and CD61 levels on more patients and healthy controls in unmanipulated whole blood, it was further observed that more PMNs in the patient cohort were positive for CD41 and CD61 compared to PMNs from healthy controls (Figure 4.3B). Interestingly, the amounts of CD41 and CD61 positive PMNs in the psoriasis cohort after Ficoll separation were decreased, resulting in comparable antigen levels as observed for healthy controls (Figure 4.3C). Similar results were obtained when investigating monocytes (Figure 6.8A-C).

These results indicate that the platelet-antigen signature previously observed in the LEGENDScreen™ is due to increased platelet-PMN aggregate formation in the blood of psoriasis patients, rather than *de novo* expression of platelet antigens by psoriasis PMNs.

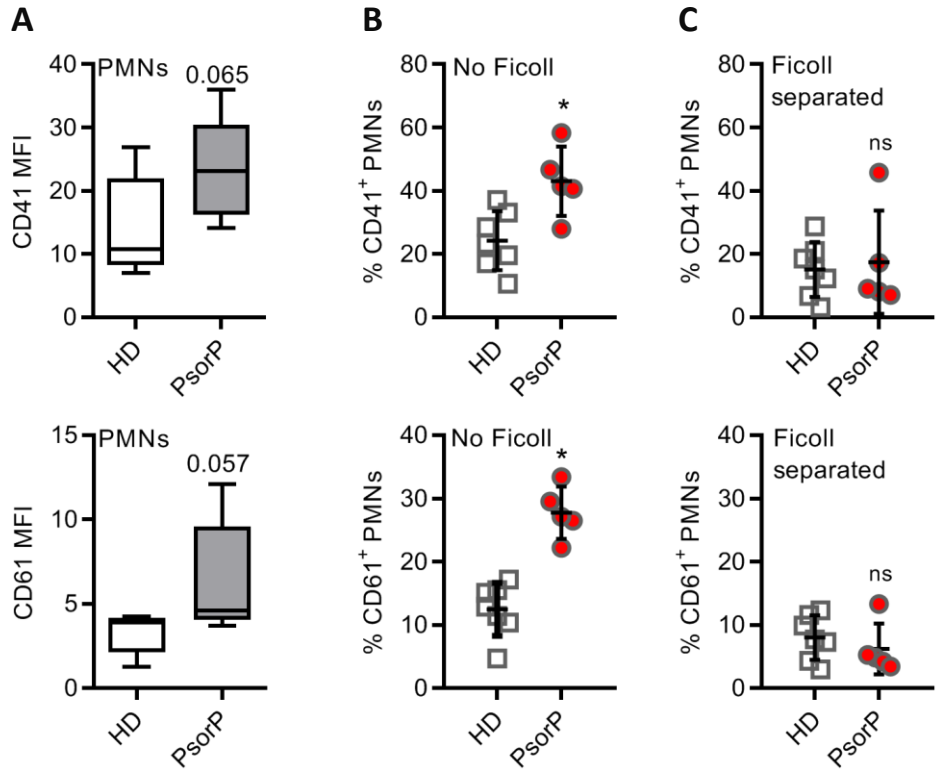


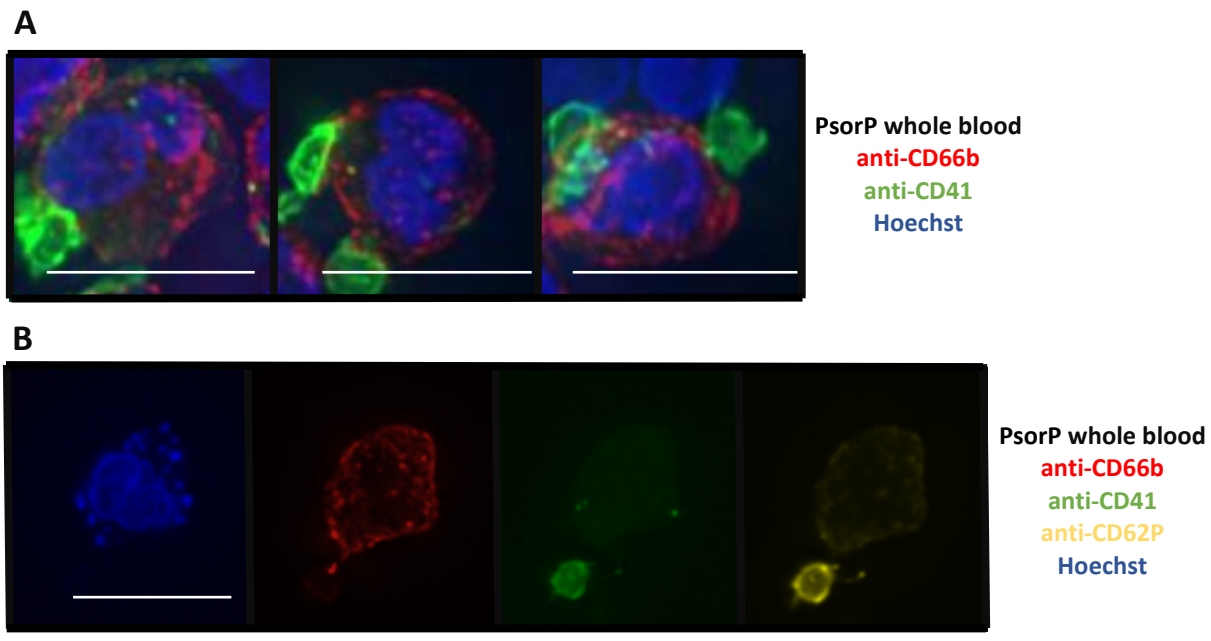
Figure 4.3: Platelets cover the surface of psoriatic PMNs in whole blood

(A) Mean fluorescence intensity (MFI) extracted from the LEGENDscreen™ for CD41 (upper panel) and CD61 (lower panel) (healthy donor (HD) and psoriasis patient (PsorP); n=5 each). FACS analysis of CD41- or CD61-positive PMNs (defined as $CD15^+CD66b^+$ and $CD41^+$ or $CD61^+$) analyzed in HDs or PsorPs (HD n=7, PsorP n=5) in whole blood samples (B) or Ficoll density gradient centrifugation (C). % of positive PMNs is shown. A-C represent combined data (mean+SD) from 'n' biological replicates, each dot represents one donor. * $p<0.05$ according to two-way ANOVA followed by Tukey's multiple comparisons correction (A) and Mann-Whitney test (B, C).

4.2.2 PMN-platelet aggregate formation can be confirmed by fluorescence microscopy and is observed in blood of IMQ-treated mice

Next, PMN-platelet aggregate formation was further confirmed using fluorescence microscopy. Whole blood from psoriasis patients was used and PMNs and platelets were labeled with CD66b and CD41 respectively. Indeed, small anucleated $CD41^+$ cells adjacent to the surface of $CD66^+$ PMNs (Figure 4.4A) were frequently observed. Interestingly, most of those $CD41^+$ cells on the surface of $CD66b^+$ PMNs also expressed the platelet activation marker CD62P (P-selectin, Figure 4.4B). This observation is in good agreement with previous studies (Sreeramkumar *et al.* 2014), where PMNs interacted with activated $CD62P^+$ platelets at the sites of inflammation. This finding further confirms that the platelet-signature of psoriasis PMNs found via the LEGENDScreen™ was due to increased numbers of aggregates which formed more prominently between PMNs and activated platelets in the blood of patients. Unfortunately, this analysis does not allow for establishing causality, i.e. answer whether psoriasiform disease activity and PMN-PLT aggregation were linked or merely coincidental. To probe for a direct link *in vivo*, the relationship between PMN-PLT aggregates and psoriatic inflammation was investigated in an Imiquimod (IMQ)- induced model of psoriasiform skin inflammation in mice (with the help of Nate Archer, group of Lloyd Miller, Baltimore, USA). In this model, application of the TLR7 agonist IMQ induces skin inflammation with PMN infiltration mimicking the human disease (Gilliet *et al.* 2004).

Interestingly, similar results could be obtained in the *in vivo* mouse model as in the human system: Even though inflammation was primarily induced in the skin, more PMN-platelet and more monocyte-platelet aggregates were found in the circulation of IMQ-treated mice compared to mock-treated controls (Figure 4.4C and D). This suggests, that aggregates of PMNs and platelets in the blood of both psoriasis patients and psoriasis-affected mice could be a result of a feedback loop of inflammation in the skin



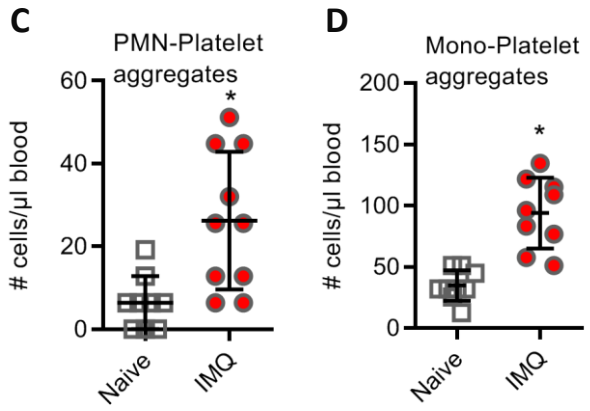


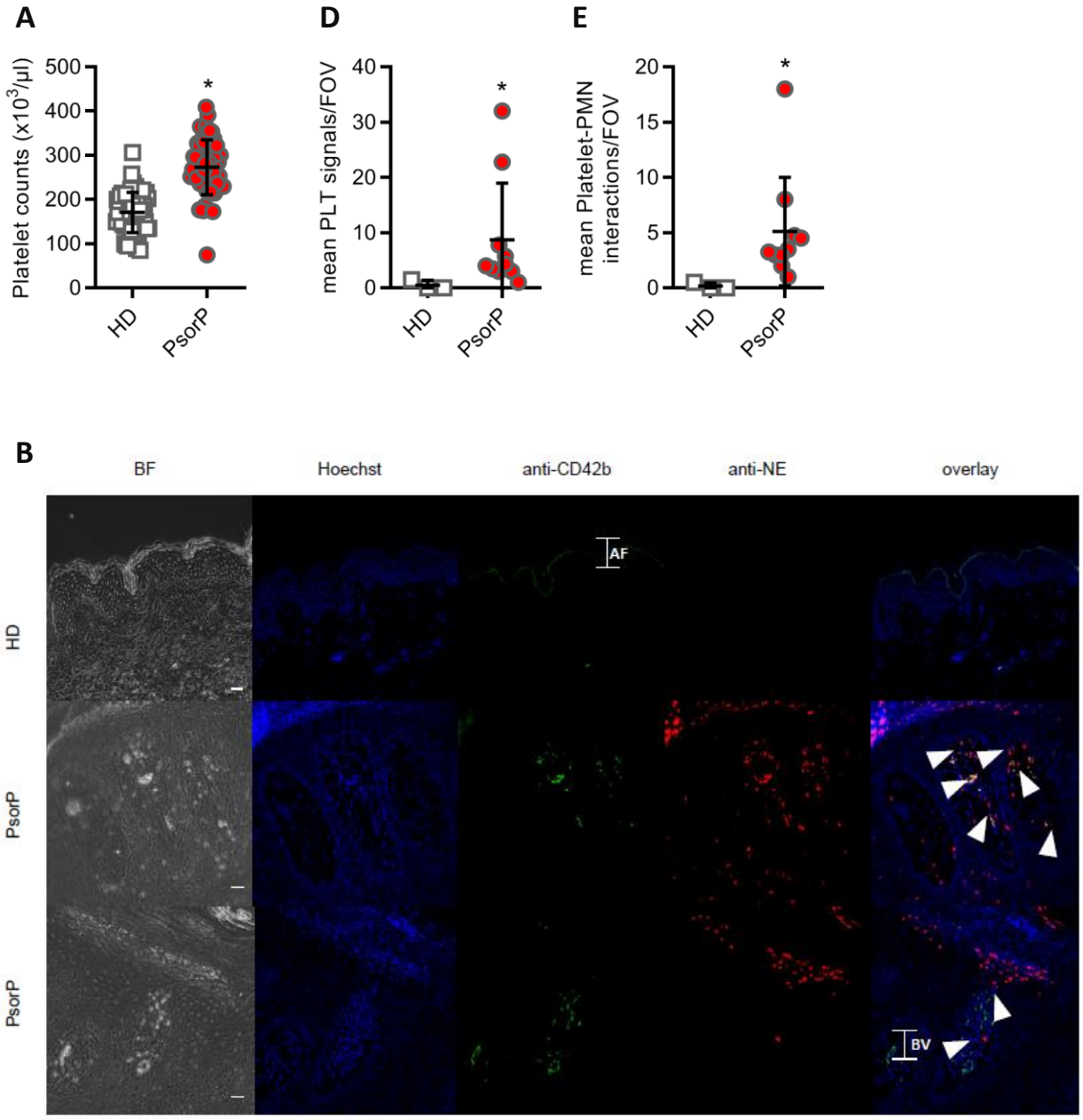
Figure 4.4*:* PMN-platelet aggregates can be found in blood from psoriasis patients and in IMQ treated mice

(A) Fluorescence microscopy of PMNs in a PsorP whole blood sample stained as indicated (scale bar = 10 µm), using CD66b AF647 (red) to label PMNs and CD41 PE (green) for platelets. (B) as in (A) but additional staining with CD62P AF488 (green, and CD41 PE depicted in yellow) as platelet activation marker is shown. The nucleus was stained in both pictures using Hoechst33342 (blue). (C and D) Mean number of PMN-platelet (C) or monocyte-platelet (D) aggregates comparing naïve (day 0) and IMQ-treated (day 5) mice (n=10 each). C and D represent combined data (mean+SD) from 'n' biological replicates (each dot represents one mouse). In A, B one representative donor is shown. * $p<0.05$ according to unpaired Student's t-test (C, D). The *in vivo* mouse experiments were performed by Nate Archer.

4.3. Platelets are found in psoriatic lesions

Previous experiments already showed that more PMN-platelet aggregates are found in the blood of psoriasis patients. Next, it was investigated if total platelet counts in general were elevated in psoriasis patients. Indeed and in keeping with literature (Unal 2016), more platelets in general in the blood of patients (62% more than for the healthy controls, Figure 4.5A) were found. This increased number of PLTs might thus facilitate the formation of PMN-PLT aggregates in the blood. Further, it was previously reported that platelets can help PMNs to infiltrate sites of inflammation (Sreeramkumar *et al.* 2014) (for psoriasis this would be the skin). Therefore, it was further investigated whether platelets may even be detectable in psoriatic lesions and potentially in proximity with PMNs. Skin sections of psoriasis patients and healthy controls were stained for PMNs using an anti-neutrophil elastase (NE) antibody and for platelets with anti-CD41 and -CD42b (only CD42b shown) antibodies. Surprisingly, platelet aggregates were found in psoriatic lesions (Figure 4.5B, quantified in D). In samples with high numbers of PMNs, platelets could additionally be found in close proximity to PMNs (Figure 4.5C, quantified in E). Conversely, neither PMNs nor platelets were found in healthy skin samples (Figure 4.5B and C).

Based on these findings, it was hypothesized that platelets infiltrate lesional skin, mainly together with PMNs. Additionally, it appeared conceivable that PMNs require the help of platelets. These results are in good agreement with previous findings, where PMNs needed platelets to infiltrate inflamed venules via interaction of P-Selectin (CD62P) on platelets and P-selectin glycoprotein ligand 1 (PSGL-1) on PMNs (Sreeramkumar *et al.* 2014).


A
Platelet counts (x10^3/µl)
500
400
300
200
100
0
HD
PsorP
*
D
mean PLT signals/FOV
40
30
20
10
0
HD
PsorP
*
E
mean Platelet-PMN interactions/FOV
20
15
10
5
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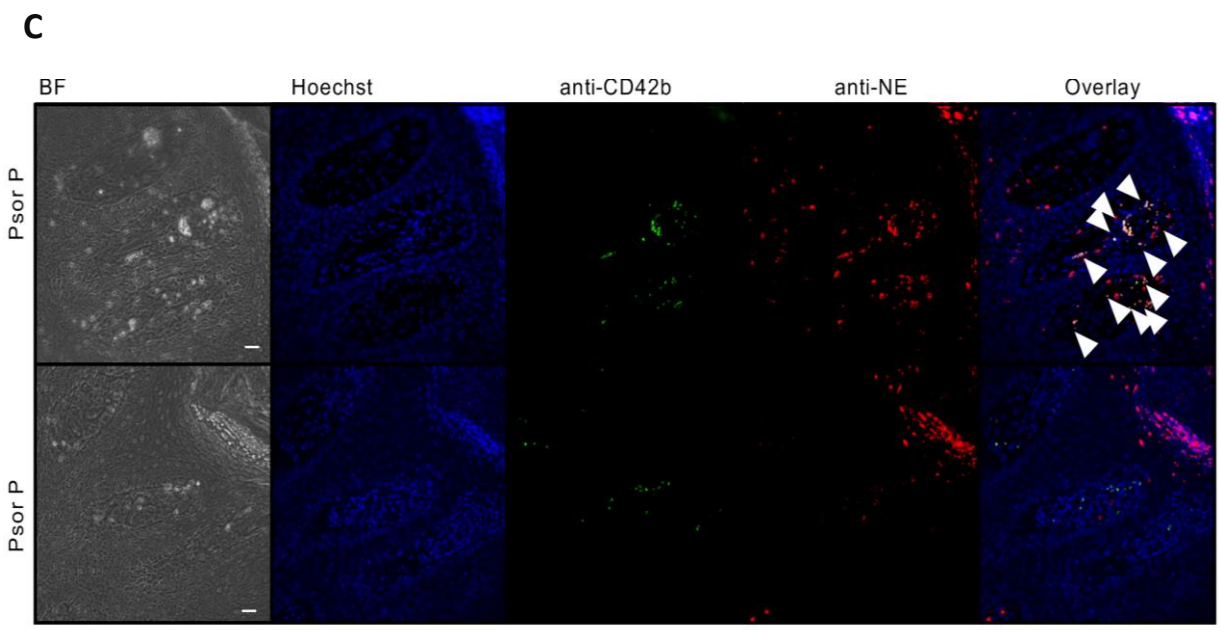


Figure 4.5: Platelet aggregates can be found in psoriatic lesions

(A) shows total platelet counts in HDs and PsorPs (A, n=52 vs 53). (B) Fluorescence microscopy analysis of skin sections stained with anti-NE (PMNs, red) (subsequent staining with anti-mouse AF594 secondary antibody), anti-CD42b (platelets, green) (subsequent staining with anti-goat AF488 secondary antibody) and Hoechst33342 (nucleus, blue), from healthy skin or psoriatic lesions (n=12 patients and 3 healthy controls, scale bar = 20 µm) and quantified in (C). (D) performed like B but showing PMNs and platelets in close proximity in psoriatic lesions, quantified in (E). AF = autofluorescence, BV = blood vessel. Arrowheads indicate platelet and PMN co-localization (B and C). A, C and E represent combined data (mean+SD) from 'n' biological replicates (each dot represents one donor or mouse). In B and D representatives of 'n' biological replicates (donors) are shown (mean+SD). * $p<0.05$ according to an unpaired Student's t-test (A) or Mann-Whitney test (C, E).

4.4. Depletion of platelets *in vivo* ameliorates skin pathology

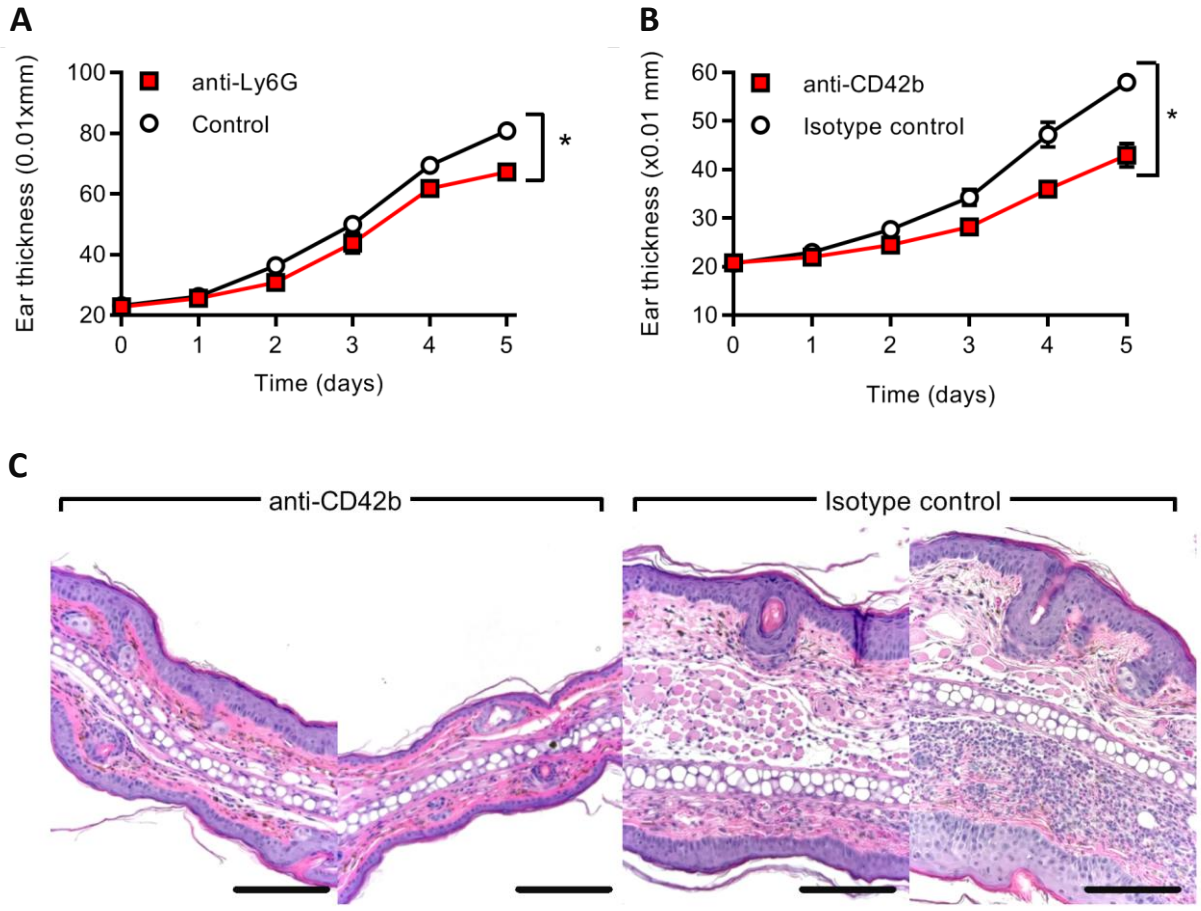
In order to get a better understanding of PMN and platelet infiltration of the skin and whether both cell populations are needed for psoriasis-like skin inflammation, collaboration partners performed PMN or platelet depletion in the IMQ-induced mouse model of psoriasiform skin inflammation. Final analysis and evaluations were further performed in our laboratory.

4.4.1. Platelet depletion reduces ear swelling and epidermal thickness

First, previous findings of Sumida et al. (Sumida *et al.* 2014) were re-evaluated. They showed that systemic depletion of PMNs using an anti-Ly6G antibody reduced ear swelling in the IMQ-induced mouse model of psoriasiform skin inflammation. As expected, significantly reduced ear swelling was observed in the PMN-depleted and IMQ-treated mice compared to isotype-treated control mice (Figure 4.6A). These results show that PMNs are relevant for the skin pathology in the IMQ-induced mouse model which resembles findings in the human situation (where PMN depletion ameliorates skin inflammation) (Ikeda *et al.* 2013). Next circulating platelets were depleted using an anti-CD42b antibody (Elzey *et al.* 2003) one day before first IMQ application and on day 4 of IMQ treatment. Unexpectedly, ear swelling was greatly

reduced in the PLT-depleted mice, even to a greater extent than seen for PMN depletion (Figure 4.6B). In H&E staining of IMQ-isotype treated skin samples, infiltration of leukocytes in the dermis of the mice, as well as epidermal thickening was observed. However, when investigating the skin of anti-CD42b treated mice, epidermal thickening was strongly reduced, almost resembling naïve skin (Figure 4.6C, quantification in D).

These results suggest that platelets are indeed of great importance for the psoriatic phenotype in IMQ-induced psoriasiform skin inflammation, because systemic platelet depletion resulted in strong amelioration of skin pathology.



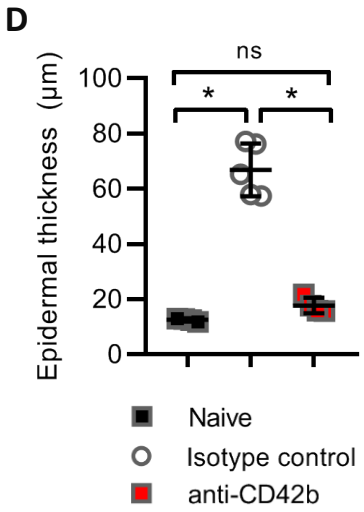


Figure 4.6: Platelet depletion decreases ear swelling and epidermal thickening in an IMQ mouse model of psoriasiform skin inflammation

(A-B) IMQ induced psoriasiform skin inflammation in BL/6 mice. Ear thickness (mm x 0.01) was measured after PMN depletion by anti-Ly6G (A) or platelet depletion by anti-CD42b (B) or respective control antibodies (n=5 in each group). (C) H&E staining of skin from mice treated with either anti-CD42b or isotype control (anti-CD42b n=4, isotype control n=5, scale bar = 180 µm). Quantification is shown in D. A, B, D represent combined data (mean+SD) from 'n' biological replicates (each dot represents one mouse). In C one representative of 'n' biological replicates (mouse biopsies) is shown * $p<0.05$ according to two-way ANOVA (A, B), unpaired Student's t-test (D). The *in vivo* mouse experiments were performed by Nate Archer.

4.4.2. Platelet depletion diminishes PMN-platelet aggregates in blood and inhibits PMN infiltration of the skin

Finally, it was investigated whether platelet depletion could potentially prevent not only platelets but also PMNs to infiltrate the inflamed skin. After showing that platelet depletion in circulation was very efficient (Figure 4.7A), it could be confirmed that concomitantly PMN-platelet aggregates in the blood were strongly reduced (Figure 4.7B). Interestingly, the total numbers of free PMNs in the blood of platelet-depleted mice were increased (Figure 4.7C). This strengthened aforementioned hypothesis that PMNs and other cells need platelets to infiltrate the site of inflammation. In this case, the platelets were missing to guide PMNs to infiltrate the skin (the site of inflammation) and therefore PMNs got "trapped" and accumulated in the blood (Figure 4.7C). To prove this hypothesis, skin biopsies of platelet-depleted and isotype- treated mice were stained with anti-MPO (PMNs) and anti-CD41 (platelets) in order to investigate whether PMN skin infiltration was affected by systemic platelet depletion. Interestingly, vast amounts of platelet aggregates, often in close proximity to PMNs in IMQ-isotype treated mouse skin, were observed. By contrast, in the platelet-depleted mice, neither platelet aggregates nor PMNs were detectable (Figure 4.7D and quantified in E and F).

Taken together, these findings indicate that PMN skin infiltration is depended on platelets in an IMQ-induced psoriasis mouse model. Platelet depletion inhibits PMN-PLT aggregates in blood and PMN infiltration of the skin and this greatly ameliorates skin inflammation. Thus, it could be speculated that in humans, inhibition of platelet-aggregation in blood can also ameliorate skin pathology in psoriasis. This may potentially be achieved as easy as the daily intake of aspirin.

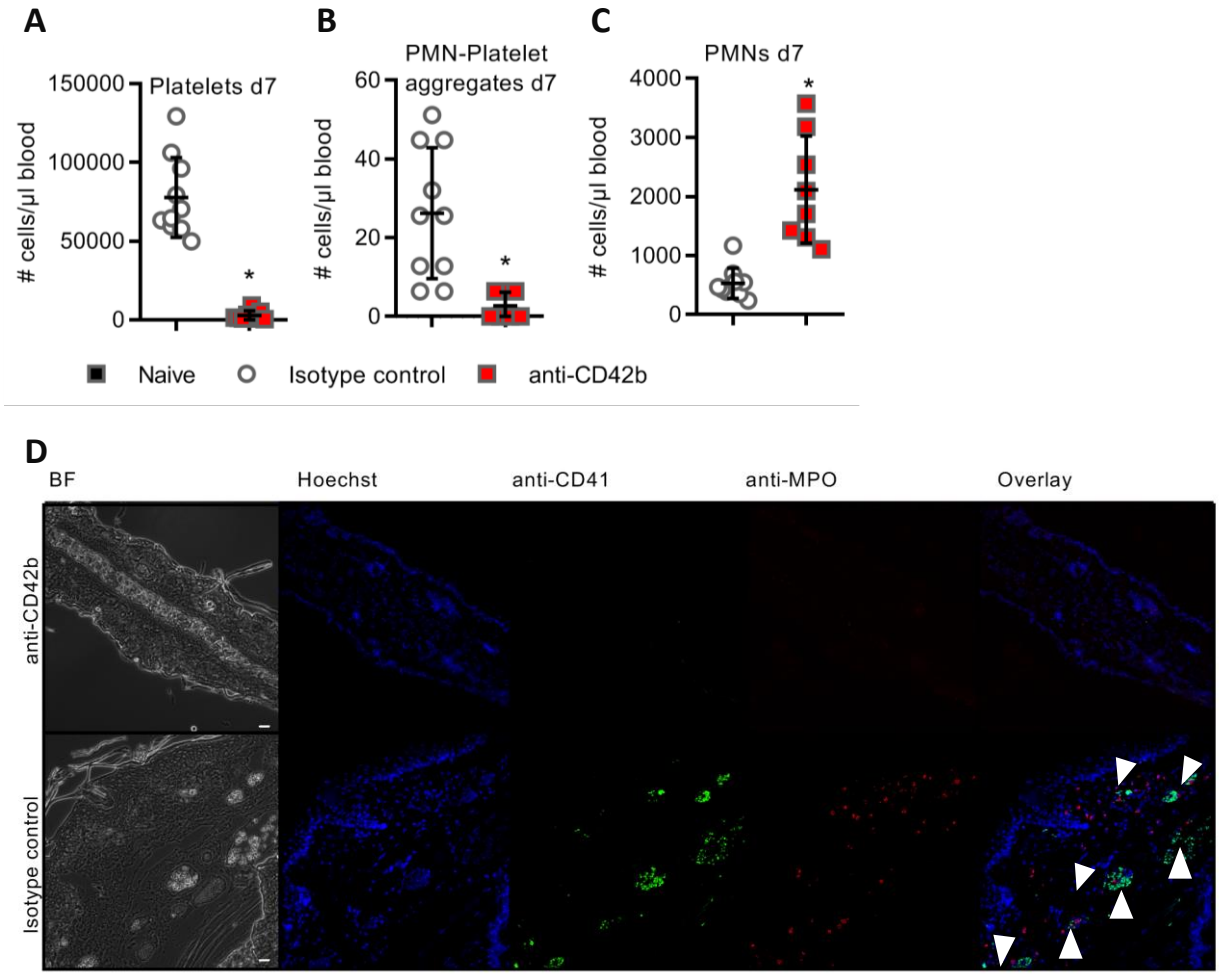


Figure 4.7: Platelet depletion diminishes PMN-platelet aggregates in blood and skin

(A-C) Flow cytometry analysis of total platelet counts on day 5 of IMQ treatment (A), PMN-platelet aggregates (B) and free PMNs (C) of blood from IMQ-treated animals with anti-CD42b antibody or isotype control (n=10 each). (D) Representative immunofluorescence staining from anti-CD42b and isotype-treated mouse skin (anti-CD42b n=4 and isotype control n=5, scale bar = 20 µm), stained with anti-MPO (PMNs, red) (with subsequent anti-goat AF594 secondary antibody), anti-CD41 (platelets, green) (with subsequent anti-rat AF488 secondary antibody) and Hoechst33342 (nucleus, blue) and the quantification of platelet aggregates in the skin (E) and platelets and PMNs in close proximity to each other (F) is shown. Arrowheads indicate close proximity of platelets and PMNs. A-C and E-F represent combined data (mean+SD) from 'n' biological replicates (each dot represents one mouse). In D one representative of 'n' biological replicates (mouse biopsies) is shown * $p<0.05$ according to Mann-Whitney test (A-C) or unpaired Student's t-test (E, F).

5. Discussion

5.1. Neutrophil extracellular trap-associated RNA (naRNA) and LL37 complexes enable self-amplifying inflammation in psoriasis

Psoriasis is an autoinflammatory disease of the skin and psoriatic skin lesions are characterized by leukocyte infiltration, dominated by PMNs. However, the triggers for inflammation and immune cell infiltration of the skin are still unknown.

The group of Michel Gilliet extensively showed that pDCs respond to complexes consisting of either RNA or DNA and the antimicrobial peptide LL37 with the production of type I interferons. Whereas protection from nucleases and uptake was promoted by LL37, signaling depended on nucleic acid sensing by endosomal TLRs, namely TLR7 for RNA- and TLR9 for DNA-LL37 complexes (Ganguly *et al.* 2009) (Lande *et al.* 2007). The authors claimed that these findings might explain an early inflammatory event in psoriasis. Although this mechanism of immune activation in psoriasis is highly plausible and congruent with the observed IFN signature in psoriatic skin (Yao *et al.* 2008), this scenario relies on a source of the immunostimulatory mediators – DNA, RNA and LL37 - that has been unknown. PMNs are the major producers (Sorensen *et al.* 1997) of LL37 and can also release nucleic acids by NETosis (Brinkmann *et al.* 2004). Also, PMN skin infiltration is a hallmark of psoriasis (Griffiths and Barker 2007) and PMNs outnumber pDCs both in human blood and in the psoriatic lesions. Additionally, PMNs are the first immune cells that respond to insult or inflammation and produce considerable amounts of pro-inflammatory cytokines and chemokines which then attract further immune cells to the sites of inflammation.

Therefore, it seemed plausible – but had not been investigated - that PMNs might be activated by complexes of nucleic-acids and LL37, and subsequently release LL37 and nucleic acids as parts of NETs as well as cytokines, thus providing the prerequisites for progredient immune infiltration and activation (e.g. including pDCs). Several observations made in the process warrant further discussion.

5.1.1 LL37 is an antimicrobial peptide which can act as a double-edged sword

The antimicrobial peptide LL37 is upregulated upon skin invasion by pathogens and exhibits antimicrobial activity against a broad spectrum of infectious species e.g. bacteria, viruses and fungi (Schauber and Gallo 2007).

LL37 is also overexpressed in psoriatic skin and acts as a double-edged sword there: on one hand it protects patients from superinfections (Marcinkiewicz and Majewski 2016) on the other hand it can also induce the uptake of self-derived DNA or RNA that in turn causes inflammatory responses in immune cells (Lande *et al.* 2007). Complexed by LL37, nucleic acids are transported into immune cells (Zhang *et al.* 2010) and bind to nucleic acid sensing receptors inducing activation of respective immune cells. In line, activation of PMNs is not induced by free RNA or DNA that do not enter PMNs (Figure 3.1 and Figure 3.2). This is in good

agreement with previous results, showing that synthetic endosomal TLR ligands like R848 (TLR7/8 agonist) or CpG (TLR9 agonist) reach the endosomes and induce robust immune stimulation via endosomal TLRs while RNA and DNA do not (Kuznik *et al.* 2011). Notably, RNA but not DNA complexed to LL37 induces substantial cytokine release from PMNs (Figure 3.1).

Interestingly only at very high concentrations of LL37 show toxicity (Wang *et al.* 1998). Thus, it can also be hypothesized that LL37 (in lower concentrations) can function as a transfection reagent to deliver (negatively charged) membrane-impermeable components into cells. Indeed, LL37 has been used for transfection and improves transfection efficiency of e.g. nanoparticles. In this setup LL37 even had two positive effects: it enhanced gene delivery and induced an antibacterial effect, thus accelerating wound healing (Wang *et al.* 2018). Interestingly, LL37 was also already used to deliver siRNA into cells. Although, transfection efficiency was largely dependent on cell type, delivery was successful in a variety of different cells (Yalcinkaya 2013). Importantly, the transfer-efficiency is largely sequence-dependent because e.g. CRAMP, the mouse ortholog of LL37 (Gallo *et al.* 1997), was less potent in binding and delivering of DNA into cells (Singh *et al.* 2013). The D-enantiomer of the human LL37 peptide however, binds and delivers DNA into cells but does not induce activation of endosomal DNA-sensing TLRs (Gestermann *et al.* 2018). Hence, transfection by the D-enantiomeric form of LL37 apparently is favorable to avoid induction of undesired immune modulation.

Of note, LL37 is also implicated in immune diseases of the skin different from psoriasis e.g. Atopic Dermatitis (AD) and Rosacea. In AD, some patients suffer from severe superinfections with bacteria from different species. In contrast to psoriasis, AD patients show defective upregulation of several antimicrobial peptides, including LL37, causing insufficient clearance of bacteria (Reinholz *et al.* 2012). In Rosacea however, (affects the centrum of the face, i.e. cheeks and nose) processing of the precursor hCAP18 into its activate form, LL37, and also smaller peptides, is elevated due to enhanced activity of cutaneous proteases. The protease activity is increased because of MAMP-sensing by TLR2 in keratinocytes in skin lesions (Reinholz *et al.* 2012). In skin lesions of Rosacea patients Demodex mites are frequently found. These mites shed chitin (a polysaccharide) from their exoskeleton (Georgala *et al.* 2001) which serves as MAMP for TLR2 (Fuchs *et al.* 2018) and hence induces increased processing of hCAP18. The interplay of activated keratinocytes with increased protease activity causing fragmentation of hCAP18 and hence the overexpression of LL37 and TLR-mediated immune reaction could elicit the pathogenesis of rosacea (Reinholz *et al.* 2012).

These examples show that balanced expression and lytic activity of LL37 is pivotal for efficient host defense against numerous pathogens, whereas the deregulation of LL37 is crucial for the pathogenesis of different autoimmune diseases of the skin.

5.1.2. Bacterial and fungal RNA might serve as inflammatory triggers in psoriasis

This study shows that not only synthetic RNA but also human (self) RNA and bacterial RNA bind to LL37 and induce immune response of PMNs (see Figure 3.3). Bacterial RNA is generally an underestimated stimulus for immune cells (Eigenbrod and Dalpke 2015) although for certain bacteria such as *Streptococcus pyogenes (S.pyogenes)* bacterial RNA is the major driver of immune response (Eigenbrod *et al.* 2015) (Eigenbrod and Dalpke 2015). Given the fact that infections with *Streptococci* (Griffiths and Barker 2007) are associated with *psoriasis guttata*, it can be imagined that in this case sensing of bacterial RNA might even drive infection-induced psoriasis, especially because PMNs can provide LL37 for complex formation in an early stage of immune defense. Furthermore, it is known that LL37 lyses bacteria (Kahlenberg and Kaplan 2013) inducing release of bacterial RNA which in turn could form complexes with LL37 and induce immune stimulation (Figure 3.3B and Figure 5.1).

Interestingly, psoriasis patients show altered commensal skin microbiota (Alekseyenko *et al.* 2013) and the abundance of certain bacteria, including *Streptococci,* is enhanced in psoriatic skin. The present data show that bacterial RNA in complex with LL37 robustly activates immune response via endosomal TLRs (TLR8 in humans and TLR13 in mice; Figure 3.11). Similarly, RNA from *Archaeon Methanosphaera stadtmanae* (a member of the human gut microbiota) induces TLR8-dependent immune reaction (Vierbuchen *et al.* 2017). These findings imply that RNA from commensals can also serve as an inflammatory mediator, especially when the composition of the microbiota is changed or dysregulated e.g. as found in psoriasis patients.

Also, commensal fungi like *Malassezia* are associated with skin diseases like atopic dermatitis or dandruff (Gioti *et al.* 2013). Furthermore, RNA from *Malassezia* is packed in extracellular nanovesicles which can in turn induce immune reactions in human keratinocytes and monocytes (Johansson *et al.* 2018). Interestingly, increased *Candida* colonization (pathogens and commensals) is found on the skin of psoriasis patients and therefore it had been proposed that fungal infections might trigger psoriatic skin inflammation (Pietrzak *et al.* 2018). Consequently, it would be interesting to investigate whether fungal RNA binds to LL37 and induces immune reactions in PMNs. It can be hypothesized that also for fungi not only pathogens but also commensals in a dysregulated skin microbiome, might provide RNA for RNA-LL37 complexation resulting in immune cell activation.

Furthermore, as RNA modifications modulate immunogenicity (Eigenbrod and Dalpke 2015) (Freund *et al.* 2019) and as these RNA modifications differ between species (Jaffrey 2014) (Edelheit *et al.* 2013) and strains it would be highly interesting to investigate RNA from different bacterial and fungal strains to identify the differences in LL37 binding and their potential to induce immune activation. Alternatively, the influence of RNA modification on immunogenicity could also be tested systematically by the use of synthetic RNA with known modifications. By using synthetic modified RNA, it would be possible to quantitatively

correlate sequence specific effects as well modulatory interplay of different RNA modifications.

Alternatively, bacterial or fungal RNA might also enter the host in psoriasis via the compromised barrier function of the skin of psoriasis patients (Ye *et al.* 2014). This would further facilitate the invasion of bacteria and fungi (RNA), either from infection or of commensal origin. First mechanisms to combat the invaders might then trigger an inflammatory feedback loop of PMN activation in psoriasis (Figure 5.1).

5.1.3. Cytokines released by PMNs in response to RNA-LL37 complexes are associated with psoriasis and act as chemo-attractants

A variety of cytokines and chemokines are upregulated in psoriasis, most importantly TNF-α, type I interferons, IL-17 and members of the IL-1 cytokine family (Baliwag *et al.* 2015). A massive release of type I interferons is induced by complexes of RNA or DNA with LL37 from pDCs (Lande *et al.* 2007) albeit most experiments to date have investigated this in an *in vitro* context. As pDCs produce IFN-α but do not release other pro-inflammatory cytokines, e.g. TNF or IL-1β which are essential for immune cell infiltration and polarization of T cells (Ghoreschi *et al.* 2007), the present study investigated whether RNA-LL37 complexes induce the release of such non-IFN cyto- and chemokines from PMNs. Indeed, PMNs release a variety of pro-inflammatory cytokines in response to stimulation with RNA-LL37 complexes, namely TNF-α, IL-6 and IL-1β (Figure 3.4). Although the amounts of cytokine release are low, one could argue that the vast amount of PMNs in psoriatic lesions might be efficient to accumulate a fulminant inflammatory reaction and induce activation of other immune cells. Also, it can be hypothesized that immuno-modulatory therapies like anti-TNF antibodies (Eberle *et al.* 2016) likely intervene already with a very early inflammatory event, targeting downstream signaling of PMNs.

Interestingly, two additional chemokines are released by PMNs in response to RNA-LL37 complexes: namely IL-16 and MIP-1β. IL-16 functions as a chemoattractant for $CD4^+$ lymphocytes (Center and Cruikshank 1982) and MIP-1β is a potent chemokine for a variety of immune cells (Menten *et al.* 2002). The release of IL-16 and MIP1β by PMNs has only scarcely been studied. This study investigated whether low amounts of IL-16 and MIP1β (as released by PMNs) can attract other immune cells, therefore transwell migration assays were performed. The transwell experiment showed migration of $CD4^+$ T cells, $CD8^+$ T cells and $CD14^+$ monocytes (Figure 3.5), but also of B cells, classical DCs (cDCs) and even pDCs (data not shown) towards IL-16 and MIP-1β. This result fits to previous findings showing that MIP-1β secretion by PMNs caused migration of dendritic cells (Chiba *et al.* 2004). Interestingly, lower concentrations of both chemokines more efficiently attract other immune cells to the lower compartments of the transwell chambers (Figure 3.5). In line, a non-linear relationship for dose and response is also described for other cytokines (Atanasova and Whitty 2012). Of note, it was also observed that RNA-LL37 complexes alone act as chemoattractant for $CD4^+$ T cells. This is in agreement with previous findings that show a chemo-attractive effect of LL37 on

neutrophils and eosinophils (Tjabringa *et al.* 2006). It would be interesting to investigate in detail which immune cells directly respond to RNA-LL37 as chemokine. Here, purified e.g. $CD4^+$ T cells could be used to investigate whether they actively migrate towards RNA-LL37 complexes or whether the observed effect was due to activated cells in the PBMC population which released chemokines leading to subsequent migration of $CD4^+$ T cells.

One could have expected to detect IL-17 production by PMNs in the Luminex screen, since IL-17 was identified as a key driver in the pathogenesis of psoriasis and anti-IL-17 therapies are clearly beneficial for psoriasis patients (Eberle *et al.* 2016). IL-17 is principally produced by Th17 cells but it was observed that PMNs contained IL-17 in the skin of psoriasis patients (Lin *et al.* 2011). Recently, it was shown that human PMNs (stimulated for 20 hours after isolation) released IL-17 upon stimulation of TLR8 by R848 (Tamassia *et al.* 2019). However, production of IL-17 by human PMNs in response to RNA-LL37 complexes or other TLR ligands e.g. LPS or R848 (Luminex analysis, data not shown) was not observed in this study. Of note, only PMNs from healthy donors, which were stimulated for 4 hours, were tested, while in other studies longer periods for cell stimulation were used (up to 20 hours). Also, IL-17 release from PMNs *in vitro* could not be reproduced by the identical group who previously reported IL-17 production by PMNs (Tamassia *et al.* 2018). Therefore, IL-17 production by human PMNs is not proven yet and still remains controversial.

Importantly, blood-derived PMNs from psoriasis patients (compared to healthy donors) release higher amounts of IL-8 and MIP-1β in response to stimulation with RNA-LL37 complexes (Figure 3.6). However, it has to be mentioned that only three patient samples were measured so far and for further confirmation more patients have to be recruited for this study. Nevertheless, in this experimental setting pre-activation of PMNs can be excluded which was investigated by the absence of CD62L shedding (example see Figure 6.1). Both, PMNs from healthy controls and psoriasis patients were not pre-activated (no CD62L shedding) and cytokine release in response to control TLR ligands like LPS was comparable between the two groups (Figure 3.6B and D). Hence, an unspecific cytokine release, due to pre-activated PMNs (e.g. due to technical issues) can be excluded. The superior response of PMNs from psoriasis patients to RNA-LL37 complexes could be explained by enhanced expression of endosomal TLRs which could bind RNA in PMNs from patients. Hence, comparative analysis of TLR expression levels in PMNs from healthy and psoriatic individuals is warranted. This could be achieved by qPCR on a mRNA level or Western blot on a protein level. Alternatively, the enhanced response of PMNs might also result from increased production of LL37 in psoriasis PMNs which can complex and stimulate further PMNs. Elevated LL37 baseline secretion by PMNs from psoriasis patients was found (Figure 3.6E). If RNA is also released by psoriatic PMNs (faster as compared to healthy controls), e.g. by cell death (NETosis) this would provide both immunomodulatory stimuli, i.e. LL37 and RNA, resulting in autoactivation of PMNs.

5.1.4. RNA-LL37 complexes trigger the release of RNA-and LL37-containing NETs

The present study shows that LL37 shuttles RNA into cytosolic compartments (Figure 3.2 and Figure 3.3) and also that RNA-LL37 complexes induce NET formation (Figure 3.7). NETs have also been detected in blood and skin of psoriasis patients (Hu *et al.* 2016), although it has to be mentioned that in tissue sections a clear demonstration is difficult. The reason for NET release, however, still remains unidentified. Therefore, RNA-LL37 complexes as potential triggers for NETs in PMNs were further investigated (in the context of psoriasis).

This study shows that LL37 alone induces NETosis in a NE-specific substrate assay (Figure 3.7A). However, it is still debated whether NE is necessary for the induction of NETosis because PMNs from mice lacking NE still induce robust NET release (Martinod *et al.* 2016). Therefore, NET formation was further investigated by fluorescence microscopy. There, RNA-LL37 complexes robustly induce NETs which in turn contain more LL37 and surprisingly also RNA (Figure 3.7 and Figure 3.8). It might be suggested that this RNA and LL37 *de novo* form complexes and induce the activation of further PMNs and the subsequent release of proinflammatory cytokines and more RNA and LL37 by NET formation. This might lead to a vicious cycle of PMN-activation, inflammation and attraction of other immune cells towards sites of inflammation. Therefore, this inflammatory loop might provide a very early event in the formation of psoriatic lesions (see Figure 1.6 and Figure 5.1 and explained below in further detail).

Importantly, psoriatic lesions contain massive amounts of RNA which is not only found in keratinocytes but also in skin infiltrating blood cells (see Figure 3.9A-C). The excessive amounts of RNA could potentially originate from hyperproliferating keratinocytes in psoriatic lesions (more mRNA is produced). Another possible explanation for these large quantities of RNA might be that LL37, which is overexpressed in psoriatic lesions, stabilizes and protects RNA from degradation (Ganguly *et al.* 2009). Hence, RNA from dying cells (keratinocytes or immune cells) might persist in psoriatic lesions, whereas it is quickly degraded in healthy individuals.

In addition, excessive amounts of RNA could potentially also originate from NETting PMNs that infiltrate the inflamed lesions (Hu *et al.* 2016). NETosis is very difficult to detect in the skin, because most PMNs and also NETs are exclusively seen in lesions that developed very recently, shortly before biopsy. Also, because of limitations in immunofluorescence microscopy (in terms of magnification and resolution), it was difficult to ascertain if DNA or RNA was detectable intra-or extracellularly. Nevertheless, potential NET formation was observed by extracellular NE staining in some biopsies, but this was not further analyzed due to limited numbers of patient samples and histone-specific antibodies, to detect NETs in tissues were not available in our group at the time of afore described experiments. Staining and subsequent analysis of NET formation in skin will be performed as soon as further patient samples are available. Furthermore, in ongoing *in vivo* experiments bacterial RNA-LL37 complexes are injected intradermally into ears of mice (Li *et al.* 2012) and the skin is analyzed for recruitment of and NET release by PMNs. These results will shed light on the relevance of the

aforementioned mechanism. Moreover, this might prove the hypothesis that RNA-LL37 complexes are physiologically relevant for driving PMN- and NET-mediated inflammation in psoriasis.

Importantly, the molecular and immunological relationships described above also provide an explanatory approach for the Koebner phenomenon. Due to a small injury, e.g. keratinocytes die or PMNs infiltrate the skin to prevent invasion of bacteria and provide inflammatory mediators. PMN-derived LL37 lyses bacteria which in turn induces the release of bacterial RNA and subsequent complex formation with LL37. RNA-LL37 complexes activate PMNs which release pro-inflammatory cytokines and chemokines to attract other immune cells to the sites of initial tissue damage. Furthermore, activated PMNs undergo NETosis and NETs contain more RNA and LL37 which form complexes and subsequently stimulate more PMNs. This mechanism might induce a vicious cycle of inflammation, subsequent chronification and development of lesions in places of minor injuries or trauma of the skin (see Figure 5.1).

5.1.5. naRNA: NET-associated RNA as a novel component of NETs

A central premise for this study was the abundance of RNA in PMA- and also RNA-LL37-induced NETs (Figure 3.7 and Figure 3.8). The presence of RNA in NETs was evidenced by two independent methods, i.e. using the RNA-specific dye SYTO RNAselect and a RNA-specific antibody, (Figure 3.7 and Figure 3.8). This NET-associated RNA was termed “naRNA” for future references. Intriguingly, the possibility that RNA could be a NET component was so far not considered, despite of the fact that more than 1900 publications (Boeltz *et al.* 2019) are dealing with NETs. To our knowledge, this is the first study that explicitly acknowledges RNA as a new NET component.

More importantly, the present study uncovers naRNA as a previously unrecognized, new immunostimulatory mediator (Figure 3.8) that triggers inflammation. The fact that isolated NETs from PMA- and RNA-LL37 (containing further naRNA and LL37) activated PMNs induced NET release from other PMNs (Figure 3.10) and the abundance of RNA and LL37 in psoriatic skin (Figure 3.9) demonstrates a potential physiological role of naRNA in the pathogenesis of psoriasis. Assumedly, this is a very early inflammatory event in psoriasis (see Figure 5.1 and described in 5.1.4.). In this scenario RNA-LL37 complexes are the first element for a spiral of increasing inflammatory responses. Because of the fact that this proposed new role of RNA-LL37 in the initiation of psoriatic lesions has far-reaching consequences for our understanding of psoriasis, rigorous testing of the hypothesis with additional control experiments are mandatory, e.g. in experiments using NET contents from RNA-LL37 or PMA stimulated PMNs for induction of further PMN responses. In the current setting the presence of the original stimulus, i.e. PMA or RNA-LL37 from isolated NETs cannot completely be excluded.

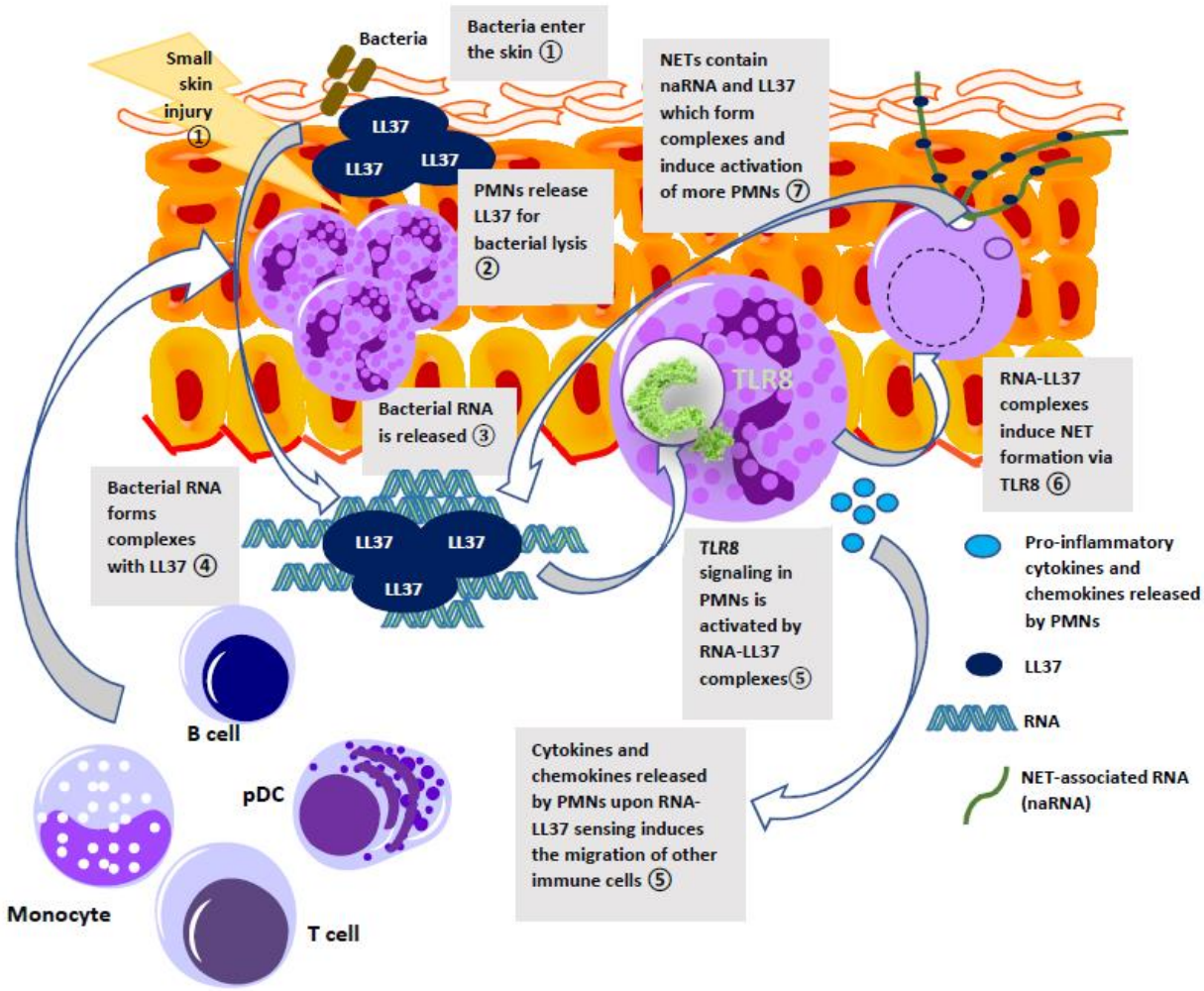


Figure 5.1: Complexes of naRNA and LL37 might drive a self-amplifying inflammatory loop in psoriasis

① A small injury of the skin weakens the skin barrier and facilitates the invasion of bacteria. PMNs infiltrate the skin to combat the invader. ② They release the antimicrobial peptide LL37 in response to bacterial invasion. LL37 induces lysis of bacteria and subsequent ③ release of bacterial RNA. ④ Bacterial RNA in turn forms complexes with LL37. ⑤ These complexes enter PMNs and induce robust TLR8 signaling. Pro-inflammatory cytokines and chemokines released by activated PMNs induce the infiltration of other leukocytes. ⑥ TLR8-dependent RNA-LL37 sensing also drives NET release. ⑦ These NETs contain NET-associated-RNA (naRNA) and further LL37 which form complexes and induce the activation of more PMNs. This highlights a TLR-mediated sensing mechanism for RNA-LL37 complexes in PMNs, inducing a self-propagating vicious cycle of inflammation and NET-associated RNA (naRNA) as a key driver of inflammation.

Of note, PMNs usually produce only little amounts of RNA (Tecchio *et al.* 2014) and RNA is normally easily degraded by RNases. However, two RNA-specific staining methods i.e using SYTO RNAselect and a Ψ-U antibody both detected considerable amounts of *de novo* synthetized RNA in PMA- and RNA-LL37-induced NETs (Figure 3.8). When using, RNase A, the RNA was robustly erased but the DNA staining (Hoechst) stayed unaffected (Figure 3.8). Controversially, the group of Klaus T. Preissner (University of Giessen) found that a variety of different RNases are present in NETs (personal communication) (Noll *et al.* 2017). There, the RNases (RNase 1 and 5) originate from PMNs, probably as a mechanism to prevent the host from overshooting immune reactions by extracellular RNA. Interestingly, RNase 7, a member

of the RNase A family, is also secreted by keratinocytes as an antimicrobial peptide. Additionally, RNase 7 is overexpressed in psoriatic lesions (Kopfnagel *et al.* 2018) and functions similar to LL37. It binds DNA and promotes IFN-α release by pDCs. However, the mechanism of the antimicrobial activity of RNase 7 is incompletely understood as the bactericidal function of RNase 7 is independent of its ribonuclease activity; mutants without functional ribonuclease still show effective bacterial killing (Rademacher *et al.* 2016). Also, RNase 7 is only secreted on the skin surface which only protects from microbial growth but not from invasion (Simanski *et al.* 2012). Thus, it is likely that self-derived RNA or bacterial RNA can still enter the host (due to a small injury) and induce immune responses, even in the presence of RNase 7. Moreover, it can be suspected that LL37 (as NET content) protects naRNA from degradation by RNases (Ganguly *et al.* 2009). In line, LL37 was shown to protect DNA from degradation by nucleases derived from bacteria. Hence, NETs containing LL37 are more robust in trapping and killing of bacteria (Neumann *et al.* 2014). This potentially also applies to naRNA.

Furthermore, it would be interesting to investigate the role of naRNA in NETs in general, e.g. to answer the question whether naRNA participates in antimicrobial functions as DNA in NETs (Brinkmann *et al.* 2004): Bacterial killing assays could be performed with naRNA containing NETs and investigate whether RNase A treatment impairs bactericidal effects. Also, it could be analyzed how naRNA can execute bactericidal functions, e.g. by trapping of bacteria or by direct interruption of bacterial function. It has to be additionally investigated how the bactericidal function of naRNA is modulated by LL37, e.g. due to stoichiometric composition or naRNA modification. Of note, NETs released by lower species like insects contains RNA rather than DNA (Altincicek *et al.* 2008). It can be therefore argued that RNA release by NETs is conserved between species, presumably acting quicker and cruder than DNA but still, similar to DNA which is important for trapping and killing of bacteria. Also, data in this study evidences accumulated RNA in granules of PMNs, ready to be released (Figure 3.8D). A similar effect was also observed for eosinophils (Behzad *et al.* 2010). Accumulation of RNA in granules might therefore be a common feature of granulocytes in general, giving another indication of a quick mechanism of RNA release.

Interestingly, NET contents are immunostimulatory for pDCs (Lande *et al.* 2011) and B cells (Gestermann *et al.* 2018) in psoriasis and SLE respectively. Both studies focused on DNA and LL37 as mediators for the induction of NETs. It would be interesting to analyze the impact of naRNA on the development of autoinflammatory diseases other than psoriasis. Interestingly, in SLE patients, anti-RNA antibodies are also frequently found (Blanco *et al.* 1991). Therefore, naRNA might be a potential source of autoantibodies in SLE but also in other autoimmune diseases. Furthermore, in other NET-related diseases like atherosclerosis (Warnatsch *et al.* 2015) (where NETs form because of cholesterol crystals) or RA (Branzk and Papayannopoulos 2013) (where NETs are found in inflamed joints) the impact of naRNA and a potential dysregulated degradation by RNases might be of interest to elucidate the role of naRNA in homeostasis and disease.

5.1.6. TLR8 is a key player in naRNA-LL37 induced signaling in PMNs

Initial experiments showed that cytokine release induced by RNA-LL37 complexes from PMNs was inhibited by chloroquine (Figure 3.3D). As chloroquine does not affect RNA-sensing by cytosolic receptors such as RIG-I, but prevents endosomal acidification and thus activation of endosomal TLRs (Matsukura *et al.* 2007) (Kuznik *et al.* 2011), it was hypothesized that for RNA-LL37 sensing endosomal TLRs are instrumental. TLR8 (human) and its equivalent TLR13 in the mouse were assumed to mediate cytokine release and NETosis, as the RNA-sensing endosomal TLRs becauseTLR3 and TLR7 are not expressed in human PMNs (Hayashi *et al.* 2003) (Janke *et al.* 2009) (Berger *et al.* 2012).

Although this study showed that RNA-LL37-mediated immune reactions where highly dependent on endosomal TLR signaling, RNA-LL37-induced NETosis might nevertheless also depend on LL37 binding to a specific receptor. Among others, the formyl-peptide receptor 2 (FPR2) (Zhang et al. 2009) was proposed to bind LL37. However, blocking of FPR2 by the selective antagonist WRW4 (Bae et al. 2004) did not affect RNA-LL37-induced NE release (measured the "NETosis kit" from Cayman Chemical). This strongly indicates that the release of NET-associated NE (and hence NET formation), induced by RNA-LL37 complexes, is not dependent on LL37-FPR2 signaling (preliminary data not shown, more donors needed for statistically relevant results; furthermore, these results were also not proven in fluorescence microscopy). Although other receptors that bind LL37 have been described, e.g. members of the EGFR (epithelial growth factor receptor) family or the human purinergic receptor P2X7 (reviewed in (Verjans et al. 2016)), subsequent experiments focused on endosomal nucleic acid-sensing receptors as potential candidates for RNA-LL37-binding and signaling.

Interestingly, cytosolic DNA (microbial and self-derived) was also reported to activate the inflammasome in a TLR-independent manner (Muruve *et al.* 2008). Also, two very recent findings show the relation of NLRP3 inflammasome, more specifically an inflammasome substrate, Gasdermin D, and NET formation (Sollberger *et al.* 2018) (Chen *et al.* 2018). Gasdermin D is a pore-forming protein and a key player in pyroptosis which is a form of cell death induced by intracellular LPS via a so-called non-canonical NLRP3 inflammasome pathway (Liu *et al.* 2016). Sollberger et al. published that Gasdermin D is activated in PMNs dying by NETosis and in a chemical screen they found a small molecule inhibitor for Gasdermin D that not only inhibits pyroptosis but also interferes with NETosis (Sollberger *et al.* 2018). The group of Kate Schroeder observed that in PMNs, Gasdermin D forms pores in granules and nuclear membranes prior to permeabilizing the plasma membrane which in turn induced the release of IL-1β and NETs – here termed "pyro-NETs" (Chen *et al.* 2018). These data show that Gasdermin D is not only implicated in pyroptosis but also links in the regulation of NETosis. Furthermore, endosomal TLR ligands like R848 are also known to prime NLRP3 inflammasome signaling (Hornung and Latz 2010). Therefore, we wondered whether "pyro-NETosis" can be mediated not only by cytosolic LPS but also by RNA which is shuttled by LL37 into endosomal compartments. Consequently, cytokine release and NET formation by BM-PMNs from one *Nlrp3*$^{-/-}$ mouse was analyzed in response to RNA-LL37 complexes. BM-PMNs from the *Nlrp3*$^{-/-}$

mouse released TNF and induced NETs to the same extend as BM-PMNs from WT mice (data not shown, IL-1β release was not analyzed). Although preliminary, these results indicate that NET induction by RNA-LL37 is most likely independent from other innate immune responses like the NLRP3 inflammasome but strongly depends on endosomal TLR signaling.

As mentioned before, this study proved that TLR8 and TLR13 are actual receptors that recognize RNA from RNA-LL37 complexes and induce immune activation in human and mouse PMNs (Figure 3.11). It was shown that PMNs are activated via these endosomal RNA-sensing receptors to release pro-inflammatory cytokines and NETosis. These NETs in turn contained further RNA and LL37 which might complex again to activate more PMNs. Interestingly, TLR8 activation of PMNs apparently switches the response from phagocytosis to NETosis (Lood 2016). This perfectly fits to the current observation: induction of NETosis by RNA-LL37 mediated activation of TLR8, rather than mere phagocytosis of the complexes. Also, the fact that TLR8-mediated NETosis differs from, for example PMA-mediated NETosis is illustrated by the different kinetics for PMA- and RNA-LL37-induced NETs: RNA-LL37 induces NET formation fast, approximately within 60 minutes post stimulation (see Figure 3.7D and E), while PMA-induced NETosis peaks at 4 hours after stimulation. The specific kinetics indicate different underlying mechanisms of NET formation. This is in accordance with published results showing fast induction of NET release when TLR2 bound its ligand *S.aureus* or by TLR4-mediated platelet activation and subsequent binding to and activation of neutrophils (Yipp *et al.* 2012) (Pilsczek *et al.* 2010) (Clark *et al.* 2007). By this means, only the nuclear membrane is degraded while the plasma membrane stays intact. In contrast, PMA induced NETosis is slower and leads to complete cell lysis (cell death is induced) (Nestle *et al.* 2009) (also see Figure 1.2). Therefore, live NETs (mostly induced by TLR signaling) and suicidal NETs (induced by PMA) have to be clearly distinguished from each other (Boeltz *et al.* 2019). However, live-cell-imaging gives the impression that the whole cell ruptures upon activation with RNA-LL37 complexes, despite of being a TLR-dependent and fast process (Figure 3.7D and E). To illustrate the underlying mechanism of NET induction by RNA-LL37 complexes it would therefore need further investigation. By plasma membrane labeling using CellMask™ (as previously described (van der Linden *et al.* 2017)), one could potentially elucidate whether RNA-LL37 complexes induce vital or suicidal NET formation, preferably by investigation using live-cell-imaging.

These data indicate that endosomal TLR8 signaling is pivotal for RNA-LL37-mediated immune reactions. The relevance of endosomal TLR signaling in RNA-LL37-induced skin inflammation could hence be further elucidated in additional *in vivo* experiments, e.g. by intradermal injection of bacterial RNA-LL37 complexes into *Unc93b1*-deficient and *Tlr13*$^{-/-}$ mouse ears. Absence or reduced ear swelling as compared to control mice, less PMN skin infiltration or NETs would verify the importance of RNA-LL37-induced activation of endosomal TLRs. Furthermore, therapeutic intervention by e.g. small molecules compounds that block TLR8-bining or signaling by TLR8 in PMNs could thus intervene with early inflammatory response in psoriasis.

5.1.7. Therapeutic implications

To date, the majority of psoriasis patients in Germany with moderate to severe disease still receive systemic therapy with methotrexate. This antimetabolite of folic acid was approved by the FDA in 1971 for the treatment of psoriasis (Menter and Griffiths 2007). As an antimetabolite, methotrexate is also used as a cytostatic drug in anti-cancer therapy to treat a variety of different solid tumors (Brown *et al.* 2016) (Abolmaali *et al.* 2013), although at considerably higher doses. As Methotrexate interferes with synthesis of purines and pyrimidines and thus with DNA synthesis (Czarnecka-Operacz and Sadowska-Przytocka 2014), its use represents a rather unspecific therapeutic intervention and long-term therapy is often associated with unwanted side-effects which can range from mild to severe; from fatigue to nausea, gastrointestinal problems and also liver dysfunction and infertility (Wang *et al.* 2018).

Due to the unspecific nature and considerable side effects of standard therapy by methotrexate it is of great importance to develop more specific therapies for psoriasis patients. As an alternative, immune-specific approaches like the use of “biologicals” (Eberle *et al.* 2016) to directly target pro-inflammatory cytokines as a potential new therapeutic intervention for psoriasis was proposed. This proposal was inspired by enhanced concentration of pro-inflammatory cytokines in psoriasis patients. In psoriatic lesions increased levels of IL-1β are found (Balato *et al.* 2013) and IL-1β and other IL-1 family members are assumedly important inflammatory mediators in psoriasis. Also, in an IMQ-induced mouse model of psoriasis IL1 receptor (IL-1R) was found to be a major driver of inflammation, however, independent from NLRP3 inflammasome signaling (Rabeony *et al.* 2015). Surprisingly, Anakinra, an inhibitor for IL-1R, which is successfully used for treatment of diseases with gain of function mutations in NLRP3, had no beneficial effect in psoriasis patients. Quite the contrary is true: upon treatment with Anakinra new onset of psoriasis was observed (Tsai and Tsai 2017). This indicates a dysregulated IL-1 production in psoriasis patients which likely is independent from activation of the NLRP3 inflammasome but unfortunately cannot be treated with IL-1R inhibition by Anakinra.

Other biologicals like anti-TNF antibodies are important new therapeutics which are now more frequently used to treat psoriasis and the FDA already approved several anti-TNF antibodies (Kircik and Del Rosso 2009). Although anti-TNF therapy is highly effective for the majority of patients, 2-5% develop paradoxical psoriasis, characterized by new inflammatory skin lesions, resembling psoriasis. These lesions form due to exaggerated type I IFN induction without T cell autoimmunity (Conrad *et al.* 2018). Another serious drawback of long-term therapy with anti-TNF antibody is the increased susceptibility to serious infections (Bongartz *et al.* 2006).

In addition to IL-1 and TNF also type I interferons were found to play a crucial role in the pathogenesis of psoriasis. Plasmacytoid DCs were shown to produce large quantities of IFN-α in response to nucleic acid-LL37 complexes and pDCs were also found in psoriatic lesions (Lande *et al.* 2007). Additionally, the type I IFN signaling cascade is greatly upregulated in psoriasis patients (van der Fits *et al.* 2004). In line, inhibition of the IFN pathway ameliorates

skin inflammation in a mouse xenograft model of psoriasis (Nestle *et al.* 2005). Blocking of type I IFN therefore has been suggested as potential new therapy for psoriasis (Yao *et al.* 2008). However, a phase I clinical trial using an anti-IFN-α antibody did not show any beneficial effects in psoriasis patients (Bissonnette *et al.* 2010). Therefore, the authors concluded that IFN-α does not significantly affect the perpetuation of psoriasis.

Yet another angle to target psoriasis derived from important observations of polymorphisms in genes associated with Th17 immune signaling in psoriasis patients. T cells of the Th17 subtype usually produce IL-17 (Eberle *et al.* 2016). The cytokine IL-17A weakens the skin barrier and amplifies inflammation by promoting the release of pro-inflammatory cytokines (e.g. IL-8 or IL-6 (Pfaff *et al.* 2017)) and chemokines from keratinocytes. Also, IL-17A induces migration of leukocytes and in association with TNF and IL-22, IL- 17A mediates upregulation of IL-1 family cytokine expression (Brembilla *et al.* 2018). Importantly, anti-IL-17 antibodies significantly ameliorate skin pathology in psoriasis patients (Hueber *et al.* 2010) (Eberle *et al.* 2016). In recent years it became evident that IL-17 production and differentiation of Th17 cells largely depends on IL-23. Therefore, the IL-23/IL-17 axis in psoriasis is considered to link innate and adaptive immunity (Schon and Erpenbeck 2018). Combination therapies that block IL-23 and IL-17 by antibodies induce impressive remission in up to 90% of patients (Kim and Krueger 2017) (Hawkes *et al.* 2017). However, these IL-23/IL-17 aimed therapies (and other biologicals) are still very cost intensive (Tsai and Tsai 2017) (Wasilewska *et al.* 2016). Also, it is still controversial whether PMNs release IL-17 (Tamassia *et al.* 2018) (Tamassia *et al.* 2019). This indicates that therapeutic intervention of the IL-23/IL-17 axis would only target chronic disease but might still miss very early, acute inflammation induced by PMNs.

Finally, the present study shows that inhibition of TLR8 by very low amounts (1-50 nM) of inhibitory oligodesoxynucleotides (iODNs) diminishes cytokine release by PMNs and completely blocks NETs (see Figure 3.12A, B and D). IODNs were proposed for the treatment of Systemic Lupus Erythematosus (SLE) (Barrat *et al.* 2005) (Barrat and Coffman 2008) and some small molecule inhibitors, similar to those used in this study, are already tested for the treatment of SLE in several clinical studies (Wu *et al.* 2015). Therefore, it might be assumed that small inhibitory molecules that block RNA-LL37-induced TLR8 signaling (in very low doses) might also be efficient as a new therapeutic intervention in psoriasis and intervening with a very early inflammatory event, by direct targeting of PMN activation in psoriasis.

5.1.8. Conclusions part I

Complexes of nucleic acids and LL37 are important immunostimulatory mediators in psoriasis. Still the origin of those inflammatory mediators has been unknown. This study provides evidence for a mechanism that activates PMNs by NET-associated RNA (naRNA) and LL37 which enables a self-amplifying inflammation in psoriasis. PMNs not only release a variety of different cytokines and chemokines (which can attract other immune cells) in response to RNA-LL37 complexes but also induce robust NET formation. These NETs in turn contain LL37 and more importantly RNA. NET contents from RNA-LL37-stimulated PMNs activate more

PMNs to release RNA-and LL37-containing NETs. In this process TLR8 (human) and TLR13 (mouse) are instrumental. Interestingly, very low concentrations of iODNs which block TLR8 signaling inhibit not only cytokine- but also NET release. This current study thus showed that PMNs, which heavily infiltrate psoriatic skin, can provide immunostimulatory components (in this case naRNA and LL37) themselves which might fuel a vicious self-propagating cycle of inflammation in psoriasis.

5.2. Platelet-PMN aggregates promote skin pathology in psoriasis (part II)

As aforementioned, a hallmark of psoriasis is the infiltration of the skin by PMNs. As PMNs are among the first cells present at sites of inflammation and attract other immune cells, it was assumed that differential expression of surface antigens on PMNs from healthy controls and psoriasis patients could be responsible for the skin homing of PMNs in psoriasis. In order to detect differences in surface antigen expression, an antibody-based screen for 332 surface proteins (LEGENDScreen™) was performed on whole blood of five psoriasis patients and five healthy controls. In this screen B cells, T cells, PMNs, dead cells were additionally labeled and monocytes were gated by size and granularity.

5.2.1. Blood cells from psoriasis patients express different surface antigens compared to healthy controls

In blood samples from healthy donors and psoriasis patients, 30 surface antigens were identified which were differentially expressed (defined by a nominal statistical significance) between the two groups. These differentially expressed antigens spread over different cell populations in the blood (see Table 4.1). However, the validity of the present results would be significantly improved by a larger sample number. Also, the mean fluorescence intensities (MFIs) in this study are low, likely owing to technical limitations, yielding in increased deviation of MFIs. Nevertheless, the MFIs of "positive hits" are considerably higher than MFIs of respective isotype controls thus indicating reliable differences. Re-evaluation of the identified differentially expressed surface antigens using another FACS device ensured comparable trends, proving reliability of results obtained by the LEGENDScreen™. Several of the identified surface antigens will be highlighted and discussed in detail in the next paragraph.

Differentially expressed surface markers on PMNs: On PMNs, five surface antigens were differentially expressed: CD209, CD143, CD235ab, CD337 and TRA-1-60R. Because CD337 is mainly expressed on NK cells (Angelo *et al.* 2015) and expression of TRA-1-60R was very low in this setting, the function and expression of only CD209, CD143 and CD235ab will be discussed in detail.

- **CD209** (DC-SIGN) is a DC marker found on monocyte-derived DCs (mDCs) which induces initial contact of DCs to resting T cells and DC trafficking by interaction with the endothelium. CD209 expression is regulated by IL-4 and IFN-γ, the major cytokines driving Th2 and Th1 responses respectively. IFN-γ acts a negative regulator of CD209 expression, whereas IL-4 induces the expression of CD209 (Relloso *et al.* 2002). This means that IFN-γ promotes Th1-mediated inflammation which correlates with downregulation of CD209. Interestingly, PMNs can act as APCs (Vono *et al.* 2017) and therefore it can be hypothesized that PMNs also express different DC markers, including CD209. Expression of CD209 was downregulated on PMNs from psoriasis patients. This might give a hint on psoriasis as an IFN-γ-Th1-driven disease (although it is now appreciated that the Th17 response is also very important) (Cai *et al.* 2012).

- **CD143**, is commonly known as **Angiotensin-converting enzyme (ACE)**. ACE converts angiotensin I into the active angiotensin II which induces increase of blood pressure and vasoconstriction (Bernstein *et al.* 2018). Millions of people take ACE inhibitors to treat cardiovascular diseases (Messerli *et al.* 2018). Although ACE is mainly found on endothelial cells, here reduced expression levels of ACE on PMNs from psoriasis patients was found compared to healthy controls. Reduced ACE levels might for example be the cause of vasodilatation is psoriasis which is a hallmark of the disease (Heidenreich *et al.* 2009). Interestingly, in mice, ACE is expressed on PMNs and overexpression of ACE enhanced the capacity to induce anti-bacterial mechanisms in PMNs. In contrast, mice lacking ACE on PMNs were more vulnerable towards infections. Thus, ACE on PMNs protects the host from infections (Khan *et al.* 2017) and psoriasis could be triggered by a small infection that is not efficiently cleared by PMNs due to their lack of ACE expression. Genetic predisposition or unknown triggers can cause the formation of psoriatic lesions after bacterial infections. Importantly, ACE inhibitors, prescribed to psoriasis patients to treat cardiovascular comorbidities, even worsened psoriasis or led to new onset of *psoriasis guttata* (Hong 2012). This indicates that inhibition of ACE on PMNs might increase susceptibility towards bacteria-triggered psoriasis.

- **CD235ab**. expression is found on terminally differentiated, anucleated erythrocytes and considered to be erythrocyte specific (Bruce *et al.* 1994). Interestingly, this study detected CD235ab on PMNs from psoriasis patients and, moreover, CD235ab was the only surface protein showing significantly enhanced expression on psoriasis PMNs. Despite this intriguing finding, CD235ab detection on PMNs should be interpreted with caution. PMNs tend to bind proteins on their surface; they are “sticky” e.g. in cancer (Olsson and Cedervall 2016) and also in autoimmune diseases. It is for example known that platelets tend to stick to the surface of PMNs (Gerrits *et al.* 2016) (Chanarat and Chiewsilp 1975). Hence, also erythrocytes might stick to PMNs and cause detection of enhanced CD235ab on PMNs. However, this would imply inefficient red blood cell lysis which is unlikely because osmolysis of erythrocytes is highly efficient and visual inspection did not indicate traces of erythrocytes (white color of cell pellet). Still, as CD235ab is a highly specific marker on erythrocytes, detection on PMNs might result from membrane-fragments from erythrocytes which stick to PMNs. These erythrocyte membrane fragments would - even in case of efficient erythrocyte lysis - cause the CD235ab-positive signal on PMNs. To exclude that CD235ab-positivity stems from erythrocyte fragments bound to PMNs from psoriasis patients, positive selection of PMNs (e.g. using magnetic beads) followed by qPCR to detect CD235ab mRNA could be performed. This approach would allow to verify *de novo* expression of CD235ab by psoriasis PMNs.

Differentially expressed surface markers on B-cells: For B cells, 7 makers were significantly different when comparing healthy control and psoriasis samples: CD11c, CD124, CD215, δ-Opioid receptor, CD261, CD267 and MSC (W5C5). For all those antigens the expression levels were considerably low and there was often a large variance either in the HD or in the PsorP

cohort between different donors. Therefore, only three markers will be discussed in more detail because they showed the most promising differences (between patients and healthy controls).

- **CD11c** is found on a variety of blood cells in humans, e.g. DCs, monocytes but also to a lower extend on PMNs and B cells (Boltjes and van Wijk 2014). CD11c was found to be important for phagocytosis and considered as an important mediator for DC antigen presentation (Sadhu *et al.* 2007) (Collin *et al.* 2013) but is also described on memory B cells in tonsils. These memory B cells were found to expand into circulation upon autoimmune reactions (Karnell *et al.* 2017). In the present study reduced expression of CD11c on B cells of psoriasis patients compared to healthy controls was found. In contrast to the detected reduced expression, it was expected that autoimmune-diseased individuals, i.e. psoriasis patients, show enhanced expression of CD11c. Because CD11c is expected to be upregulated on B cells from psoriasis patients, as an indicator of autoimmune responses, there is no conclusive explanation for the result.

- **CD261** belongs to the TNFR superfamily. In healthy individuals only weak expression of CD261 is mainly found on immature B cells of the bone marrow (Rudolf-Oliveira *et al.* 2018). **CD267**, frequently referred to as TACI (Transmembrane Activator and CAML Interactor), was shown to mediate inhibition of B cell activation. $TACI^{-/-}$ mice show strong B cell activation and accumulation in the spleen that causes splenomegaly (Yan *et al.* 2001). In B cells of psoriasis patients, the expression of CD261 is enhanced whereas the expression of TACI is reduced indicating the presence of hyperactive and immature B cells in psoriasis, well in agreement with the higher risk of psoriasis patients of developing a lymphoproliferative disease like lymphoma or leukemia (Gelfand *et al.* 2006).

Differentially expressed surface markers on T-cells: For T cells only two surface antigens were found to be differentially expressed in psoriasis patients: CX3CR1 and CD244. Both are decreased for patient-derived T cells.

- **CX3CR1** is a known mediator for both leukocyte adhesion and migration (Imai *et al.* 1997). The observed decrease of CX3CR1 on T cells from psoriasis patients would indicate that psoriasis T cells have attenuated ability to adhere and migrate. Opposite results were expected and therefore there is no conclusive explanation for these findings.

- Interestingly, low expression of **CD244** is implicated in activating NK and CD8 T cell immunity (Chlewicki *et al.* 2008). Hence, low expression of CD244 on psoriatic T-cells is well in line with a hyperactive immune system in psoriasis in general.

Differentially expressed surface markers on monocytes: The majority of differentially expressed antigens was detected on monocytes (see Table 4.1 and Figure 6.7D). Due to technical limitations the analysis did not include a specific antigen labeling for monocytes and therefore these cells were identified by size and granularity. Morphological characteristics are

of low specificity and it must therefore be assumed that some of the identified differentially expressed markers are false positives. Still it is worth to highlight a few which could then be confirmed in a setup that includes CD14 and CD16 staining to unequivocally identify different monocyte populations:

- **CD13** is was found to be upregulated on monocytes from psoriasis patients. Interestingly, CD13 is also upregulated on monocytes after trauma (Huschak *et al.* 2003). In line with the pathogenesis of plaque psoriasis lesions usually form at the sites of trauma and pressure (Koebner phenomenon).

- Interestingly, CD162/**PSGL-1** was found to be upregulated on psoriatic monocytes. PSGL-1 is essential for monocyte homing and initial tethering (Huo and Xia 2009). Surprisingly, PSGL-1 is therefore the only homing receptor found to be upregulated in this screen, and therefore possibly indicating the tendency of psoriasis monocytes to infiltrate the skin.

- Furthermore, CD154/**CD40L**, is overexpressed on monocytes of SLE patients and its expression correlates with disease severity. As an explanation, Katsiari et al. suggested a T cell independent CD40L-mediated cell-cell interaction that induces humoral immunity in SLE (Katsiari *et al.* 2002). The present study identified enhanced expression of CD40L on monocytes which assumingly has a similar function as in SLE.

- Most importantly, compared to healthy controls, monocytes from psoriasis patients express elevated levels of platelet antigens, namely **CD41** and **CD61** (see Table 4.1 and Figure 6.7D). In this study, this finding was due to aggregate formation between monocytes and platelets in the blood of psoriasis patients (see Figure 6.8). Monocyte/PMN-platelet aggregates will further be elucidated in the next paragraph.

Due to sample limitations in general, all these findings still need further validation using more patient samples before initializing follow-up studies to elucidate the exact role and function of the described surface molecules in psoriasis. Analysis in the present study focused further on PMNs as suspected cellular key drivers in psoriasis.

5.2.2. Blood-derived PMNs from psoriasis patients have a defined "platelet surface antigen signature"

In order to identify additional differences in blood samples from healthy individuals and psoriasis patients, principal component analysis (PCA) of data generated by the LEGENDScreen™ was performed. PCA uncovered specific clusters of protein expression in healthy individuals and psoriasis patients, i.e. certain combinations of surface antigens allowing discrimination between the two groups. Importantly, most significant results in PCA were obtained for PMNs, revealing strong clustering of patients' PMNs. The five antigens which in combination define "psoriasis PMNs" in whole blood are: CD6, CD11c, CD41, CD61 and CD235ab (Figure 4.2). This study further focused on the expression of CD6, CD41 and CD61 on psoriasis PMNs.

CD6 is important for T cell activation (Carrasco *et al.* 2017) and CD6-ligands like CD166 (Bowen *et al.* 1995) and CD318 (Enyindah-Asonye *et al.* 2017) are expressed on epithelial cells e.g. keratinocytes. Importantly, anti-CD6 antibodies are currently tested in clinical trials as a new T cell-based therapy for psoriasis in India (Srivastava 2017). Assuming a high expression of CD6 on PMNs of psoriasis patients, it can be hypothesized that anti-CD6 therapy not only intervenes with chronic manifestation of psoriasis induced by T cells but also prevents acute events driven by PMNs. Therefore, the investigation of CD6 expression on psoriasis PMNs was also of therapeutic interest and a potential role of CD6 on PMNs in psoriasis was assumed. Surprisingly, after re-gating of obtained LEGENDScreen™ data, it became apparent that 4 out of 5 psoriasis patients had very high CD6 expression levels on PMNs whereas 4 out of 5 healthy controls had no CD6 surface expression on PMNs (data not shown). Unfortunately, these findings could not be verified in additional patient samples. CD6 expression was neither detected on PMNs from psoriasis patients nor from healthy controls (data not shown). Therefore, it can be assumed that this "positive hit" was due to a kit effect, showing a false positive CD6 staining for psoriasis PMNs. Actually, during further evaluation of the results obtained by the LEGENDScreen™, it became apparent that mainly patients and healthy controls were measured with the same kit respectively. Only for the last patient and healthy control, the same kit was used, showing negative staining for CD6 on PMNs for both. For this reason, involvement of CD6 in pathology of psoriasis was not followed up in this study (see above) and further investigations focused on platelet-PMN interactions in psoriasis.

CD11c is a known marker found on different cell subsets in human blood, e.g. DCs, monocytes but also granulocytes (Boltjes and van Wijk 2014) and is considered to be important for phagocytosis and for DC antigen presentation (Sadhu *et al.* 2007) (Collin *et al.* 2013). As PMNs are able to act as APCs (Vono *et al.* 2017) it can be hypothesized that CD11c is also found on PMNs (see 5.2.1., similar to CD209) and displays similar functions there. Of note, CD11c expression and function on psoriasis PMNs was not further evaluated in this study. Furthermore, enhanced **CD235ab** expression was already detected as individual marker and has already been discussed in detail (see 5.2.1.).

Notable, two of the five combination markers, namely **CD41** and **CD61**, are known platelet antigens which usually act in concert and are necessary for platelet adhesion and aggregation (Mateo *et al.* 1996) (Anderson *et al.* 1991). The relevance of surface antigens CD41 and CD61 (in the context of psoriasis) was evidenced by further analysis (see 4.2.-4.4.) and hence will be discussed in the following sections (5.2.3.-5.2.6.).

PCA analysis of LEGENDScreen™ data revealed that blood-derived cells from psoriasis patients and healthy controls can be distinguished by specific surface antigens that are differentially expressed in these two groups. Importantly, PCA shows strong clustering especially for psoriasis PMNs, additionally uncovering a clear platelet signature on PMNs from psoriasis patients.

5.2.3. Platelet-PMN aggregates - a novel requisite for skin and cardiovascular manifestations of psoriasis?

Intriguingly, the present study identified a distinct “platelet signature” for circulating PMNs in psoriasis with two dominant markers: CD41 and CD61 (Figure 4.2). Expression of CD41 is commonly associated with CD61 and both proteins are required for platelet adhesion and aggregation (Mateo *et al.* 1996) (Anderson *et al.* 1991). CD41 and CD61 expression in this study was detected on PMNs due to platelet-PMN aggregates in circulation (confirmed by FACS and fluorescence microscopy, see Figure 4.3 and Figure 4.4). Importantly, the presence of platelet-PMN aggregates found in blood of psoriasis patients was also evident in IMQ-challenged mice (Figure 4.4), an established model system of psoriasiform skin inflammation. Of note, expression of the adhesion molecule P-selectin was shown to correlate with psoriasis severity (Ludwig *et al.* 2004). Although the present screen did not show enhanced expression of P-selectin (CD62P) in psoriasis patients (data not shown) further immunofluorescence microscopy identified platelets in platelet-PMNs aggregates which were predominantly activated and expressed CD62P (Figure 4.4). Although these findings are in good agreement with previous results (Ludwig *et al.* 2004), platelet-PMN aggregates were so far not studied in psoriasis and their impact on disease pathology has not been investigated: a potential etiology of platelet involvement was proposed - but not proven experimentally (Tamagawa-Mineoka 2015).

The present study experimentally evidences that platelet counts in psoriatic blood are greatly increased compared to blood from healthy controls. This is in agreement with previous findings which show an increased platelet mass index and volume in psoriasis furthermore correlating with arthritis. Thus, in psoriatic arthritis platelet mass index and volume can serve as a clinical marker (Canpolat *et al.* 2010) (Unal 2016). The elevated number of platelets might explain the increase in platelet-PMN aggregates found in psoriatic blood. Nevertheless, elevated platelet counts could be a secondary effect of increased hematopoiesis in response to inflammation, including megakaryocyte production in the bone marrow (Masamoto and Kurokawa 2016).

Also, a specific role of platelets in the development of skin lesions in psoriasis has not been reported yet. Here, in an IMQ mouse model of psoriasis significant amelioration of skin inflammation was reported upon platelet depletion: reduced ear swelling, decreased epidermal thickness, undetectable platelet-PMN/monocyte aggregates in the blood and also no detectable immune infiltrates in the skin (Figure 4.6 and Figure 4.7). In contrast, in human psoriatic lesions, PMNs are found in close proximity or even co-localized with platelets (Figure 4.5). This strongly indicates that PMNs rely on platelets to extravasate and cause inflammation in psoriatic lesions. Potential mechanisms of platelet-PMN extravasation are discussed in the following section (5.2.3.).

In summary, the present study for the first time provides solid evidence that platelet-PMN aggregates are highly relevant for skin pathology in psoriasis. Given the debated role of T-cells,

PMNs and endothelial cells in psoriasis it is worthwhile to investigate the role of platelets as new players in the pathogenesis of psoriasis as well as the mechanism underlying platelet-PMN aggregate formation and infiltration of the skin.

5.2.4. Potential mechanisms of platelet-PMN extravasation

Importantly, platelet aggregates are present in psoriatic skin (Figure 4.5), frequently accompanied by PMNs. These results are well in line with the recently published process of PMNs actively scanning for activated platelets prior to extravasation (Sreeramkumar *et al.* 2014). Also, platelets adhere at sites of inflammation and subsequently "capture" neutrophils or monocytes and promote extravasation (Zuchtriegel *et al.* 2016). Ludwig et al. showed that leukocyte rolling depends on PSGL1-P-selectin interactions and that rolling is enhanced by platelet-leukocyte aggregate formation (Ludwig *et al.* 2004). In combination with active migration of platelets (Gaertner *et al.* 2017) it is assumable that platelet-leukocyte aggregates form in the blood and then collectively migrate through the blood vessel. However, the scenario of platelets actively assisting leukocytes extravasation and its specificity for psoriatic skin remains to be validated. Currently ongoing experiments are focusing on shedding light to the mechanism of PMN-platelet aggregate formation and extravasation. The most important interaction of PMNs and platelets was shown for PSGL-1 (PMNs) and P-selectin (platelets). The impact of PSGL-1-P-selectin interactions on PMN extravasation in an IMQ-induced psoriatic mouse model is currently investigated by neutralizing PSGL-1 as previously described (Sreeramkumar *et al.* 2014). It is assumed that disruption of platelet-PMN interactions can prevent PMN infiltration of psoriatic skin.

Another component involved in leukocyte and platelet extravasation is the endothelium which is activated in psoriasis (Lee *et al.* 1994). Activated endothelium imparts enhanced attraction of platelets and other blood cells in turn augmenting extravasation. Interestingly, adherence of neutrophils is greatly increased in psoriasis which depends on activated endothelial cells (Wetzel *et al.* 2006). Of note, endothelial leakage is known for a long time in acute and chronic inflammation, facilitating extravasation of blood cells (McDonald *et al.* 1999). This also applies for psoriasis which, in rare cases, is associated with largely increased capillary permeability causing a hypovolemic shock; this phenomenon is commonly referred to as *leaky syndrome* (Bressan *et al.* 2017). There is evidence that endothelial permeability largely controls and affects angiogenesis. In line with this, angiogenesis is increased in psoriasis and new micro-vessels form in psoriatic lesions. Activated endothelium and angiogenesis of new micro-vessels would facilitate access of platelets and blood cells to sites of inflammation. In line, the vascular endothelial growth factor (VEGF) which regulates angiogenesis is associated with psoriasis. Keratinocyte-derived VEGF induces angiogenesis and supports hyperproliferation of the keratinocytes in the epidermis of psoriatic lesions. Furthermore, a pro-angiogenic role is attributed to IL-17, an accepted key mediator in the pathogenesis of psoriasis (Heidenreich *et al.* 2009). IL-17 induces expression of VEGF (Pan *et al.* 2015) (You *et al.* 2017) and angiogenesis. Thus, VEGF inhibitors (blocking angiogenesis) are considered to be efficient for treatment of psoriasis (Li *et al.* 2014).

Furthermore, in arthritis, platelets were shown to increase vascular permeability which is strongly regulated by serotonin (Cloutier et al. 2012). Current studies investigate, whether PMN influx of the skin is mediated by serotonin-driven vascular permeability or if platelet-PMN interaction/aggregate formation is necessary for PMN skin infiltration in an IMQ-induced psoriasis mouse model. As disease progression in psoriasis is improved by serotonin re-uptake inhibitors (Thorslund et al. 2013), the application of serotonin re-uptake inhibitors in IMQ-treated mice will further elucidate the mechanistic interrelationship of enhanced vascular permeability and PMN infiltration of the skin.

A specific role of platelets in the development of skin lesions in psoriasis has not been reported yet. As previously mentioned, this study describes significant amelioration of psoriatic skin inflammation accompanied with disappearance of PMNs in the skin upon depletion of platelets in an IMQ-induced mouse model of psoriasiform inflammation (Figure 4.6 and Figure 4.7). These data are consistent with results from experiments performed for another inflammatory disease of the skin, namely atopic dermatitis (AD): platelet-leukocyte aggregates are also found in the blood of mice suffering from AD and platelet depletion drastically reduced inflammation of the skin (Tamagawa-Mineoka *et al.* 2007). In humans, AD patients (the majority are children) suffer from severely itchy skin which could originate from pruritus-inducing chemical mediators. Such chemical mediators, e.g. histamines, can be provided by platelets (Tamagawa-Mineoka 2015). The fact that other inflammatory skin diseases like AD also profit from platelet-depletion, shows that the beneficial platelet depletion in the IMQ-induced mouse model of psoriasis might be of broader interest. Therefore, it would be interesting to investigate skin and blood from patients with other autoimmune diseases of the skin, such as AD, Rosacea, Sweet Syndrome, Behçet's Syndrome or contact dermatitis (Dainichi *et al.* 2014) (Villarreal-Villarreal *et al.* 2016). These analyses could reveal similar results as seen for psoriasis patients and uncover platelet-PMN aggregation as a common mechanism in inflammatory skin diseases. Thus, reduced platelet aggregation could also ameliorate skin pathologies in a broader sense.

This study for the first time presents solid data that instigate intervention with platelet-physiology as an opportunity to ameliorate pathologic skin condition in psoriasis. Data in this study show that platelets infiltrate the skin (Figure 4.5 and Figure 4.7) and might therefore either independently contribute to disease severity or by formation of platelet-PMN aggregates (Li *et al.* 2015). Interestingly, enhanced numbers of "free" PMNs are present in the blood of platelet-depleted mice, whereas skin infiltration of PMNs is absent (Figure 4.7). The absence of PMNs in the skin in combination with enhanced numbers of PMNs in the blood indicates that PMNs get "trapped" in the blood likely because the signal for skin infiltration is lacking due to loss of platelet-mediated skin homing. Thus, it is assumed that PMNs rely on platelets to infiltrate the skin in psoriasis and both, platelets and PMNs, are inevitable for a fulminant skin inflammation in psoriasis.

To further elucidate the underlying mechanisms of platelet-PMN skin infiltration, it would be interesting to characterize the molecular composition of platelets that aggregate with PMNs

in psoriasis patients. Regarding proteins at the platelet surface an antibody-screen (LEGENDScreen™) including additional platelet and PMN markers could be performed focusing on differentially expressed markers on platelets that are bound by PMNs. This screen may potentially identify antigens that reveal the molecular basis for platelet-assisted skin homing of PMNs. Alternatively, proteome analysis would be a promising, however also challenging, approach because separation of PMNs and platelets that already formed aggregates is hard to accomplish

As previously discussed, in RA, platelet accumulations in synovial fluid attract PMNs to the site of injury and PMNs subsequently are trapped in the fibrin network of platelets (Habets *et al.* 2013). These trapped PMNs become activated and release pro-inflammatory cytokines or even NETs. A similar mechanism can be assumed for psoriasis. Given the fact that NETs are found in blood and skin of psoriasis patients (Hu *et al.* 2016) and that platelets evoke NETosis (Caudrillier *et al.* 2012), it can be hypothesized that platelets might activate NET formation in psoriatic lesions. Interestingly, in NETs coagulation factors are detected (Healy *et al.* 2016) and there is emerging evidence that NETs participate in thrombosis (Kimball *et al.* 2016) – establishing a second line of evidence for a close relationship of platelets and PMN in immune responses of both, injury and autoimmune diseases. The interplay of platelets and PMNs might be essential to induce inflammation. Presuming that platelets are the first cells recruited into the skin, early inflammatory responses in psoriasis could potentially arise from a small injury that induces platelet activation at the leaky endothelium. Active platelets then attract PMNs or platelets already are accompanied by PMNs due to preformed aggregates in blood (proposed hypothesis see Figure 5.2). Then, PMNs get trapped and activated, release pro-inflammatory mediators or NETs which in turn attract and activates more PMNs and other blood cells. This mechanism would establish the formation of new lesions at the site of trauma or pressure.

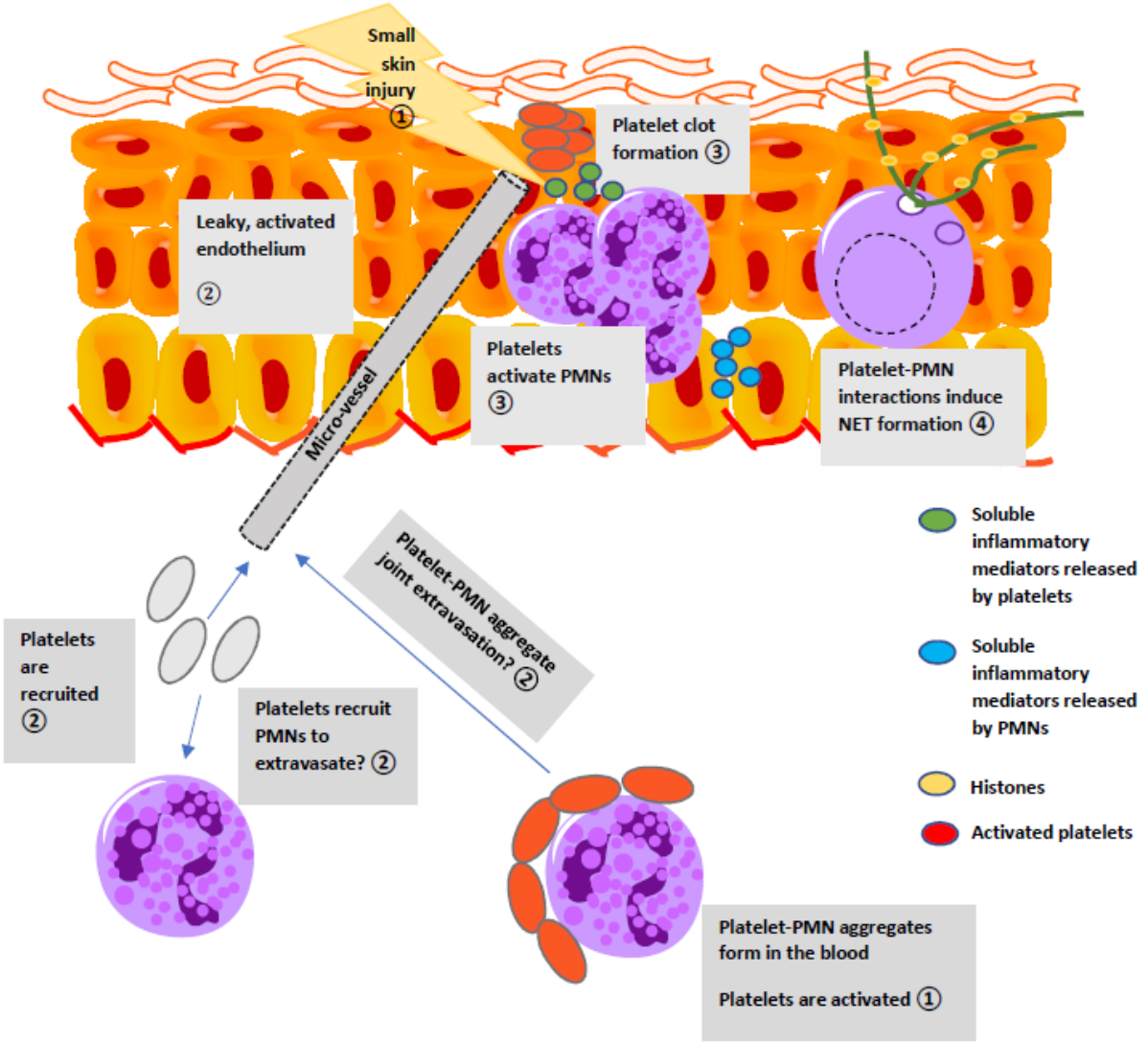


Figure 5.2: Platelet-PMN interactions potentially cause the development of psoriatic lesions

① A small injury of the skin actives platelets to close the wound by clot formation. In psoriasis activated platelets either recruit PMNs to the site of injured skin or platelet-PMN aggregates already form in blood and migrate as complex. ② Due a leaky and activated endothelium, platelet-PMN aggregates might easily reach the injured skin. ③ After skin infiltration, platelets can activate PMNs to induce NET formation which potentially leads to skin inflammation.

5.2.5. Potential role of platelet-PMN aggregates in CVDs found in psoriasis patients

Platelet-aggregates detected in this study offer an explanation for psoriasis associated cardiovascular comorbidities. Activated platelets form aggregates and recruited PMNs establish inflammation. This platelet-PMN driven mechanism has been described for RA, where activated platelets trap PMNs in the synovium, mediating PMN activation resulting in inflammation (Habets *et al.* 2013). Dysregulation of hyperactivity of platelets yields in thrombosis and/or cardiovascular diseases. Interestingly, suffering from severe psoriasis serves as an independent risk factor for the occurrence of major cardiovascular events

(Gelfand *et al.* 2006), substantiating the outlined interrelation of platelet-PMN aggregates and psoriasis. Therefore, it can be assumed that platelets attract PMNs also to other tissues (e.g. the heart) and induce inflammation. Hence, it would be interesting to investigate whether platelet-PMN aggregates are found e.g. in the aortas of mice with psoriatic skin inflammation and whether this infiltration is inhibited upon PMN-or platelet-depletion in circulation. This might serve as a potential link between psoriatic skin inflammation and CVDs seen in psoriasis patients and intervention with platelet-aggregation might simultaneously ameliorate psoriatic skin lesions and co-morbidities like CVDs.

Interestingly, the cytokine IL-17 was not only identified as a driver for psoriatic inflammation and but also for cardiovascular co-morbidities (Karbach *et al.* 2014). Antagonization of IL-17 expectedly reduced inflammation but also attenuated vascular diseases in a psoriatic mouse model (Schuler *et al.* 2018). Given that IL-17 increases platelet aggregation (Maione *et al.* 2011), it was hypothesized that IL-17 might also be a potential target to ameliorate CVDs in psoriasis. This could be further investigated using IL-17R conditional-platelet specific (Pf4-Cre) knock-out mice in the IMQ-induced psoriatic mouse model. Presumably, these mice would develop less CVDs compared to WT controls in psoriasiform inflammation.

Therefore, it can be assumed that targeting IL-17 in psoriasis is not only beneficial for chronic T cell driven psoriasis but also for cardiovascular comorbidities and potentially also reduces early inflammatory events induced by platelets.

5.2.6. Therapeutic implications

In this study, platelet depletion drastically ameliorates skin inflammation in an IMQ-induced psoriatic mouse model. This was likely due to strong interactions and aggregate formation with PMNs (Figure 4.6 and Figure 4.7).

It was hypothesized that platelet depletion is causative for reduced skin infiltration which might be supported by reports on beneficial effects on psoriatic skin lesions in the human system, by disrupting platelet aggregation e.g. using aspirin. Frequently, aspirin is prescribed to prevent thrombosis. However, aspirin inhibits cyclooxygenase, synthesis of prostaglandins and the formation of thromboxane A2. Therefore, it reduces pain, has an anti-inflammatory effect and, most importantly in the current context, it prevents platelet activation and aggregation (Mistry *et al.* 2017). Furthermore, aspirin reduces platelet-leukocyte aggregates in thrombosis (Trelinski *et al.* 2009) indicating that disruption of platelet-leukocyte aggregates indeed intervene with the induction of platelet-driven complications. Interestingly, beneficial effects of aspirin are also assumed for a variety of different immune diseases like RA, MS, SLE etc. (Habets *et al.* 2013).

Further investigations revealed case reports (reference see Table 6.1 and 6.3.2.) documenting improvement of psoriatic skin conditions upon aspirin therapy. Although aspirin was primarily prescribed to psoriasis patients to prevent cardiovascular comorbidities, it was also found to

induce regression of skin lesions. These findings are intriguing, because aspirin is a widely used drug, with marginal side-effects at appropriate doses (Berkel 1999) (Kwok 2010). To our knowledge, there is no data from systematic clinical studies on the effects of aspirin on psoriasis available so far. Nevertheless, it would be helpful to further investigate which functional mechanism underlies aspirin mediated amelioration of skin inflammation and whether the inhibition of platelet activation in this context is causative.

5.2.7. Conclusions part II

To date, the initial trigger for immune infiltration of the skin in psoriasis is unknown. Here, surface antigen expression of various cell populations was investigated using whole blood of psoriasis patients and healthy controls. It was assumed that differential expression of surface receptors might explain skin homing. Interestingly, circulating PMNs from psoriasis patients do not present an altered expression of chemokine- or adhesion-receptors as could be expected but a platelet signature, due to aggregate formation of platelets with PMNs, was found. Additionally, platelets were detected in psoriatic lesions, frequently in close proximity to PMNs. Strikingly, skin inflammation was greatly ameliorated and platelet-PMN-skin infiltration was inhibited upon platelet depletion in an IMQ-induced psoriatic mouse model. Thus, the present study is the first to provide solid evidence for an essential function of platelets in the pathogenesis of psoriasis. Future experiments should aim to enhance our understanding on the underlying mechanism of platelet-PMN aggregates in skin infiltration and resulting inflammation in psoriasis. Undoubtedly, it would be worthwhile to investigate the impact on interfering with platelet aggregation e.g. by using anti-coagulants like aspirin in the context of psoriasis.

6. Appendix

6.1. Neutrophil extracellular trap-associated RNA (naRNA) and LL37 complexes enable self-amplifying inflammation in psoriasis

6.1.1. PMNs are not pre-activated and are viable in this experimental setting

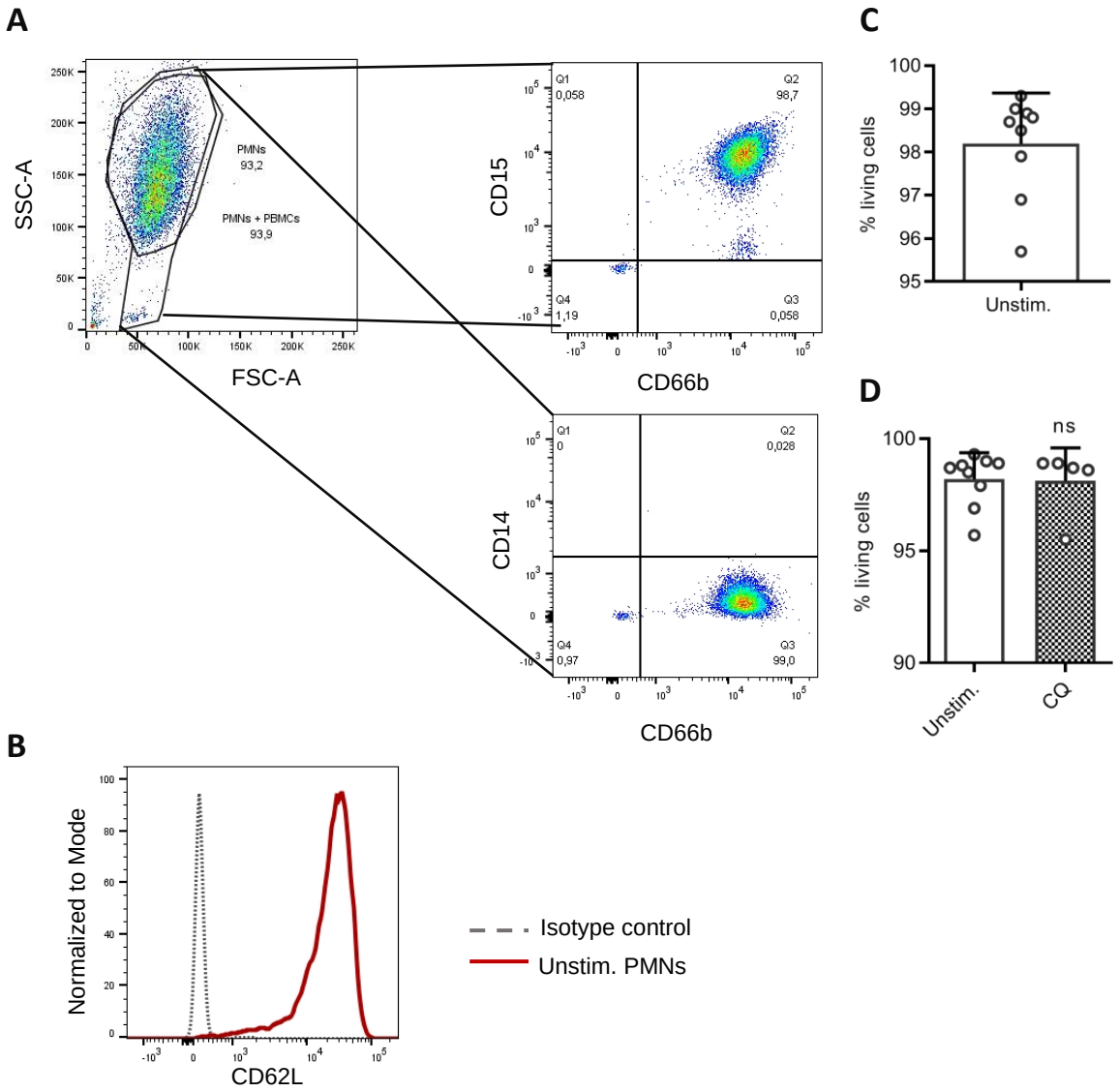


Figure 6.1: PMNs are not pre-activated and viable in this experimental setting

(A) FACS analysis of PMNs using anti-CD15, anti-CD66b, anti-CD14 and anti-CD62L antibodies against surface antigens. Gating strategy is shown. $CD15^{+}CD66^{+}$ and $CD14^{-}$ cells were considered as PMNs. (B) Histogram of one respective donor for CD62L is shown. PMNs are not activated. (C) Aqua Live-dead flow cytometric viability analysis of unstimulated PMNs after 4 h in culture (n=9 combined from several experiments) and (D) including 10 µM chloroquine (CQ) 30 min pre-incubation (n=5-8). C and D represent combined data (mean+SD) from 'n' biological replicates. A and B show results from one respective donor. * $p<0.05$ according to Wilcoxon signed rank sum (D).

6.1.2. Luminex analysis reveals a variety of cytokines and chemokines which are released by PMNs upon stimulation with RNA-LL37 complexes

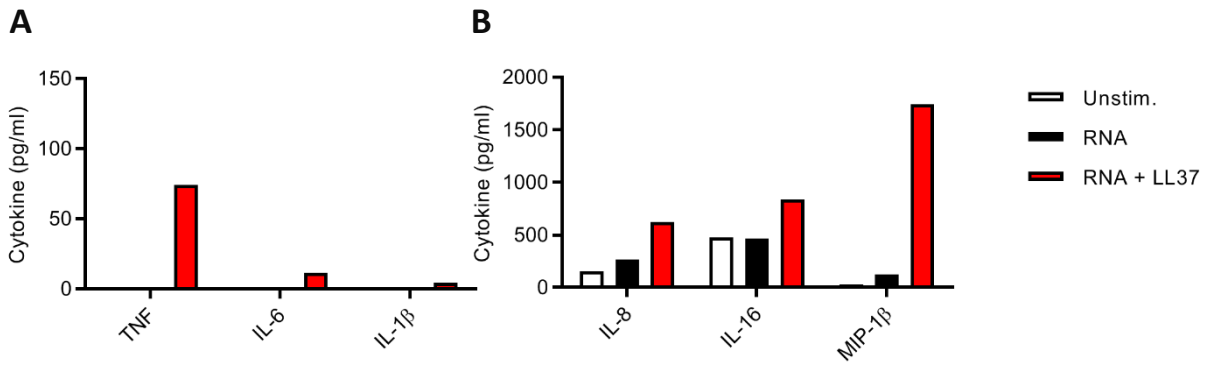


Figure 6.2: Luminex analysis of PMNs from two healthy donors

This analysis was performed at the NMI in Reutlingen (by Nicole Schneiderhan-Marra and Thomas Knorpp). Luminex multiplex cytokine analysis from supernatants of PMNs from two donors. (A) PMNs release more TNF-α, IL-6 and IL-1β when stimulated with RNA-LL37 complexes for 4 h (n=2). (B) PMNs release more IL-8, IL-16 and MIP-1β when stimulated with RNA-LL37 complexes for 4 h (n=2).

6.1.3. T cells migrate towards SDF-1α

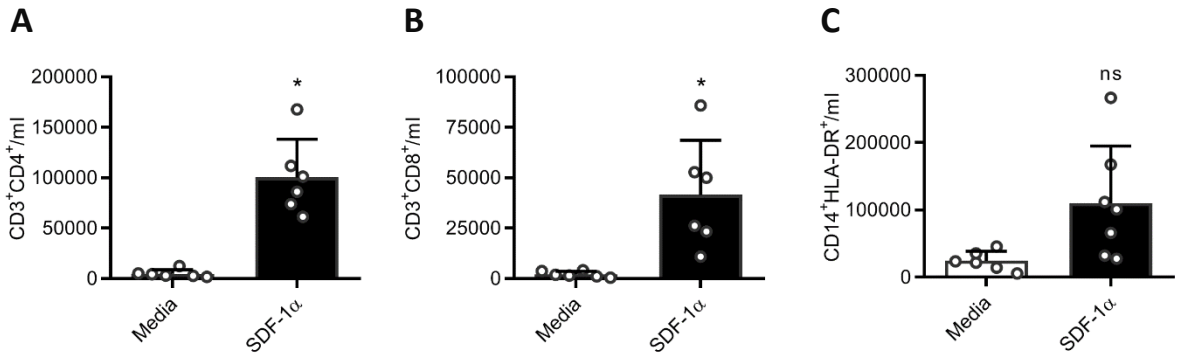


Figure 6.3: Migration control of transwell migration assay

Total amount of migrated cells in transwell migration assays with total PBMCs in the upper and SDF-1α (100 ng/ml positive control) in the lower compartment (n=6-7, p>0.05 for treatments vs. media). (A) total migrated $CD4^+$ T cells, (B) $CD8^+$ cells and (C) $CD14^+HLA\text{-}DR^+$ monocytes are shown. Combined data (mean+SD) from 'n' biological replicates (each dot represents one donor) throughout. * p<0.05 according to Friedmann test with Dunn's correction (A, B), one-way ANOVA with Dunnett's correction for multiple testing (C).

6.1.4. IRS661 and IRS954 (in nanomolar concentrations) are not toxic for PMNs

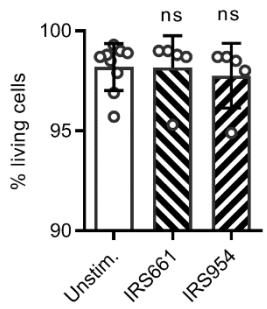


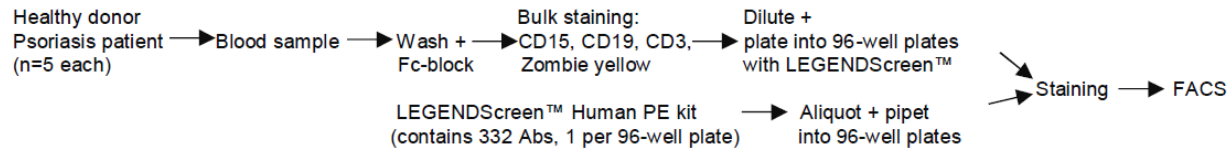
Figure 6.4: IRS661 and IRS954 are not toxic for PMNs

Aqua Live-dead flow cytometric viability analysis of PMNs pre-treated with IRS661 (1 nM) and IRS954 (50 nM) for 30 min and subsequent culturing for 4 h (n=5-9). * p<0.05 according to Kruskall-Wallis test with Dunn's correction.

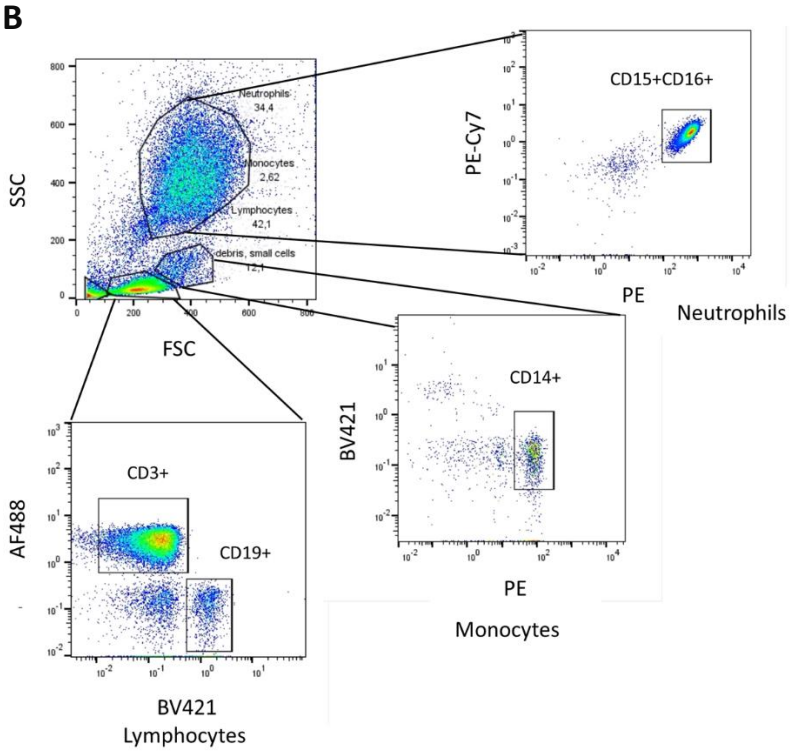
6.2. Platelet-PMN aggregates promote skin pathology in psoriasis

6.2.1. General workflow and gating strategy

A



B



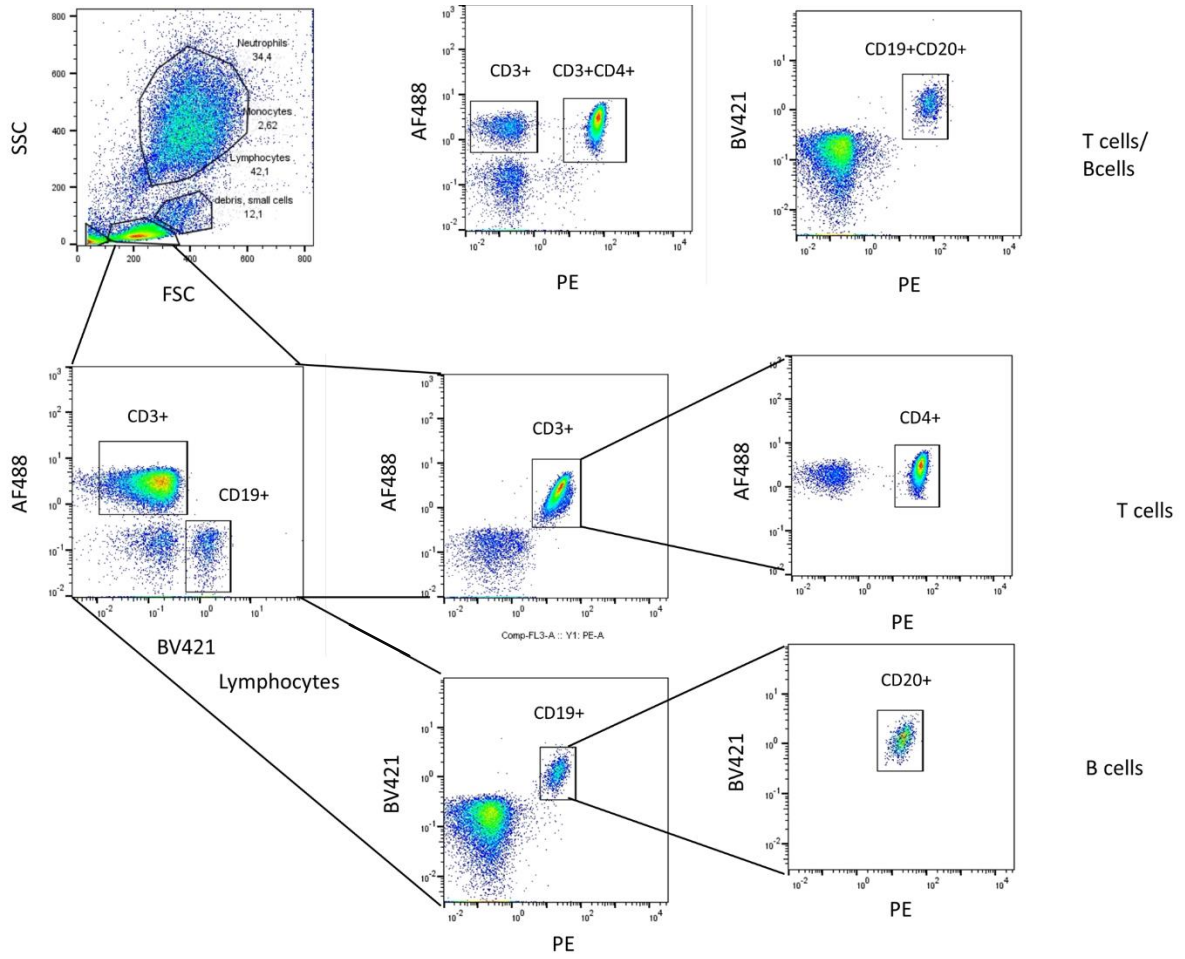
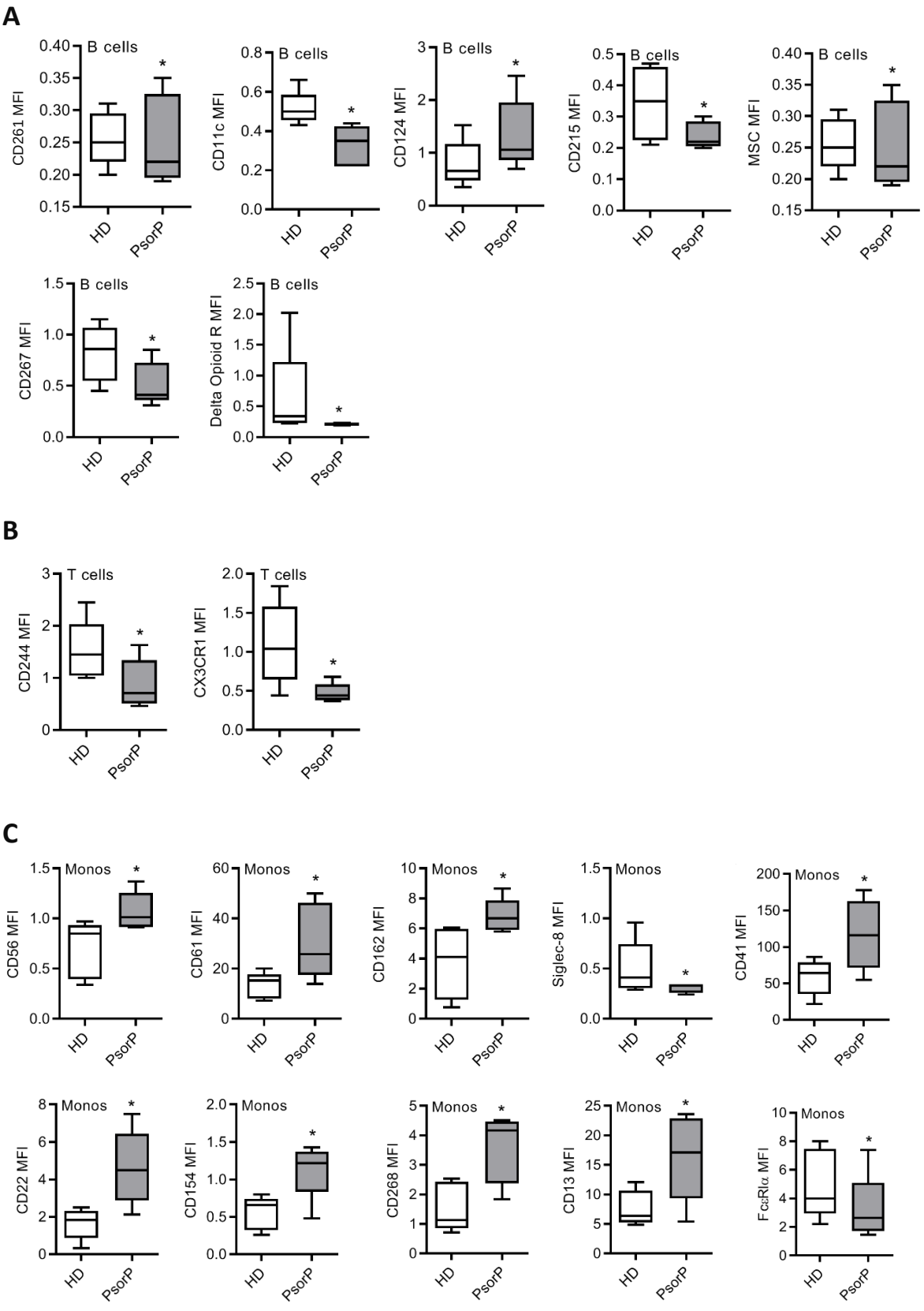


Figure 6.5: Workflow and gating strategy for LEGENDScreen™ from BioLegend

(A) shows the general workflow of the LEGENDScreen™ using whole blood from 5 psoriasis patients and 5 sex- and age-matched controls. After short erythrocyte lysis, the cell suspension was stained with CD15 PE-Cy7 (PMNs), CD3 AF488 (T cells), CD19 BV421 (B cells) and with Zombie yellow (dead cells). Then the screen was performed following the manufacturer's instructions (for further information see Materials and Methods). (B) shows the gating strategy. PMNs were gated by $CD15^+$ and subsequently checked for $CD16^+$ (PE marker provided in the kit) expression. For B and T cells, the lymphocytes were gated and checked for $CD19^+$ and $CD3^+$ (or $CD19^+CD20^+$ double positives and $CD3^+CD4^+$ double positives, with CD4 PE and CD20 PE provided by the kit). Monocytes were gated by size and granularity but were checked for $CD14^+$ (PE provided by the kit) expression.

6.2.2. Significant differences in surface antigen expression on B cells, T cells and monocytes found in LEGENDScreen™ analysis



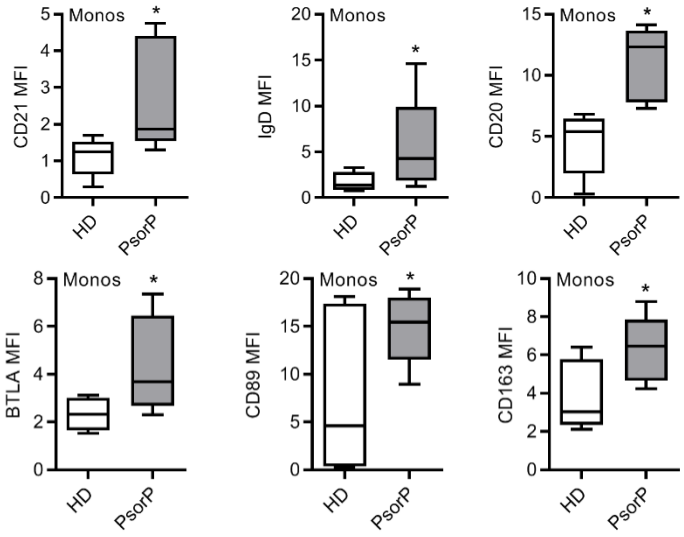


Figure 6.6: Significant differences in surface antigen expression between psoriasis patients and healthy controls

(A) Surface antigen expression (MFI) on B cells which are significantly different (CD261, CD11c, CD124, CD215, MSC, CD267 and δ- Opioid receptor; see Table 9 for further information) between healthy donors (HD) and psoriasis patients (PsorP) n=5 each. (B) the same as (A) but for T cells (CD244 and CX3CR1). (C) the same as (A) but for monocytes (CD56, CD61, CD162, Siglec-8, CD41, CD22, CD154, CD268, CD13, FcεRIα, CD21, IgD, CD20, BTLA, CD89 and CD163). A-C represent combined data (mean+SD) from 'n' biological replicates. Statistical analysis was performed by Marius Codrea and Simon Heumos from QBIC. * $p<0.1$ nominal by two-way ANOVA followed by Tukey's multiple comparisons correction.

6.2.2. Surface antigen signature of monocytes, B and T cells (psoriasis patients vs. healthy controls)

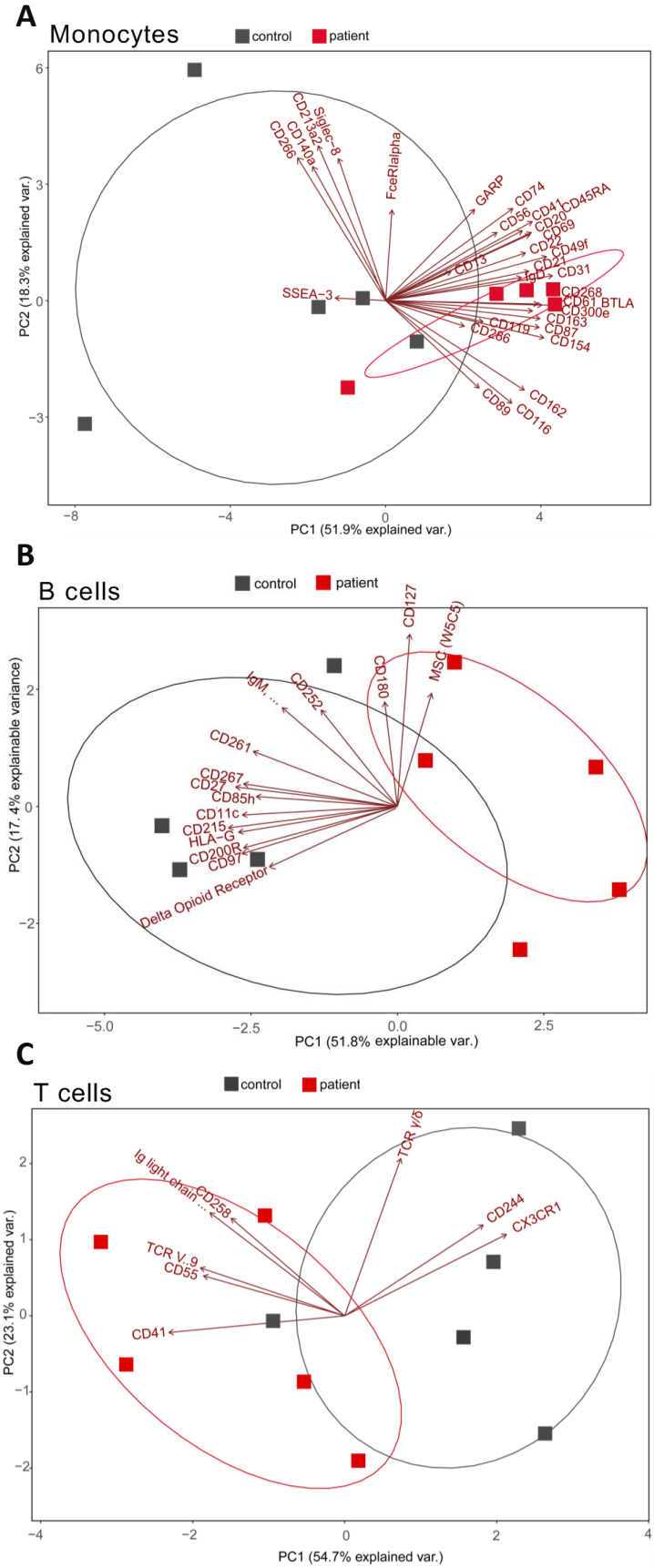


Figure 6.7: Monocytes, B and T cells from psoriasis patients have a different surface antigen signature compared to healthy controls

Principal component analysis of monocytes (A), B cell (B) or T cell (C) surface antigens for healthy donors (red) and psoriasis patients (blue) (data obtained by LEGENDScreen™). The top significant markers (based on nominal p-value <0.1) which contribute to a separation between patients and healthy donors (n=5). A-C represent combined data (mean+SD) from 'n' biological replicates (each dot represents one donor).

6.2.3. Platelet-monocyte aggregates are found in the blood of psoriasis patients

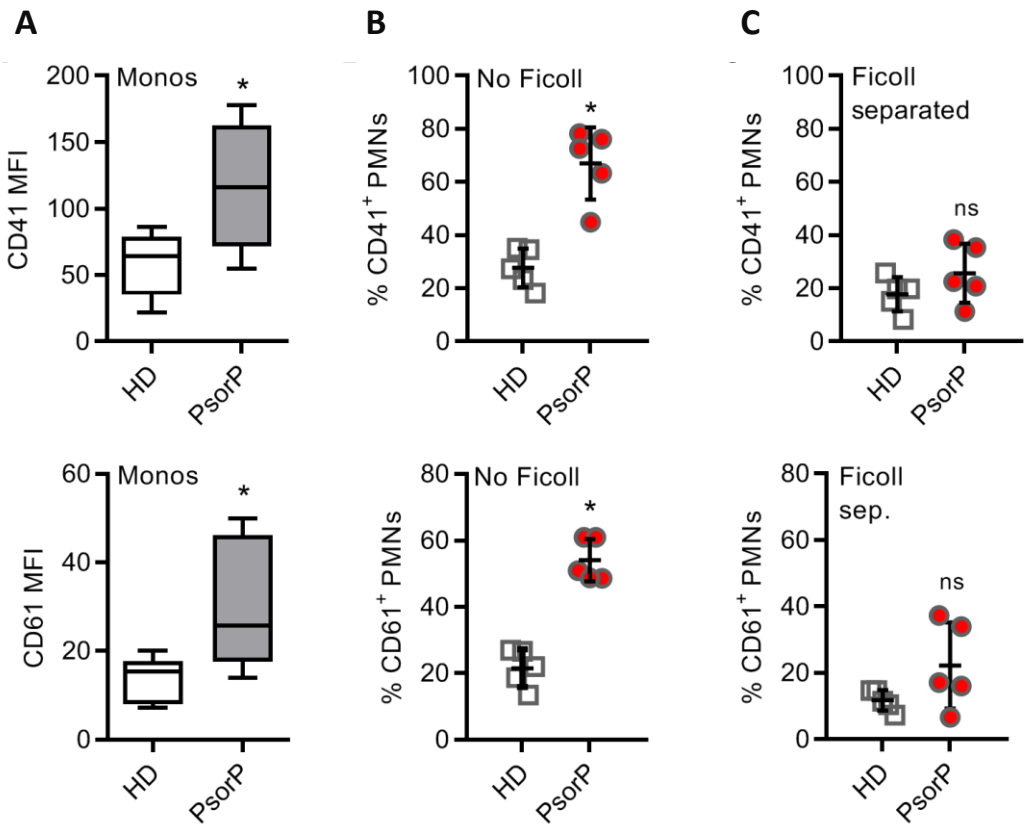


Figure 6.8: Platelets cover the surface of monocytes in the blood of psoriasis patients

(A) Mean fluorescence intensity (MFI) extracted from the LEGENDscreen™ for CD41 (upper panel) and CD61 (lower panel) (healthy donor (HD) and psoriasis patient (PsorP); n=5 each). (B) FACS analysis showing % of CD41- or CD61-positive PMNs (defined as $CD15^+CD66b^+$ and $CD41^+$ or $CD61^+$) analyzed in HD or PsorP (HD n=7, PsorP n=5) in whole blood samples or (C) after Ficoll density gradient centrifugation. In A * $p<0.1$ nominal by two-way ANOVA followed by Tukey's multiple comparisons correction, in B and C * $p<0.05$ by unpaired Student's t-Tests.

6.3. Supplementary references

Table 6.1: Supplementary references

homepage	paragraph
https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death	1.5.4.
https://www.clinicaladvisor.com/home/consultations/aspirin-for-psoriasis/	5.2.5
https://www.everydayhealth.com/news/surprising-uses-for-aspirin/	5.2.5.

6.3.1. Leading causes of death worldwide (WHO, 2016)

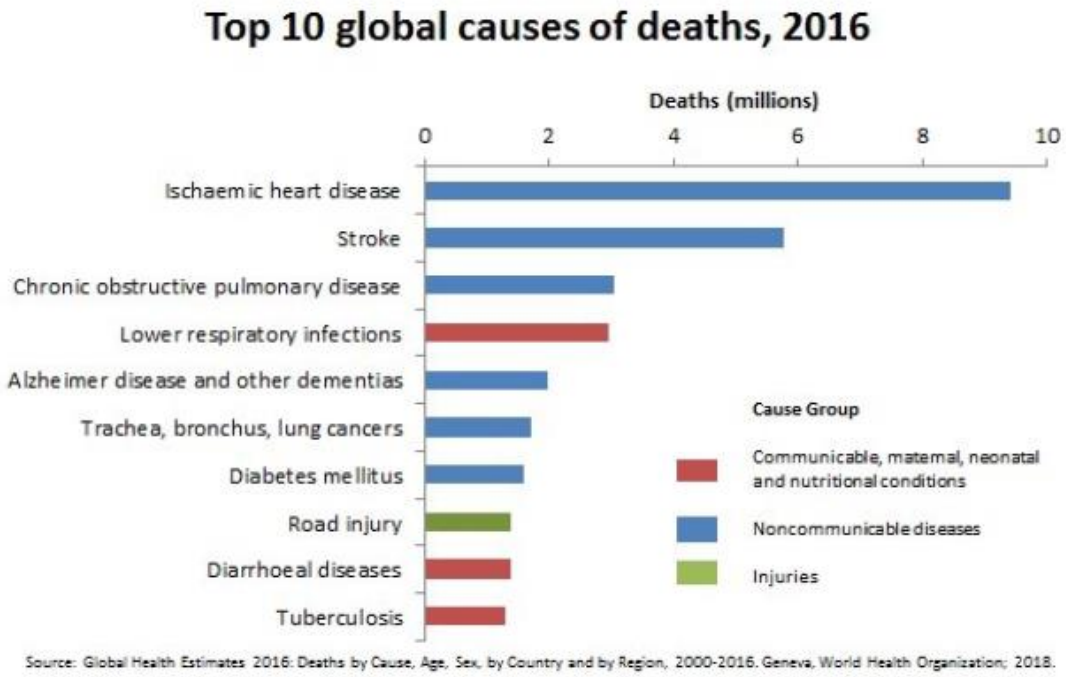
24 May 2018

Of the 56.9 million deaths worldwide in 2016, more than half (54%) were due to the top 10 causes. Ischaemic heart disease and stroke are the world's biggest killers, accounting for a combined 15.2 million deaths in 2016. These diseases have remained the leading causes of death globally in the last 15 years.

Chronic obstructive pulmonary disease claimed 3.0 million lives in 2016, while lung cancer (along with trachea and bronchus cancers) caused 1.7 million deaths. Diabetes killed 1.6 million people in 2016, up from less than 1 million in 2000. Deaths due to dementias more than doubled between 2000 and 2016, making it the 5th leading cause of global deaths in 2016 compared to 14th in 2000.

Lower respiratory infections remained the most deadly communicable disease, causing 3.0 million deaths worldwide in 2016. The death rate from diarrhoeal diseases decreased by almost 1 million between 2000 and 2016, but still caused 1.4 million deaths in 2016. Similarly, the number of tuberculosis deaths decreased during the same period, but is still among the top 10 causes with a death toll of 1.3 million. HIV/AIDS is no longer among the world's top 10 causes of death, having killed 1.0 million people in 2016 compared with 1.5 million in 2000.

Road injuries killed 1.4 million people in 2016, about three-quarters (74%) of whom were men and boys.



6.3.2. Aspirin ameliorates psoriatic skin inflammation

April 30, 2011

Aspirin for psoriasis?

Have there been any reports in the literature linking improvement of psoriasis vulgaris with initiation of long-term, low-dose aspirin therapy, even when usual therapy has failed? I have seen marked improvement in three patients taking aspirin; results were even better than with halobetasol (Ultravate) or calcipotriene (Dovonex) therapy. One patient who stopped aspirin temporarily noticed an immediate flare-up. Restarting the aspirin brought renewed improvement. One possible mechanism: Aspirin slows cell growth and may therefore inhibit the hyperproliferation or accelerated growth of skin cells that is characteristic of psoriasis. — Watson Gutowski, DO, Newtown Square, Pa.

I was unable to find any reports or studies on the utility of aspirin in psoriasis. According to the National Psoriasis Foundation website, aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) do not change psoriasis skin lesions, although such agents do sometimes cause worsening. Certain NSAIDs, such as indomethacin, have been reported to trigger flares of psoriasis. — Jeffrey M. Weinberg, MD (150-10)

5 Surprising Uses for Aspirin

The common over-the-counter pain reliever can do wonders you probably don't know about.

By Aleksandra Mencel

Last Updated: 7/29/2014

The benefits of aspirin far exceed temporary relief from fever, aches, and pains. Most people know that it can protect you from a heart attack or stroke, if taken properly. Research suggests that it can reduce the risk of some cancers and Alzheimer's disease. But aspirin has many other potential health, beauty, and personal benefits. Discover some of the amazing things that bottle in your medicine cabinet can do for you.

1. Treats skin conditions. Used as far back as the fifth century B.C. by Hippocrates to ease aches and pains, salicylic acid, a derivative of acetylsalicylic acid (aspirin), is a powder extracted from the bark of a willow tree. It "helps remove excess oil and exfoliate dead cells on the skin's surface," says Joshua Zeichner, MD, director of cosmetic and clinical research in dermatology at Mount Sinai Hospital in New York City, adding that it's "extremely useful in treating acne."

By dissolving dead skin and helping to shed the skin's top layer, salicylic acid reduces the possibility of clogged pores – the common culprit behind breakouts. It also clears up redness and decreases swelling, which can alleviate symptoms of rosacea. People with psoriasis may also benefit from the salicylic acid in aspirin. "It can make psoriasis lesions thinner and can help reduce itch," says Dr. Zeichner.

RELATED: 8 Easy Home Remedies for Acne

To make your own aspirin-based skin mask, crush up five uncoated aspirin pills and drop them into a quarter-cup of warm, distilled water. Stir well until the concoction develops a paste-like consistency. If you want, you can add a teaspoon of honey to the mix to reap the benefits of this natural antibacterial moisturizer. Once the mixture is about the same temperature as your skin, apply to any parts of the body affected by acne or other kinds of inflammation, and leave on for about 10 minutes. Wash off with warm water.

2. Gets rid of dandruff. Because of its moisturizing qualities, the same salicylic acid contained in face washes is also an ingredient in many dandruff shampoos. "Applying aspirin mixed with shampoo to your scalp may help reduce scalp inflammation that causes dandruff and help exfoliate flakes on the skin," says Zeichner.

To make the concoction, crush two or three uncoated aspirins and mix them into the normal amount of shampoo used each time you wash your hair. Focus on your scalp as you massage the mixture into hair and let it sit for three to five minutes before rinsing.

3. Soothes stings and bites. Zeichner says aspirin, with its anti-inflammatory properties, may help reduce the redness, swelling, and pain from bug bites. Make an aspirin paste similar to the one used for problem skin or just dampen an uncoated aspirin and apply it to the affected area for a few minutes.

4. Removes stains. The salicylic acid in aspirin helps to neutralize stains by attacking the compounds found in sweat and breaking them down. Mix three crushed aspirins and half a cup of warm water in a bowl. Submerge the stained portion of the clothing into the bowl for a few hours. Afterwards, wash the shirt as usual. You can use any of the leftover mixture to remove nicotine or grass stains from hands by applying the solution and letting it sit for 15 minutes before washing hands thoroughly.

5. Makes your garden grow. Aspirin works just as well outside as it does on your skin by activating the plants' natural defenses and preventing the formation of fungus. It also increases growth rate. Dissolve an uncoated aspirin in one gallon of water and spray onto any indoor and outdoor plants. It can also help prolong the life of fresh cut flowers. According to Judy Jernstedt, professor of plant sciences at UC Davis, the "salicylic acid reduces ethylene production, and with less ethylene present, floral senescence is delayed and the flowers last longer. The anti-fungal properties of salicylic acid dissolved in the vase water may also slow growth of mold, which if it enters the cut stem, can damage or clog the vascular tissue." So be sure to add a ground aspirin to the water for your floral arrangement.

Statutory Declaration

Hereby I affirm that I wrote this Doctoral thesis with the topic

“PMNs, naRNA-LL37 complexes and platelets – a vicious inflammatory ‘trio’ in psoriasis “

independently and that I used no other aids than those cited. In each individual case, I have clearly identified the source of the passages that are taken paraphrased from other works and the linked manuscript. Moreover, I acknowledge the work done by collaborators. I affirm that I performed the scientific studies according to the principles of good scientific practice.

Franziska Herster

_____

Tübingen, Germany

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