NOD2-RIPK2 signaling mediates psoriasis-associated gene expression by naRNA from neutrophil extracellular traps in primary human keratinocytes

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Summary

Psoriasis is a common inflammatory skin disease affecting an estimated 2-3% of the population. This skin disease is a complex disorder that can be triggered by genetic, environmental and immunological factors, but the exact cause is still unknown. Psoriasis is characterized by persistent inflammation associated with uncontrolled keratinocyte hyperproliferation and abnormal differentiation. The inflammation is triggered by a broad spectrum of cytokines, such as IL-8, IL-23, IL-17, and IL-36. These cytokines are produced by many different cell types, but one of the most important producers in psoriasis are the keratinocytes. In this process, the keratinocytes produce not only cytokines, but also chemokines and antimicrobial peptides to attract and activate immune cells, which then also release cytokines that activate the keratinocytes and lead to hyperproliferation of these cells. This feedback loop amplifies the response and increases the complexity and severity of psoriasis. Furthermore, the severity of psoriasis is associated with the frequency of neutrophil extracellular traps (NETs). These NETs can not only capture and kill microorganisms, they also promote inflammation. In this context, NET-associated RNA (naRNA) was first described by Herster et al. in 2020. This naRNA is a potential trigger of the proinflammatory processes, however, not much is known about naRNA or its influence on immune responses. Therefore, the aim of this work was to investigate the influence of naRNA on cells in the skin, to identify the receptors responsible for it and to analyze the possible role in the progression of psoriasis.

It could be shown that naRNA leads to self-amplification of NET-formation in neutrophils and that this is mediated by the receptor TLR8 in human or TLR13 in mice. Thereby this work could show that naRNA recognition in macrophages leads to TLR8-dependent IL-8 release. Human keratinocytes, the most abundant cell type in skin, respond to naRNA in a dose-dependent manner and subsequently release IL-8. This has also been demonstrated in physiologically relevant 3D models of the skin. This recognition of naRNA is not only TLR8, but MyD88-, NLRP1- and MAVS-independent. This activation of keratinocytes is mediated by the NOD2-RIPK2 signaling pathway. This was demonstrated by inhibition of RIPK2 and knockdown of NOD2 using siRNA. NOD2-mediated activation by naRNA thereby induces the expression of proinflammatory cytokines and antimicrobial peptides associated with psoriasis, such as *IL17C*, *IL36G*, *CXCL8*, *CAMP* and *RNASE7*. The activation of these genes could also be detected in 3D models of the skin. Furthermore, the induced expression of *IL17C*, *IL36G*, *CXCL8*, and *CAMP* was found to be mediated by NOD2, but not *RNASE7*. In addition, inhibition of IκBζ was found to lead to a reduction in many of these cytokines, suggesting that IκBζ lies downstream of NOD2. NaRNA-mediated activation of mouse keratinocytes was also examined. This also revealed NOD2-dependent activation of mouse keratinocytes by RNA, leading to induction of psoriasis-associated genes. Fibroblasts, the second most abundant cell type in the skin, also respond to RNA in NETs, but the cell line used responded in a RIPK2 independent manner.

In summary, naRNA was shown to be a major proinflammatory component of NETs that is recognized by all cell types tested. However, the signaling pathways for recognition differ. The NOD2-mediated activation of keratinocytes and the resulting activation of psoriasis-associated genes could be a key to further understanding this disease but also provide new potential therapeutic approaches.

Zusammenfassung

Psoriasis ist eine weit verbreitete, entzündliche Hautkrankheit unter der schätzungsweise 2-3% der Bevölkerung leiden. Diese Hautkrankheit ist eine komplexe Erkrankung, die durch genetische, umweltbedingte und immunologische Faktoren ausgelöst werden kann, deren genaue Ursache jedoch noch unbekannt ist. Charakteristisch für Psoriasis ist dabei eine anhaltende Entzündung in Verbindung mit einer unkontrollierten Keratinozyten-Hyperproliferation und einer abnormen Differenzierung. Die Entzündung wird durch ein breites Spektrum von Zytokinen ausgelöst, wie z.B. IL-8, IL-23, IL-17 und IL-36. Diese Zytokine werden von vielen verschiedenen Zelltypen produziert, aber einer der wichtigsten Produzenten bei Psoriasis sind die Keratinozyten. Die Keratinozyten produzieren dabei nicht nur Zytokine, sondern auch Chemokine und antimikrobielle Peptide, um Immunzellen anzulocken und zu aktivieren, die dann ebenfalls Zytokine freisetzen, die die Keratinozyten aktivieren und zu einer Hyperproliferation dieser Zellen führen. Diese Rückkopplungsschleife verstärkt die Reaktion und erhöht die Komplexität und den Schweregrad der Psoriasis. Darüber hinaus ist der Schweregrad der Psoriasis mit der Häufigkeit von neutrophilen extrazellulären Fallen (neutrophil extracellular traps (NETs)) assoziiert. Diese NETs können dabei nicht nur Microorganismen fangen und töten, sie fördern auch Entzündungen. Dabei wurde erstmals 2020 von Herster et al. die NET-assoziierte RNA (naRNA) beschrieben. Diese naRNA ist ein potenzieller Auslöser der Entzündungsprozesse, jedoch ist nicht viel über naRNA oder ihren Einfluss auf Immunreaktionen bekommt. Ziel dieser Arbeit war es daher, den Einfluss von naRNA auf Zellen in der Haut zu untersuchen, die dafür verantwortlichen Rezeptoren zu identifizieren und die mögliche Rolle beim Fortschreiten von Psoriasis zu analysieren.

Es konnte gezeigt werden, dass naRNA zu einer selbst Amplifikation der NET-Bildung bei Neutrophilen führt und dies durch den Rezeptor TLR8 bei Menschen bzw. TLR13 in Mäusen vermittelt wird. Diese Arbeit konnte dabei zeigen, dass die Erkennung von naRNA in Makrophagen zu einer TLR8 abhängigen IL-8 Freisetzung führt. Die humanen Keratinozyten, in der Haut der am häufigsten vorkommende Zelltyp, reagieren dosisabhängig auf naRNA und schütten daraufhin IL-8 aus. Dies konnte auch bei physiologisch relevanten 3D Modellen der Haut gezeigt werden. Diese Erkennung von naRNA ist dabei nicht nur TLR8, sondern MyD88-, NLRP1- und MAVS-unabhängig. Diese Aktivierung der Keratinozyten wird durch den NOD2-RIPK2 Signalweg vermittelt. Dies konnte durch Inhibition von RIPK2 und Ausschaltung von NOD2 mittels siRNA gezeigt werden. Die NOD2-vermittelte Aktivierung durch naRNA induziert dabei die Expression entzündungsfördernder Zytokine und antimikrobieller Peptide die mit Psoriasis assoziiert sind, wie z.B. *IL17C*, *IL36G*, *CXCL8*, *CAMP* und *RNASE7*. Die Aktivierung dieser Gene konnte auch in 3D-Modellen der Haut detektiert werden. Darüber hinaus konnte festgestellt werden, dass die induzierte Expression von *IL17C*, *IL36G*, *CXCL8* und *CAMP* durch NOD2 vermittelt wird, nicht jedoch von *RNASE7.* Darüber hinaus konnte festgestellt werden, dass eine Inhibition von IκBζ ebenfalls zu einer Reduktion vieler dieser Zytokine führt, was vermuten lässt, dass IκBζ nachgeschaltet von NOD2 liegt. Es wurde auch die naRNA vermittelte Aktivierung von Maus-Keratinozyten untersucht. Dabei konnte auch eine NOD2 abhängige Aktivierung der Maus-Keratinozyten durch RNA festgestellt werden, die zu einer Induktion von Psoriasis assoziierten Genen führt. Fibroblasten, die in der Haut der am zweithäufigsten vorkommende Zelltyp, reagieren ebenfalls auf die RNA in NETs, jedoch reagiert die genutzte Zelllinie auf eine RIPK2 unabhängige Weise darauf.

Zusammenfassend konnte gezeigt werden, dass naRNA ein bedeutender entzündungsfördernder Bestandteil von NETs ist, der von allen getesteten Zelltypen erkannt wird. Die Signalpfade für die Erkennung unterscheiden sich jedoch. Die durch NOD2-vermittelte Aktivierung der Keratinozyten und

die daraus resultierende Aktivierung von Psoriasis assoziierten Genen könnte ein Schlüssel zum weiteren Verständnis dieser Krankheit sein aber auch neue mögliche Therapieansätze liefern.

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1. Introduction

1.1 The immune system

Over a century ago, the immune system started to be characterized as a complex network of components for the protection of our human body during infectious diseases (Sattler 2017). The beginnings of the immunology, with all functions we know by now and the extrinsic studies that are ongoing, are attributed to Louis Pasteur, who suggested that germs cause diseases (Plotkin 2005), and Robert Koch, who proved that infectious diseases arise from microorganisms (King 1952). Our world is heavily populated by microbes which can be either pathogenic or non-pathogenic. The host has to differentiate between the pathogens and beneficial, commensal microorganisms, that have to be eliminated or tolerated, respectively, in order to maintain normal organ and tissue functions (Chaplin 2010). This is essential as our body is constantly surrounded by microbes, which are in contact with the body surface, inhaled, swallowed or actively penetrate our mucosal membranes and skin (Parkin and Cohen 2001). Apart from infections, the immune system is also involved in further processes such as anti-tumor responses but certainly host defense is a key function.

The immune system has a variety of mechanisms that are rapid and highly specific to protect the body against pathogens, such as bacteria, viruses, fungi and other microorganisms and their biologic products to maintain health (Medina 2016). To be rapid, as well as highly specific, the immune system consists of three major functional entities. The first are the barriers that form a first line of defense of the immune system and prevent the penetration of foreign substances. The second is termed innate immunity, a nonspecific defense response that is direct and rapid (Medzhitov and Janeway 1997). The other component is the adaptive immune system that represents an acquired long-term response against specific antigens derived from pathogens (Pancer and Cooper 2006). However, the three parts of the immune system are closely linked and adaptive immunity is regulated in part by the innate immune system and vice versa (Medzhitov and Janeway Jr 1997; Iwasaki and Medzhitov 2015). The interaction is a highly regulated and complex interplay between different humoral mechanisms and cell types which communicate and interact (Rieckmann et al. 2017). The mechanisms of defense by innate and adaptive immunity are explained in more detail in the following chapters.

1.1.1 Barriers

The human body is not only constantly endangered by countless microorganisms around us, but it also needs to be protected from physical damage, chemicals, environmental factors, like heat and UV-radiation, and uncontrolled water loss (Proksch, Brandner, and Jensen 2008). For this the epithelia forms the basis for physical, chemical, and microbiological barriers, which contribute to homeostasis and maintain organ functionality (Natsuga 2014; Turksen 2017). Physically speaking, by tight junctions epithelial barriers provide a physical lining of the body's internal organs, tissue and cells but with the capacity for regulating with a selective permeability (Crawford and Dagnino 2017). Chemical barriers consist of chemical substances released by epithelia and cells in the underlying tissues, that are directly antimicrobial (e.g. β-defensins and cathelicidins) (Hans and Madaan Hans 2014) or create conditions adverse for microorganisms (e.g. low pH). Finally, barriers are ecosystems for commensal microbiota which either inhibit growth of pathogens or trigger a protective reaction that prevent invasion and colonization of pathogens by acting on the host's immune system (Khan, Petersen, and Shekhar 2019).

Three main interfaces between the environment and a human exist. For each of them the structure and function of the barrier differs. The first is the skin, the second is the respiratory tract and the third is the gastrointestinal system (Şenel 2021). Although the specific structure and functions between the barriers differ, they all have four main components. The first are epithelial cells, the second are structural proteins like filaggrin or proteins that form tight and adherent junctions, the third are products secreted by the epithelial cells, like antimicrobial peptides (AMPs), mucus and a lipid rich matrix. The fourth one is the epithelial microbiota, which consists of commensal microorganisms (Mitamura et al. 2021). In these barriers the microbiome and host cells, especially epithelial cells and cells of the immune system, can interact in multifaceted ways (Turksen 2017). The communication between the host and the microbiome shapes the immune system to react appropriately to antigens and enables a tolerance between the commensal microorganisms and effector immune functions (Takiishi, Fenero, and Câmara 2017). Changes in the microbiome or pathological conditions that may be related to immune-overactivation can alter the integrity of the epithelial barrier or lead to dysfunctional immune responses (Yu, Khodadadi, and Baban 2019).

1.1.2. Innate immunity

Vertebrates are the only organism to have an adaptive immunity, which means that for most of the organisms on earth innate immunity alone is sufficient for survival. Innate immunity can detect invading microbes by only a limited number of receptors that bind to conserved molecular components of microbes. These germline-encoded receptors are called pattern recognition receptors (PRRs) and cannot be altered in terms of specificity. That is why innate immunity leads to a relatively unspecific immune reaction. (Akira, Uematsu, and Takeuchi 2006; Kawai and Akira 2010). These PRRs can detect two distinct classes of molecules. The microbe-associated molecular patterns (MAMPs) also referred to as pathogen-associated molecular patterns (PAMPs), associated with components from microbial pathogens. The other class are damage-associated molecular patterns (DAMPs), associated with components from damaged or dead cells (Gulati et al. 2018). Furthermore, a defining characteristic is that the innate immune system reacts to a microbe exposure within minutes and may be able to start effector responses aimed at clearing these microbes and generate a protective inflammatory response (Turvey and Broide 2010). PRRs are discussed in greater detail in section 1.3.

Physical and chemical barriers are also augmented by components of the innate immune system, consisting of both humoral and cellular parts (Janeway Jr and Medzhitov 2002). The humoral part includes MAMP-binding proteins (e.g. lipopolysaccharide binding protein, C-reactive proteins or lectins), antimicrobial peptides and complement proteins. These proteins can be found in the plasma and on the cell surface. They are not only involved in the sensing of microbes but also assist in the clearance of infections by effector mechanisms such as cellular phagocytosis or direct lysis (Turvey and Broide 2010). The complement system was first found over 100 years ago, referring to plasma factors that are important for host defense and are involved in removal of microbes (Morgan 1998). The complement system consists of around 30 proteins and protein regulators and can be differentiated into three pathways, the classical, the lectin and the alternative pathway (Nordahl et al. 2004). These three pathways signal through a series of different proteins but all of them activate the central plasma protein C3. The subsequent signaling cascade involves soluble anaphylatoxins and potent chemoattractants like C3a and C5a that are formed by cleavage of the proteins C3 and C5. Moreover, C3b is formed, which interacts with the microbial surface and enables an easier recognition of the microbe by phagocytes or allows pore formation through additional complement factors. C3b, like

other soluble factors such as C-reactive protein, also acts as an opsonin, facilitating the uptake of opsonized microorganisms by phagocytes (Murphy and Weaver 2016). Dysregulation or defects in the complement system are associated with various diseases like lupus erythematosus and asthma (Sarma and Ward 2011).

The cellular part of the innate immune system consists of cells from hematopoietic and non-hematopoietic origin. The hematopoietic cells include white blood cells of myeloid origin, such as neutrophils, eosinophils, basophils, mast cells, monocytes, macrophages and dendritic cells. Certain immune cells of lymphoid origin are also considered to be part of the innate immune system, such as natural killer (NK) cells. All these cells derive from multipotent, hematopoetic stem cells in the bone marrow (Morrison, Uchida, and Weissman 1995). In addition to these cells, also non-hematopoietic, e.g. tissue, cells are involved in innate immune responses. This includes skin cells such as keratinocytes but also epithelial cells of other tissues, for instance the respiratory and gastrointestinal tracts (Turvey and Broide 2010).

Neutrophils are the most abundant leukocytes (white blood cells) in human blood and usually recruited first to the site of an innate immune reaction. They are involved in the pathogen eradication by phagocytosis, release of granules (degranulation) and release of so called neutrophil extracellular traps (NETs) (Witko-Sarsat et al. 2000). Neutrophils together with the far less abundant eosinophils and basophils are part of the granulocyte family. Eosinophils are involved in the parasite clearance (Rosenberg, Dyer, and Foster 2013) and basophils appear in many inflammatory reactions during an immune reaction, especially those for allergic reactions (Siracusa et al. 2013). Mast cells, another type of hematopoietic cells, are only found in connective tissue and contain granules, which are rich in cytokines, histamine and heparin, and are key players in inflammatory processes (da Silva, Jamur, and Oliver 2014). Monocytes constitute the largest type of leukocyte that are circulating in the blood and can migrate into tissues. Within the tissue they differentiate into macrophages and monocyte-derived dendritic cells (Gordon and Taylor 2005). Macrophages can be found in every tissue and have several functions. From phagocytosis to antigen presentation and production of massive amounts of pro- and anti-inflammatory cytokines (Wynn, Chawla, and Pollard 2013). Dendritic cells are also able to produce cytokines to attract and activate other immune cells, but their main function as an antigen-presenting cell (APC) is to process antigens and present those to naïve T-cells. With these functions, especially dendritic cells are able to connect the innate and adaptive immune system (Steinman 1991; Banchereau et al. 2000). In contrast, cytotoxic NK cells are able to kill cells with a virus infection, intracellular pathogens and tumor cells via release of lytic granules upon recognition of alterations in self-surface molecules on target cells (Vivier et al. 2011). As aforementioned several non-hematopoietic cells have innate immune functions. Especially those at barrier epithelia are the main producers of antimicrobial peptides and can produce cytokines to attract and activate immune cells (Mai et al. 2013). Innate immune cells with all their functions are critical regulators of inflammatory diseases. Dysregulation or defects can lead to the development of asthma, atopy and autoimmune disorders, like inflammatory bowel disease, type 1 diabetes and systemic lupus erythematosus (SLE) (Turvey and Broide 2010)

1.1.3 Adaptive immunity

The adaptive immune system is found only in vertebrates. The key elements of this system are the B- and T-cells, two types of lymphocytes that respond in an antigen-specific manner. Thereby, the B-cells,

via secreted antibodies, provide the humoral immune response and T-cells the cellular response. To recognize a specific antigen, each cell type possesses an antigen-specific receptor on the cell surface, the so-called B-cell receptor (BCR) and T-cell receptor (TCR), respectively (Arden et al. 1995).

In order to achieve the greatest possible variability of antibodies and receptors and thus to be able to specifically bind almost any antigen, the process of somatic recombination ensures random gene segment rearrangements and therefore a staggering combinatorial repertoire of receptor specificities. This process is also called V(D)J (V: variable; D: diversity; J: joining) recombination. This allows an almost uncountable number of different antibodies and receptors to be encoded by a limited number of genes (Alt et al. 1992; Gellert 2002). Further, variation is generated by junctional diversity and, for B-cells, by somatic hypermutation of rearranged receptor genes. Thereby, each B-cell and T-cell has only one specific BCR and TCR, respectively.

The characteristic feature of the humoral immune response is the production of secreted immunoglobulin (Ig) antigen receptors or antibodies such as IgG and IgM. However, for the release of antibodies, B-cells must be activated first. This is achieved through the membrane bound antigen receptor BCR, which has the same specificity as the secreted antibody. BCR engagement leads to engulfment of the antigen, parts of which can be processed and presented to T cells via major histocompatibility complex (MHC) II molecules. T helper (Th) cells, MAMPs and secreted cytokines can promote the process. The antigen must be presented to the Th cell by an APC via a MHC class II molecule to activate it. This cell then secretes cytokines that are co-stimulatory and causes B-cell activation. These activated B-cells differentiate into antibody-producing plasma cells and memory B-cells (Noelle et al. 1992). The BCR is essential for B-cell function. It consists of the antigen-binding transmembrane immunoglobulin molecule and the non-covalently associated Igα-Igβ heterodimer as a signal transduction module. Both Igα and Igβ contain an immunoreceptor tyrosine-based activation motif (ITAM), which provides tyrosine phosphorylation upon antigen binding to the BCR (Hobeika, Nielsen, and Medgyesi 2015).

T-cells are responsible for the cellular immune response. Here, T-cells are divided into two groups depending on whether they express cluster of differentiation (CD) 4 or CD8 on the cell surface. The CD8+ T-cells bind to the MHC class I molecules and the CD4+ T-cells bind to the MHC class II molecules. The CD4+ T-cells can further be divided into Th cells and T regulatory (Treg) cells. Th cells can be involved in the activation of B-cells. Furthermore, through their secretion of proinflammatory cytokines, they promote not only parts of the adaptive immune system, but also the innate immune system. In contrast, Treg cells suppress the activity of lymphocytes by secreting anti-inflammatory cytokines and thereby regulate the immune reaction (Kondelková et al. 2010). The CD8+ T-cells are also called cytotoxic T-cells and can eliminate target cells presenting the appropriate antigen by apoptosis induction (Bennett et al. 1998; Kojima et al. 1994).

For full T-cell activation, three signals are required (Chen and Flies 2013). The first is an antigen-specific signal that occurs when an antigen binds to the TCR/CD3 complex via a MHC complex (Smith-Garvin, Koretzky, and Jordan 2009). The second signal comes from co-stimulatory receptors such as CD28 (June et al. 1987; Mueller, Jenkins, and Schwartz 1989), while the third signal comes from inflammatory cytokines and is required to promote a strong immune response (Curtsinger and Mescher 2010). The TCR consists of an α- and β-chain, neither of which has the ability to transduce signals. Signal transduction requires the CD3, which consists of one γε- and one δε-heterodimer and has ITAMs in the

cytoplasmic region, which contains tyrosine residues that are phosphorylated after activation for further signal transduction (Blumberg et al. 1990; Weiss and Littman 1994).

A great advance of the adaptive immunity is that it is not only possible to create specificity against antigens but some of the B- and T-cells differentiate into memory cells. If a memory cell encounters the same antigen a second time, rapid reactivation and development into effector cells takes place. This results in a faster response against the pathogen and is the basic principle behind vaccination (Ahmed and Gray 1996).

1.2 Specific innate immune cells and mechanisms

1.2.1 Neutrophils

The immune system consists of several different immune cells but the predominant immune cell type in the blood are neutrophils with 50-70% of all circulating leukocytes (Liew and Kubes 2019). Polymorphonuclear neutrophils (PMNs) are highly abundant in the body with $1.7x10^{9}$ cells/kg but have a short life span of only several hours (Liew and Kubes 2019). During infections, the life span of activated PMNs is extended and additionally the production rate can be upregulated to 10^{12} neutrophils per day (Edwards 1994). Also 55-60% of the bone marrow cells are specifically dedicated for the neutrophil production. The development of neutrophils is called granulopoiesis in which hematopoietic stem cells from the bone marrow differentiate into mature neutrophils. A characteristic for mature neutrophils is their segmented nucleus and granules, filled with more than 700 different proteins (Kruger et al. 2015). These PMNs are the leukocytes that are usually recruited first to a site of infection and are crucial for the clearance of bacteria, fungi, and other microorganisms. Moreover neutrophils are key players during acute inflammation (Kolaczkowska and Kubes 2013).

As PMNs have a high turn-over rate and get constantly produced in the bone marrow, their maturation is regulated by several transcription factors, proteins, cytokines and receptors but a master regulator for this is the granulocyte colony-stimulating factor (G-CSF) (Lieschke et al. 1994). The complete mechanism behind G-CSF is not fully understood. However, it leads to lineage commitment of progenitor cells to their myeloid character (Richards et al. 2003). It further supports proliferation of the precursors of neutrophils, reduction of the transit time and the final release of the mature neutrophils from the bone marrow into the blood (Lord et al. 1989). During their differentiation they can be divided into three populations. The first is the stem cell pool with their hematopoietic stem cells. Another population is the mitotic pool with their proliferating and differentiating granulocyte progenitor cells. The third is the postmitotic pool, build by the mature und fully differentiated PMNs which can get released (Borregaard 2010). Moreover, production of G-CSF and with that also the maturation of neutrophils is highly regulated by serval cell types and cytokines. Macrophages and dendritic cells produce interleukin (IL) 23 which regulates IL-17A production by γδ T-cells and natural killer T-cells, which then regulates G-CSF production. Neutrophils undergo apoptosis during the resolution of an inflammation and are removed by phagocytic cells like macrophages. This process is called efferocytosis and has the consequence that the production of IL-23 is reduced, which in turn leads to a reduced G-CSF production (Stark et al. 2005; Ley, Smith, and Stark 2006). Therefore G-CSF is a therapeutic agent for neutropenic diseases in which patients have very low numbers of PMNs (Kruger et al. 2015).

Neutrophils of the blood stream can be identified by specific CD markers on their surface. These mature PMNs are $CD11b^+$, $CD14^-$, $CD15^+$ and $CD66b^+$. The proteome on the cell surface is also changing if a cell gets activated. A potent marker for activation is CD62L which gets downregulated after activation (Lakschevitz et al. 2016).

During an inflammation the PMNs must translocate from the blood stream into the tissue and further to the site of infection or injury. The migration from the blood stream into the tissue is a multistep process including tethering and rolling, followed by adhesion and crawling and finally transmigration (Liew and Kubes 2019). The migration is initiated through the activation of PMNs by chemokines like IL-8, keratinocyte-derived chemokine (KC), C-X-C motif chemokine (CXCL) 2 also called macrophage-inflammatory protein 2 (MIP-2) and CXCL5 (Sadik, Kim, and Luster 2011; Sanz and Kubes 2012). These chemokines are sensed through CXCR2 on PMNs (Williams et al. 2011). Inflammatory mediators like cytokines and histamines are released by tissue-resident leukocytes and endothelial cells after contact with a pathogen sensed by PRRs and lead not only to activation of PMNs but also to the activation of endothelial cells. Within minutes, this results in the upregulation of P-selectin and E-Selectin (Petri, Phillipson, and Kubes 2008). Those adhesion molecules bind their glycosylated ligands like P-selectin glycoprotein ligand 1 and binding allows the tethering of neutrophils to activated endothelial cells. PMNs then begin to roll on the cell surface of the endothelial cells in the direction of the blood flow (Zarbock et al. 2011). The next step is the adhesion were the chemokines trigger signaling pathways in the PMNs that lead to F-actin polymerization in front of the cell, as well as actin-myosin contractions and a membrane retraction at the back end. Actin dynamics support the crawling of the PMN along the luminal side of the endothelial cells (Williams and Chambers 2014). This process of adhesion and crawling along the endothelium is necessary for PMNs to get in position for transmigration from the vasculature into the tissue. PMNs have two possibilities for this migration. Either transcellular through the endothelial cell or paracellular between two endothelial cells (Voisin and Nourshargh 2013). For the paracellular way through junctions between those cells, the PMNs need integrins that can bind the respective cellular adhesion molecule, like intercellular adhesion molecule (ICAM) 1, ICAM2 or vascular cell adhesion molecule 1 (VCAM-1) (Petri, Phillipson, and Kubes 2008). After the migration out of the vasculature, the PMNs are not at the site of infection or injury where they are needed. The chemokine gradients were essential for this transmigration process but in the next step the PMNs have to leave this site of the primary chemokine gradient. Hence, a new gradient of presumably different chemoattractants is necessary for proper cell navigation (Zimmerman, McIntyre, and Prescott 1996). This process of activation, migration and guiding of PMNs towards the inflammation site is a complex system of interacting signaling pathways that are induced by several chemoattractants, which are released by an interplay of several cell types (Liew and Kubes 2019).

If the infection is cleared, the neutrophilic inflammation process has to be terminated. This is also highly regulated to prevent that an acute inflammation progress develops to a chronic inflammation and therefore to prevent host damage. This resolution can be divided into two different processes. The first is an anti-inflammatory process, the other one is a proresolving process (Liew and Kubes 2019). For the first phase, anti-inflammatory cytokines, like IL-4 and IL-10 are released. This leads to a downregulation of proinflammatory cytokines and the transcription factor nuclear factor κB (NF-κB), which leads to transcription of cytokines (Schottelius et al. 1999; Iribarren et al. 2003). In addition to anti-inflammatory cytokines, receptor antagonists like IL-1RA can function to limit the inflammation. This antagonist IL-1RA, which gets upregulated by anti-inflammatory signals, like glucocorticoid hormones, binds to the IL-1 receptor (IL-1R), but does not lead to an activation of the proinflammatory

pathway and further blocks binding of IL-1 to the receptor (Bourke et al. 2003). The other phase is a switch of lipid mediators to the release of proresolving lipid mediators. For example, the release of lipoxin A4 inhibits cytoskeleton organization in PMNs and therefore prevents PMN migration. Furthermore, lipoxin A4 also leads to a recruitment of macrophages, which can phagocytose the apoptotic neutrophils at the site of inflammation (Serhan, Chiang, and Van Dyke 2008).

1.2.1.1 Functions of neutrophils

Neutrophils have a wide range of functions. They are involved in pathogen clearance, tissue repair and connecting innate and adaptive immunity. Their antimicrobial effector functions include phagocytosis, degranulation, and the formation of neutrophil extracellular traps (NETs) (Liew and Kubes 2019). The choice of which of these antimicrobial functions is activated, depends on several settings. The first is that the serum in the blood supports phagocytosis but it inhibits the formation of NETs (Hakkim et al. 2010). The second is that transmigration of PMNs triggers the release of cytotoxic components from secretory vesicles and granules. The third is that adherend neutrophils have a lower threshold for an integrin-dependent activation (Kettritz et al. 2004). Moreover, it is shown that PMNs are able to fulfill several tasks at the same site of infection, which shows that multiple pathways can be active during host-pathogen interactions (Yipp et al. 2012).

PMNs are potent phagocytes. After the opsonization of a pathogen, the respective opsonic receptor like Fcγ-receptors, complement receptors or C-type lectins of the PMN binds and the PMN starts to engulf it (Dale, Boxer, and Liles 2008). This process is mediated by cytoskeletal rearrangements and regulated through a complex interplay between pathways of membrane proteins and cascades of intracellular signaling. Phagocytosis can even be enhanced by the complement system, cell priming or IgG antibodies and it is a fast process that occurs within minutes (Segal 2005). During the phagocytosis the pathogen is trapped in a phagosome and primary and secondary granules fuse to these to release their antimicrobial contents into the phagosome. Additionally, the nicotinamide adenine dinucleotide phosphate (NADPH), which is essential for microbial killing, assembles from membrane bound flavocytochromes. THE NADPH oxidase produces oxygen radicals, so called reactive oxygen species (ROS). ROS are released into the phagosome and enhance microbial killing (Segal 2005). A dysfunction or lack of NADPH oxidase leads to chronic granulomatous disease (CGD) (Song et al. 2011). Myeloperoxidase (MPO), another oxidant-producing enzyme in PMNs, is a di-heme protein that consists of two identical heterodimers. Together with the cofactor heme it produces hypochlorous acid (HOCl) from H_2O_2 that also facilitates microbial killing (Davies 2010).

Another mechanism for microbial killing is the degranulation of PMNs. PMNs have four different types of granules in their cytoplasm (Lacy 2006). The first granules, produced during the development of the cell, are termed azurophil granules. These granules contain MPO, defensins and neutral proteases like elastase and proteinase 3 that can kill and digest pathogens. The second type of granules are the secondary or specific granules that consist mainly of lactoferrin, a protein that sequesters copper and iron which are important for microbes. Third, gelatinase granules can release several gelatinase proteins, like matrix metalloproteinase-9 (MMP-9), that are involved in degradation of the extracellular matrix and IL-1β activation. Last but not least, the secretory granules which consist of serum albumin and several preformed cytokines (Sheshachalam et al. 2014). The content of these granules is highly toxic and can damage the host. That is why the release is highly regulated. It needs two binary signals. The first is in an adhesion dependent process that β2-integrins initiate (Lacy 2006). The next process depends on an interaction between immune receptors and ligands which includes binding of a formylated peptide to the formyl peptide receptor 1 that then is able to trigger the Src

kinases with release of Ca^{2+} from intracellular stores (Futosi, Fodor, and Mócsai 2013). Furthermore, the four types of granules get also released by a hierarchical order to control the degranulation process and limit the exposure of the host to those cytotoxic reagents. The first ones to be released are secretory vesicles, followed by tertiary granules, secondary granules and, finally, primary granules (Sengeløv, Kjeldsen, and Borregaard 1993).

Neutrophils have a third function to trap and kill microbes. Thereby, they are able to form so called neutrophil extracellular traps (NETs) in a process called NETosis (Brinkmann et al. 2004). These NETs have a web-like structure that consists of DNA decorated with proteins and other molecules (Kolaczkowska et al. 2015). As NETs are of special interest for this thesis, they are explained in more detail in 1.2.1.2.

Besides their function in microbial killing PMNs exert other functions. They are also involved into tissue repair. Although it is not completely understood how they contribute to the healing process. They can cause tissue damage with their granule contents and the formation of NETs and are able to produce an inflammatory milieu. Furthermore, release of DAMPs leads to increased PMN recruitment which then further can potentiate the tissue damage caused by PMNs (Kono, Onda, and Yanagida 2014). It is therefore postulated that tissue damage which is due to neutrophil infiltration can cause persistent inflammation and that this prevents a proper healing of the injury (Liew and Kubes 2019). But it is shown as well, that a lack of neutrophil recruitment leads to delayed repair of injuries (McDonald and Kubes 2016). In thermal haptic injury it is further shown that PMNs remove injured or dead vessels and help to build channels for the vascular regrowth (Wang et al. 2017). These demonstrates that neutrophils are necessary for the healing process.

Furthermore, neutrophils also modulate the adaptive immunity by regulating B- and T-cells. PMNs are able to produce cytokines that are required for survival and activation of B-cells like B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) (Scapini, Bazzoni, and Cassatella 2008). T-cells get regulated by PMNs at different levels. Production of ROS and presents of arginase 1 from azurophilic granules inhibit proliferation and activation of T-cells (Pillay et al. 2012; Tosello Boari et al. 2012). PMNs do not only inhibit T-cells, but they also serve as antigen-presenting cells. Their MHC class II is upregulated together with other proteins after an interferon-γ stimulation. This leads to an facilitated differentiation of Th1 and Th17 cells (Abi Abdallah et al. 2011). Also NETs contribute by lowering the threshold for the activation of CD4+ Th cells and by presenting antigens (Tillack et al. 2012).

1.2.1.2 Neutrophil extracellular traps

Since the first discovery of neutrophil extracellular traps in 2004 (Brinkmann et al. 2004) numerous studies have been focusing on the composition, triggers and functions of NETs (Ravindran, Khan, and Palaniyar 2019). During the formation of NETs, the PMNs release large structures in the extracellular matrix that look like a web. These structures have a decondensed chromatin scaffold with histones as well as cytosolic and granule proteins assembled to these (Brinkmann et al. 2004). The DNA within NETs mainly consists of DNA from the nucleus but mitochondrial DNA was also found in NETs (Lood et al. 2016). The composition of the NETs is very diverse. After PMN treatment with phorbol 12-myristate 13-acetate (PMA), a non-physiological tool compound that has been used in the vast majority of NET-related studies, it leads to an activation of protein kinase C (PKC), production of ROS and finally NET-formation. The protein mix that is assembled to the DNA backbone of the NETs includes histones, MPO,

the serine protease neutrophil elastase (NE), antimicrobial peptides like the cathelicidin LL37, defensins, calprotectin and actin (Urban et al. 2009). LL37 is an amphipathic, antimicrobial peptide (AMP), which functions against fungal, bacterial, and viral pathogens and it can form a complex with DNA and RNA. Furthermore, this complex of either DNA or RNA with LL37 is able to activate cells and trigger the release of IL-8 and interferon (IFN) α (Lande et al. 2007; Ridyard and Overhage 2021). Further studies showed that are even more proteins assembled to NETs and that these can vary dependent on the stimulus that led to the formation of NETs. Different strains of *Pseudomonas aeruginosa* led to NETs that have 33 common proteins and 50 proteins that are variable (Dwyer et al. 2014). These list of assembled proteins that are common to all NET inducers or variable are further increasing as a lot of research is investigating the composition of NETs (Papayannopoulos 2018). Recently, it was demonstrated by our group that RNA is a common component of NETs. These RNAs within NETs are called NETs-associated RNA (naRNA) (Herster et al. 2020). But how these differences in the NET composition impact on their function remains unclear and was one of the objectives relating to this thesis.

NETs do not only trap but also lead to a neutralization and killing of bacteria (Brinkmann et al. 2004), viruses (Saitoh et al. 2012), fungi (Urban et al. 2006), parasites (Abi Abdallah et al. 2012) and furthermore prevent fungal and bacteria dissemination (Walker et al. 2007; Branzk et al. 2014). For the formation of NETs PMNs need to depolarize and the actin dynamics need to be arrested (Metzler et al. 2014). Furthermore, ROS and their pathways are essential for the process of NETosis. MPO, activated by ROS from the NADPH oxidase, leads to a translocation and activation of NE from the azurophilic granules into the nucleus (Papayannopoulos et al. 2010). But the activity of the NADPH oxidase can also be redundant as mitochondrial ROS, which is e.g. produced in response to immune complexes as stimuli, is sufficient to activate the NETosis process (Lood et al. 2016). In the nucleus, NE leads to a disrupted chromatin packaging by proteolytically processing histones. Additionally, MPO has more functions in NET formation, as it can also bind to chromatin and synergize with translocated NE to decondense chromatin (Papayannopoulos et al. 2010), independently of its enzymatic activity. But inhibition of MPO activity only leads to a delay of NETosis but does not block it (Metzler et al. 2011). Oxidative activation of NE by MPO is important as NE in the cytoplasm binds and then degrades F-actin filaments, which allows NE to enter the nucleus (Metzler et al. 2014). Many stimuli, like fungi, induce this MPO and NE pathway (Papayannopoulos 2018). The importance of these two proteins in the formation of NETs and protection of the body is supported by patients with CGD, as these patients have a mutation in the NADPH oxidase and are unable to generate ROS and induce NET-formation (Fuchs et al. 2007) and by mouse models with MPO or NE deficiency, which suffer from frequent and more severe infections (Metzler et al. 2011; Papayannopoulos et al. 2010). Another protein that is involved in the chromatin decondensation process by histone deamination or citrullination is protein-arginine deiminase type 4 (PAD4). Transcriptionally most of the DNA in PMNs is inactive and condensed into heterochromatin within the nucleus. Nucleosomes are histones wrapped with DNA and these nucleosomes then are organized further into chromatin (Sørensen and Borregaard 2016). The decondensation of the heterochromatin is mediated by PAD4, a nuclear enzyme that is able to catalyze the reaction of arginines to citrullines in histones by changing amine groups to ketones (Wang et al. 2004; Rohrbach et al. 2012). This conversion is weakening the binding of the histone to DNA by reducing the complementary positive charge of the histones, and leads to an unwrapping of the nucleosomes, which is necessary for the formation of NETs (Wang et al. 2009). For its activation PAD4 needs a reducing environment (Damgaard et al. 2016). Thus, citrullination is decreased when the NADPH oxidase is inhibited. This is due to the fact that PAD4 can be activated by NADPH oxidase-

dependent H_2O_2 (Neeli et al. 2009; Li et al. 2010). This process also needs calcium and is mediated by protein kinase C (PKC) ζ (Neeli and Radic 2013; DeSouza-Vieira et al. 2016). The kinase PKCζ is involved in the ROS burst. Even if the entire pathway is not yet fully understood, it demonstrates that ROS, MPO, NE and PAD4 are essential factors for the formation of NETs (Papayannopoulos 2018).

Additionally, for the formation of NETs the nuclear envelope disassembles and the decondensation of the nuclear chromatin leads to a mixture of chromatin with components of the cytoplasm and granules (Fuchs et al. 2007). The final step is then the permeabilization of the plasma membrane, so that the NETs can be released into the extracellular matrix. In humans this NET forming process takes up 2-4 hours after the activation of the PMNs (Papayannopoulos 2018). The pathways upstream of ROS that induce NET formation are also not fully understood. But in response to different stimuli some kinases and ROS-inducing receptors have been shown to be involved in NETosis. These are e.g. extracellular-signal-regulated kinase (ERK), MAPK/ERK kinase (MEK), phosphoinositide 3-kinase (PI3K), IL-1 receptor-associated kinase (IRAK) and AKT (DeSouza-Vieira et al. 2016; Hakkim et al. 2011; Gabriel et al. 2010; Behnen et al. 2014; Douda et al. 2009). An overview of this NET forming process is shown in figure 1.

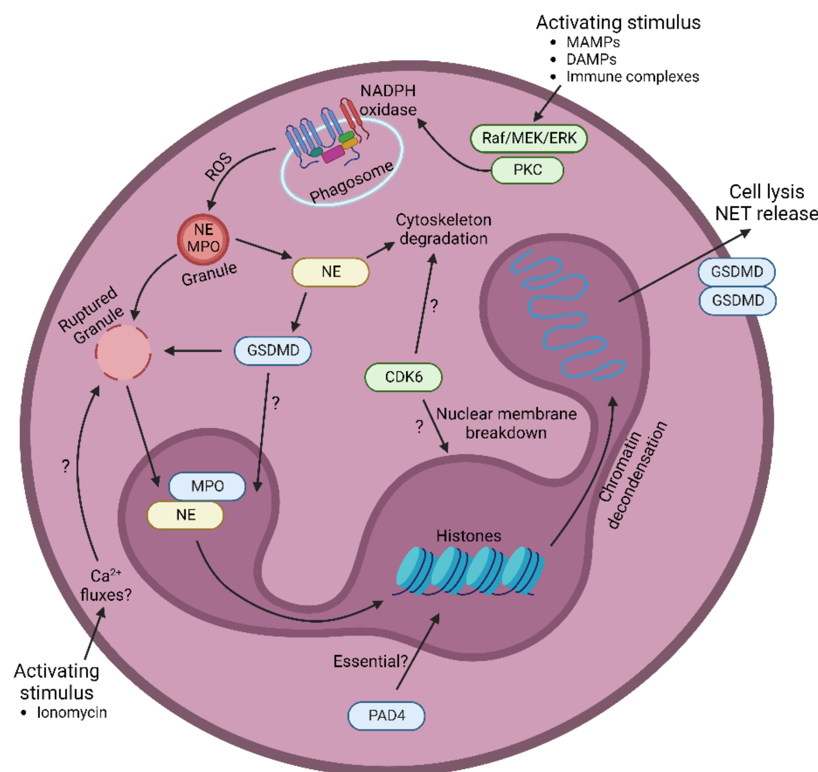


Figure 1: NET formation pathways

Dependent on the stimulus a NADPH oxidase dependent or independent NET formation is triggered. Activation of the Raf/MEK/ERK pathway leads via PKC to NADPH oxidase dependent ROS production. Then ROS triggers the release of NE, which activates GSDMD and degrades the actin cytoskeleton. GSDMD is then acting in a feed-forward loop by releasing more NE. In another step, NE migrates to the nucleus, where it mediates chromatin expansion by cleaving histones. After CDK6 mediated nuclear membrane, chromatin fills the cell and gets released as a NET after cell lysis. In the NADPH oxidase independent formation of NETs, activation of the neutrophil triggers Ca^{2+} fluxes, resulting in granule rupture and release of NE. Furthermore, Ca^{2+} leads to activation of PAD4, resulting in citrullination of histones, decondensation of chromatin and finally release of a NET. Created with BioRender.com, adapted from Rosazza et al. (2021) (Rosazza, Warner, and Sollberger 2021).

There is also another type of NET formation that does not lead to cell death. This mechanism is called non-lytic NETosis. It also leads to the release of NETs out of chromatin and granule components. But this process does not lead to the death of the PMN. Moreover, intravital microscopy demonstrated that the NET formation of this non-lytic NETosis lead to an anucleated cytoplast that is still able to crawl and kill bacteria by phagocytosis (Yipp et al. 2012). However, the vital NETs are formed in an oxidant independent process that is suggested to be mediated by TLR2 signaling. In contrast to suicidal NET formation, vital NET-formation occurs within minutes after induction by certain pathogens, like *Staphylococcus aureus* (Tan, Aziz, and Wang 2021).

To prevent excessive NET-formation the process of NETosis is tightly regulated. One major factor among several others is the size of the microorganism. Sensing the size of the pathogen depends on the access to NE and leads to a competition between phagocytosis and NETosis. Small pathogens can be taken up by the PMNs into the phagosome and there azurophilic granules release their content into these phagosomes. As a result, NE cannot be transported into the nucleus and thus cannot lead to decondensation of chromatin (Branzk et al. 2014). This mechanism allows the PMNs to preferentially form NETs against big microorganisms that are too big to be phagocytosed (Papayannopoulos 2018). Some of the pathogens are able to escape from the phagosomes (Stevens and Galyov 2004) which suggests that these small virulent pathogens, that are able to interfere with the phagocytosis, also induce NET formation for clearance. Taken together, this demonstrates that NETosis is modulated by several factors of the microorganism, like size and expression of virulence factors, to obtain optimal pathogen clearance with minimal damage to the host (Papayannopoulos 2018).

The formation of NETs is an efficient way of clearing pathogens, but it can also lead to tissue damage, delay in tissue repair, chronic inflammations and is associated with autoimmune diseases, like SLE (Papayannopoulos 2018). It is reported that NETs can cause tissue damage by directly killing epithelial cells (Saffarzadeh et al. 2012). Furthermore, excessive NETosis plays an important role as it is damaging the epithelium during pulmonary fungal infections (Papayannopoulos 2018) or the endothelium during transfusion-related acute lung injury (Thomas et al. 2012). Moreover, NETs also regulate directly or indirectly the release of inflammatory cytokines by modulating immune cells. In atherosclerosis it was shown that NETs induce transcription of IL-6 and pro-IL-1β in macrophages, resulting in Th17 cell differentiation and increased recruitment of myeloid cells to atherosclerotic lesions (Warnatsch et al. 2015). In addition, in autoimmune diseases with autoantibodies against neutrophil-derived proteins, NETs serve as self-antigen source (Papayannopoulos 2018). Thus, it was demonstrated that patients with antineutrophil cytoplasmic autoantibody-associated vasculitis have autoantibodies against proteinase 3 and MPO, which are part of NETs (Kessenbrock et al. 2009). Further, rheumatoid arthritis (RA) patients do not only have antibodies against citrullinated proteins, also NET-components were found in the synovial fluid, that act as source for citrullinated proteins (Khandpur et al. 2013). Similarly, SLE patients were reported to have autoantibodies against extracellular nucleic acids (Pisetsky 2020). These and more NET-mediated pathologies are summarized in figure 2, demonstrating their crucial role among others in various sterile inflammations.

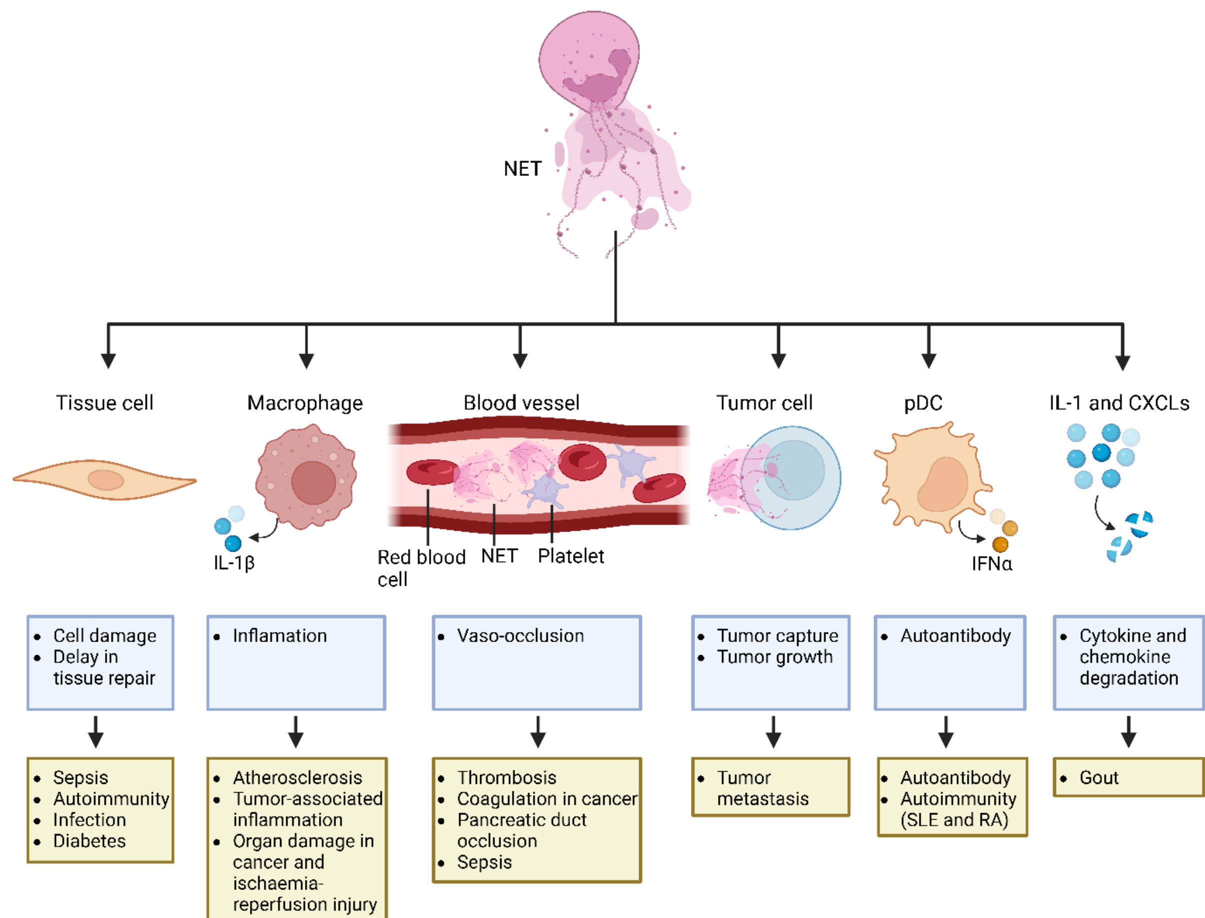


Figure 2: Overview of NET-mediated pathologies
Neutrophil extracellular traps (NETs) are associated with several pathologies through different mechanisms, e.g. direct cell damage in infection, sepsis and diabetes. In addition, NETs contribute to atherosclerosis, tumor-associated inflammation and organ damage by licensing macrophages for inflammation. Furthermore, in vaso-occlusion NETs lead to coagulation and activate platelets, leading to thrombosis, for example. NETs were also found to promote tumor metastasis and serve as autoantigens for autoantibodies in autoimmune diseases. Moreover, NETs can also lead to degradation of cytokines and chemokines, which contributes to gout disease progression. Created with BioRender.com, adapted from Papayannopoulos (2018) (Papayannopoulos 2018).

1.2.3 Macrophages

Macrophages and their role in host defense were first described in 1882 by Metchnikoff (Gordon 2008). It is known by now that they have major roles in inflammation, infection but also in tissue homeostasis (Orecchioni et al. 2019). These cells derive from hematopoietic stem cells, which first differentiate into a progenitor cell, called macrophage dendritic cell precursor. They then differentiate into monocytes which circulate in the blood stream. According to signaling monocytes can differentiate into dendritic cells or macrophages (Fogg et al. 2006; Wang et al. 2021). This differentiation into tissue macrophages is initiated when the $Ly6C^{+}$ subset of monocytes migrate through the endothelial membranes (Randolph et al. 1998) while sensing macrophage-colony stimulating factor (M-CSF) (Martinez et al. 2008). Additionally, macrophages can also undergo a self-proliferation, which is a mechanism to replenish the number of macrophages in the tissue (Geissmann et al. 2010). Macrophages are professional phagocytes that have a broad range of functions within the tissue, like in the steady state removal of cellular debris and apoptotic cells, clearance and recycling of erythrocytes and tissue remodeling, but are also involved in the host response to infections and inflammations by phagocytosing pathogens, releasing pro- and anti-inflammatory cytokines and

serving as APC (Erwig and Henson 2007; Verschoor, Puchta, and Bowdish 2012). Tissue macrophages can be differentiated into two major subsets. They polarize according to their environment into M1 macrophages or M2 (Martinez and Gordon 2014). The polarization into the M1 phenotype is induced e.g. by the microbial component lipopolysaccharide (LPS) and the M2 phenotype by IL-4 (Sica and Mantovani 2012). M1 macrophages are strongly bactericidal and responsible for proinflammatory responses and they release proinflammatory cytokines, like IL-6, IL-8, IL-12 and tumor necrosis factor (TNF). The M2 macrophages are responsible for anti-inflammatory responses, repair of the damaged tissue, angiogenesis and metabolism (Van Dyken and Locksley 2013; Murray et al. 2014). Therefore, first macrophages get polarized into M1 in infected tissue to assist in pathogen clearance and subsequently, when the pathogen is removed, the macrophages are polarized into M2 to generate an anti-inflammatory response and repair the damage within the tissue (Yunna et al. 2020).

The primary function of macrophages is to maintain tissue homeostasis by providing defense against pathogens, clearance of apoptotic and necrotic debris and remodeling of tissue after an injury (Shapouri-Moghaddam et al. 2018). To sense their local environment, macrophages have cell surface and intracellular PRRs. Furthermore, macrophages express various Toll like receptors (TLRs) (Kumar, Kawai, and Akira 2011). Additionally, they produce complement proteins and express complement and Fc receptors to bind circulating opsonized microbes and antibodies that are bound to an antigen (Varin and Gordon 2009). Macrophages are able to sense either exogenous signals like pathogens or endogenous signals like modified host proteins or debris. Dependent on the signal and the polarization of the cell, this leads either to a pro- or anti-inflammatory response. For example, the Tyro3, Axl, and Mer (TAM) receptor family can bind to phosphatidylserine associated proteins like ProS and Gas6. These proteins are associated with the recognition as well as uptake of apoptotic cells (Lemke and Burstyn-Cohen 2010), which then leads to phagocytosis but can also lead to release of immunoregulatory and anti-inflammatory cytokines like IL-10 or transforming growth factor (TGF) β (Savill et al. 2002). Another basic function of the macrophages is chemotaxis. Upon sensing a pathogen, macrophages secrete chemokines, like CC-chemokine-ligand (CCL) -3, CCL-4, CCL-5, IL-6, TNFα, CXCL-9, CXCL-10 and more, that lead to a recruitment of more macrophages but also Th1 lymphocytes, natural killer cells, neutrophils, and other cells. Especially for PMNs the secretion of the potent chemoattractant IL-8 leads to a strong recruitment (Mantovani et al. 2004; Benoit, Desnues, and Mege 2008). Other functions of macrophages are phagocytosis and tissue repair. Despite the major role in clearance of pathogens via phagocytosis, it is essential to clear redundant or damaged tissue and material to return the tissue to homeostasis. After identification of a target, this unwanted pathogen or material is engulfed within a phagosome. In a next step a lysosome is fusing with the phagosome to release their highly reactive and toxic content to facilitate the destruction of the engulfed materials. As in PMNs, in this process of destruction in macrophages ROS play a major role. Moreover, in macrophages nitric oxide (NO) is a crucial immunomodulator of phagocytosis. After activation of the macrophage, NO is produced in large amounts despite their constitutively but very low levels of NO production. NO then regulates the reactivity as well as the levels of ROS and moreover the production of reactive nitrogen species (RNS), which is highly toxic as RNS can interact directly with ROS (Wink et al. 2011). Furthermore, M2 macrophages are mainly responsible to return the tissue to homeostasis by repairing and remodeling the local environment, especially the extracellular matrix but also by induction of cell growth, angiogenesis, and collagen production (Shapouri-Moghaddam et al. 2018). Moreover, macrophages also have a function in the stimulation of the adaptive immunity. This function is also linked to phagocytosis as this leads to the generation of peptide antigens that are presented to

T-cells on the cell surface of macrophages by MHC class II. Furthermore, they release cytokines like IL-12 and IL-4, for additional signaling that lead to antigen specific T-cell expansion (Jensen 2007).

1.2.4 Innate immune mechanisms involving non-hematopoietic cells (tissue immunity)

Non-hematopoietic cells not only form barriers but are also involved in immune reactions. Epithelial cells, endothelial cells and fibroblasts are non-hematopoietic cells, which build organs like the skin that can detect environmental changes and are crucial for the host defense. They are expressing several PRRs to sense pathogens and epithelial cells are usually the first cell type that recognize when pathogens overcome a barrier (Royer and Cook 2021). Once a MAMP or DAMP binds to these PRRs, they rapidly initiate an immune response e.g. by releasing cytokines or chemokines to attract and activate immune cells. Furthermore, they also express cytokine receptors to sense secreted cytokines, which allows them to respond to them properly by releasing more cytokines, regulate further immune cell activation (Hewitt and Lloyd 2021) and release anti-microbial peptides to prevent overgrowth of pathogenic microbes (Okumura and Takeda 2017). Epithelial cells, especially in the lung, are moreover involved in a process called immune exclusion. In this process secretory IgA, which are produced by plasma cells, are transported by the polymeric immunoglobulin receptor of epithelial cells to the apical surface of the airway, where secretory IgAs prevent the adherence of airborne microbes (Johansen and Kaetzel 2011). Barriers are formed among other things to prevent microorganisms to get in the body. This also means that the epithelial cells in these barriers are always in contact with these but some of them are commensal and should not lead to an immune reaction. Therefore, epithelial cells mediate between signals of the microbes and the immune cells. They produce mediators after sensing these microorganisms, metabolites and secreted proteins of them. These mediators can be chemokines and pro- or anti-inflammatory cytokines, but they also can induce T-cell responses or transport antigens to APCs (Okumura and Takeda 2017). Additionally, apoptotic cells are often cleared by professional phagocytes like macrophages but epithelial cells are also able to do that as they are so called non-professional phagocytes (Juncadella et al. 2013). Furthermore, certain studies suggest that epithelial and endothelial cells are able to present antigens to T-cells as they are expressing MHC class II molecules (Mai et al. 2013; Heuberger, Pott, and Maloy 2021), although an activation of T-cells through this mechanism could not be shown yet. Additionally, endothelial cells are reported to also express nucleotide-binding oligomerization domain (NOD) like-receptors (NLRs) like NOD1 or NOD2. These receptors can be activated by a microbial stimuli, resulting in a release of cytokines, like IL-6 and IL-8 (Opitz et al. 2005; Davey et al. 2006) and they can further induce $CD4^+$ T helper cell-17 (Th17) polarization (Manni et al. 2011). Fibroblasts, are another type of non-hematopoietic cells that have diverse immunological properties, involving the regulation of immune responses and induction of an inflammatory environment (Chambers and Vukmanovic-Stejic 2020; Davidson et al. 2021). For example, a TLR4 signaling in fibroblasts induces production of IL-6, IL-8 and CCL2 (Wang et al. 2011) but they can also induce the production of immunoregulatory cytokines, like IL-10, by T-cells or suppress T-cell proliferation via production of indoleamine 2,3-dioxygenase (Haniffa et al. 2007).

A dysregulation or defects of immune responses by those non-hematopoietic cells, like sensing innocuous antigens in allergic diseases, can lead to a so-called type 2 immune response. In these typically alarmins like thymic stromal lymphopoietin (TSLP), IL-25 or IL-33 are secreted which are considered as important mediators of the inflammation in allergic diseases. This type 2 inflammation can in allergic diseases drive food allergies in the gastrointestinal tract or allergic rhinitis and asthma in the respiratory system and in the skin atopic dermatitis (Roan, Obata-Ninomiya, and Ziegler 2019).

This collection of functions of non-hematopoietic cells and especially epithelial cells demonstrates that they not only build barriers but are potent innate immune associated cells that act for the host defense as a vital component, as they are regulating immune responses to maintain homeostasis of the tissue in many ways (Iwasaki, Foxman, and Molony 2017).

1.3 Pattern recognition receptors

An essential mechanism for innate immune cells is that they are able to recognize the presence of microbes. They sense these microbes by germline-encoded PRRs. This basic mechanism is highly conserved in innate immunity among species from insects to mammals and even in plants (Akira, Uematsu, and Takeuchi 2006). All innate immune cells express these PRRs and certain non-professional immune cells, like epithelial cells, fibroblasts and endothelial cells (Takeuchi and Akira 2010), also do. PRRs have three common characteristics. The first one is that they recognize so called microbe-associated molecular patterns (MAMPs) (Janeway 1992). Whereas most MAMPs are from non-pathogenic microbes, there are truly pathogen-associated molecular patterns (PAMPs), i.e. MAMPs that relate to a pathogenicity trait. MAMPs are molecular patterns that are highly conserved and essential for microorganisms what makes it difficult for the microbes to alter it and evade the PRR recognition. The second characteristic is that PRRs are constitutively expressed in the host. The third group of characteristics is that these PRRs are germline encoded and therefore nonclonal and all cells of a given cell type express the same receptors (Akira, Uematsu, and Takeuchi 2006; Li and Wu 2021). Furthermore, cells do not exclusively recognize non-self patterns by certain PRRs but are also able to sense endogenous molecules from damaged cells, so-called damage associated molecular patterns (DAMPs), via these receptors (Zindel and Kubes 2020).

Currently, the PRR family consists of four main classes, but additional receptors exist that do not fit into these four groups. Two of these consist of transmembrane proteins and are called Toll-like receptors (TLRs), the best characterized PRRs to date, and C-type lectin receptors (CLRs). The other two families consist of cytoplasmic proteins and are called nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs). An overview of the PRRs and their ligands is shown in table 1. The activation of these receptors activates signaling cascades resulting in the induction of genes, but the expression patterns differ among the different PRRs and the cell type. The activation of PRRs via MAMPs or DAMPs leads to the induction and upregulation of inflammation-associated genes like proinflammatory cytokines, chemokines, type I interferons, anti-microbial peptides but also proteins that are involved or itself modulate PRR signaling, and many other characterized and uncharacterized proteins (Takeuchi and Akira 2010; Li and Wu 2021). Certain PRRs, oftentimes NLRs, activate a non-transcriptional pathway by forming so-called inflammasomes that activate a proteolytic signaling pathway involving caspase-1 activation and subsequent processing of IL-1 cytokine family members and an inflammatory cell death, pyroptosis (Wicherska-Pawłowska, Wróbel, and Rybka 2021). Additionally to these groups, several cytosolic sensors of DNA have been described to be PRRs. These DNA sensors include Absent in Melanoma 2 (AIM2), Interferon-inducible 16 (IFI16) and cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS) (Chen, Sun, and Chen 2016). The may activate inflammasomes (e.g. AIM2) or regulate interferon and/or cytokine transcription (Briard, Place, and Kanneganti 2020).

Table 1: PRRs and their ligands
Adapted from Takeuchi and Akira (2010) and Wicherska-Pawłowska, Wróbel and Rybka (2021) (Sabbah et al. 2009; Takeuchi and Akira 2010; Chen, Sun, and Chen 2016; Briard, Place, and Kanneganti 2020; Wicherska-Pawłowska, Wróbel, and Rybka 2021; Bauernfried et al. 2021)

PRRs		Localization	Ligand	Origin of the ligand
TLR				
TLR1		Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2		Plasma membrane	Lipoprotein	Bacteria, virus, parasite, self
TLR3		Endolysosome	dsRNA	Virus
TLR4		Plasma membrane	LPS	Bacteria, virus, self
TLR5		Plasma membrane	Flagellin	Bacteria
TLR6		Plasma membrane	Diacyl lipoprotein	Bacteria, virus
TLR7		Endolysosome	ssRNA	Bacteria, virus, self
TLR8		Endolysosome	ssRNA	Bacteria, virus, self
TLR9		Endolysosome	CpG-DNA	Bacteria, virus, protozoa, self
TLR10		Endolysosome	Unknown	Unknown
TLR11		Plasma membrane	Profilin-like molecule	Protozoa
RLR				
RIG-I		Cytoplasm	Short dsRNA, 5'triphosphate dsRNA	RNA virus, DNA virus
MDA5		Cytoplasm	Long dsRNA	RNA Virus
LGP2		Cytoplasm	Unknown	RNA virus
NLR				
Subgroup			Ligands and/or function	
NLRA	CIITA	Cytoplasm	Regulation of MHC class II expression	-
NLRB	NAIP	Cytoplasm	Flagellin, components of the type-3 secretion system	Bacteria, self
NLRC	NOD1	Cytoplasm	iE-DAP	Bacteria
	NOD2	Cytoplasm	MDP, dsRNA	Bacteria, virus
	NLRC3-5	Cytoplasm	Flagellin, or regulators of immune responses	Bacteria
	NLRX1	Cytoplasm	ROS production, autophagy induction	-
NLRP	NLRP1	Cytoplasm	dsRNA, MDP	Virus, bacteria
	NLRP2-14	Cytoplasm	MAMPs, DAMPs and for some it is unknown; some just have regulatory functions	Bacteria, fungi, self
CLR				
Dectin-1		Plasma membrane	β-Glucan	Fungi
Dectin-2		Plasma membrane	β-Glucan	Fungi
MINCLE		Plasma membrane	SAP130	Fungi, self
Alternative DNA sensors				
cGAS		Cytoplasm	DNA	Bacteria, virus, parasite, self
AIM2		Cytoplasm	DNA	Bacteria, virus, parasite, self
IFI16		Cytoplasm	DNA	Bacteria, virus, parasite, self

1.3.1 Toll-like receptors

1985 marked the discovery of the *Toll* gene in *Drosophila melanogaster* (Anderson, Bokla, and Nüsslein-Volhard 1985), a seminal finding that led to the discovery that this *Toll* gene plays a role in the immunity against fungi in *Drosophila* (Lemaitre et al. 1996). Then in 1997 the first Toll-like receptor was described in mammals (Medzhitov, Preston-Hurlburt, and Janeway Jr 1997).

TLRs are characterized as type 1 transmembrane proteins in which the recognition of MAMPs and DAMPs are mediated by ectodomains that contain leucine-rich repeats. Furthermore, they consist of

transmembrane domains and an intracellular Toll-Interleukin 1 receptor (TIR) domain, which is essential for signal transduction and bears witness to the similarity between the Drosophila Toll protein and the human IL-1 receptor in its name (Gay and Keith 1991). By now 10 functional TLRs have been identified in humans and 12 functional TLRs in mice (Wicherska-Pawłowska, Wróbel, and Rybka 2021). The activation of each TLR leads to an individual immune response, even though many TLR pathways share proteins in their signaling cascade, like MyD88 (Duan et al. 2022). Crystal structure analysis of TLR ectodomains suggested that each TLR can bind its cognate MAMP ligands (Jin and Lee 2008). These recognized ligands can be proteins, lipids, lipoproteins and nucleic acids derived from basically all microorganisms like bacteria, fungi, viruses and parasites (Akira, Uematsu, and Takeuchi 2006) but they can also derive from the host itself as DAMPs (Murao et al. 2021). The TLRs are located in different parts of the cell as this is important to mediate the ligand accessibility and signal transduction and at the same time to ensure a tolerance to self-molecules, especially nucleic acids (Kawai and Akira 2010). This self-tolerance is important as the cell e.g., is producing RNAs during transcription but in a healthy cell this RNA should not be recognized by the receptors and activate an immune response. To get a tailored immune response to infecting pathogens, individual TLRs selectively recruit TIR domain–containing adaptors via homotypic TIR-TIR interactions on their cytoplasmic side (Li and Wu 2021).

TLRs can be divided upon their localization within the cell and the ligands they bind into two subgroups. The first group consists of cell surface TLRs that mainly bind proteins, lipids and lipoproteins and consists of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11. The TLR2 binds to a broad range of different MAMPs and also DAMPs from bacteria, viruses, fungi and parasites. This includes lipoteichoic acid from gram-positive bacteria (Duan et al. 2022) lipopeptides from the host itself (Lorey, Öörni, and Kovanen 2022) and bacteria, lipoarabinomannan from mycobacteria, and many more. Thereby, formation of heterodimers of TLR2 with TLR1 or TLR6 are described. The TLR1-TLR2 complex is able to recognize ligands from mycoplasma and triacylated lipopeptides (Jin et al. 2007). In contrast, TLR2-TLR6 heterodimers sense diacylated lipopeptides from mycoplasma and gram-positive bacteria (Kang et al. 2009). Gram-negative bacteria have lipopolysaccharides (LPS) in their outer membrane. The major LPS-binding component is TLR4 that is able to form a complex on the cell surface with TLR accessory molecule MD2 (Akashi-Takamura and Miyake 2008). The TLR5 is able to bind to the flagellin protein which builds up the bacterial flagella (Uematsu et al. 2008). Finally, TLR11 recognizes profilin-like molecules from protozoa like *Toxoplasma gondii* (Yarovinsky et al. 2005).

The other subgroup of TLRs senses nucleic acids and is exclusively located in intracellular vesicles, like lysosomes, endosomes, endolysosomes and the endoplasmic reticulum (ER) (Kawai and Akira 2010). This localization allows a tolerance against the nucleic acid sensing, as they are only sensing nucleic acids that entered the cell. Otherwise, they would sense all extracellular nucleic acids. Structural analysis of the ectodomain of TLR3 revealed that it recognizes double-stranded RNA (dsRNA), which lead to a production of proinflammatory cytokines and type I interferon (Choe, Kelker, and Wilson 2005). A common ligand for TLR3 activation studies is the synthetic analog polyinosinic-polycytidylic acid (Poly(I:C)) but it is also thought to bind dsRNA which results from the replication of single-stranded RNA (ssRNA), genomic RNA from retroviruses and many other viruses, as well as small interfering RNAs (Kawai and Akira 2008; Chen et al. 2021). The antiviral immune responses triggered by TLR3 activation suggest that TLR3 has a crucial role during virus infections (Chen et al. 2021). TLR7 and TLR8 show high similarities in the structure and both recognize degradation products of ssRNA with distinct sequence preferences (Duan et al. 2022). They can sense RNA viruses like human immunodeficiency virus and

influenza A virus but also endogenous RNA, synthetic ssRNA and small interfering RNAs (Hornung et al. 2005; Duan et al. 2022). While TLR7 is specialized in the recognition of ssRNA fragments that are rich in guanosine, TLR8 is specialized in the recognition of ssRNA fragments with uridine (Murphy and Weaver 2016). Furthermore, it is shown that TLR8 is recognizing the degradation products of the lysosomal endoribonucleases RNase 2 and RNase T2 which can also cooperate together (Greulich et al. 2019; Ostendorf et al. 2020) – most likely the same is true for TLR7. The sensing of RNA viruses is in a replication-independent manner, as the virus gets first internalized and then recruited to the site of the TLRs which initiate antiviral responses after activation (Kawai and Akira 2010). TLR9 is the only TLR that can sense DNA. Furthermore, TLR9 recognizes unmethylated cytosine-phosphate-guanine (CpG) DNA motifs. These CpG motifs are rarely found in mammals, but they are very common in bacteria and viruses. There are some results suggesting that the DNA phosphodiester backbone also contributes to recognition, at least for natural DNA (Haas et al. 2008). In general, most of what is known for the activity of nucleic acid sensing TLRs has been derived by using synthetic oligoribo- (ORN) or -deoxyribonucleotides (ODN). These typically do not contain a natural phospho-diester backbone but employ phospho-thioate linkages for added nuclease-resistance *in vitro* and *in vivo*. From these results mechanisms for the sensing of natural RNA and DNA are extrapolated but oftentimes were not investigated in detail directly.

The intracellular TLRs have to be transported to the endosomes as their final recognition sites to activate an immune response. Responsible for this translocation is the ER-localized multi-transmembrane-domain-containing protein UNC93B1 (Kim et al. 2008). A single mutation within the *UNC93B1* gene results in a complete silencing of intracellular TLR signaling and subsequently abolished cytokine release and high bacterial and viral infection rates (Tabeta et al. 2006). Activation of a TLR receptor triggers individual signaling cascades. For example, activation of TLR5 or the complexes of TLR1 and TLR2 or TLR2 and TLR6 lead to inflammatory cytokine induction, whereas TLR3 and TLR4 lead to inflammatory cytokine and type I interferon production. The induction of different immune responses by the activation of different signaling pathways is mediated by TIR domain–containing adaptor molecules. This includes the proteins MyD88, TIR-domain-containing adaptor protein-inducing IFN-β (TRIF), TIR-associated protein (TIRAP) and TRIF-related adaptor molecule (TRAM). The adaptor protein MyD88 is able to mediate the signaling of all cell surface and intracellular TLRs except TLR3 (Fitzgerald and Kagan 2020). After activation MyD88 leads to induction of inflammatory cytokines via direct binding to IL-1R-associated kinase (IRAK)-4 which finally leads to activation of the transcription factor NF-κB and mitogen-activated protein kinases (MAPKs) (Tabeta et al. 2006). In contrast to MyD88, TRIF mediates the signaling of TLR3 and TLR4, the only TLR that can use both. The activation of the TRIF pathway leads to signal transduction that results in the activation of transcription factors NF-κB and interferon regulatory factor (IRF) 3 and finally lead to inflammatory cytokine and type I interferon production (Kawai and Akira 2008; Fitzgerald and Kagan 2020). The other two adaptor proteins TIRAP (also known as Mal, MyD88-adaptor-like) and TRAM are sorting adaptors that are responsible to recruit either TRIF or MyD88 to TLR4. Therefore, the signaling pathways of the TLRs can be classified either as TRIF-dependent or as MyD88-dependent pathway (Fitzgerald and Kagan 2020). The regulation of TLR sensing and signaling needs to be highly regulated to prevent chronic and autoimmune diseases. So far, several negative regulators of the signaling pathways have been identified to prevent defects and dysregulation. These negative regulators can be deubiquitinases (Kayagaki et al. 2007), ubiquitin ligases (Shi et al. 2008), splice variants of adaptor proteins and their related proteins (Carty et al. 2006; Palsson-McDermott et al. 2009), microRNAs or transcriptional regulators (Taganov et al. 2006).

1.3.2 Nod-like receptors

The biggest family within the PRRs are the NLRs with 22 receptors in humans, which are all located in the cytoplasm (Chou et al. 2023). The NLRs can be characterized by the organization of three common domains. The first is centrally located and contributes to the auto-oligomerization with its nucleotide-binding NACHT domain. This domain is crucial for the ATP-dependent activation. The second characteristic domain is the N-terminal effector domain. This domain is essential for transmission of receptor signals by binding adaptor proteins. The third one is the C-terminal domain, which may be responsible for the recognition of their ligands and/or by conferring an auto-inhibition mechanisms, mediated by a differing number of leucine rich repeats (LRRs) (Wicherska-Pawłowska, Wróbel, and Rybka 2021). Depending on the structure of the N-terminal domain the NLRs are further divided into four subgroups. The subgroup with an acidic transactivation domain as NLRA. The second subgroup is NLRB with a baculoviral inhibitory repeat-like domain. The third is NLRC with a caspase activation and recruitment domain (CARD). Last but not least NLRP have pyrin domains as their N-terminal domains (Kim, Shin, and Nahm 2016). Not all of the NLR receptors are PRRs as a few of them do not respond to MAMPs or DAMPs but cytokines or are transcriptional regulators (Wicherska-Pawłowska, Wróbel, and Rybka 2021).

The NLRA consists of such a non-PRR called class II histocompatibility complex transactivator (CIITA). In the C-terminal domain it contains four LRR repeats and a GTP-binding domain, which mediates the transport of this protein to the cell nucleus. There it is involved in the activation of the MHC class II expression by intrinsic acetyltransferase activity (Huang et al. 2016). Furthermore, the NLRB subgroup also consists of only one receptor, called NLR family apoptosis inhibitory protein (NAIP). Besides its inhibitory effects on caspase 3 and 7, NAIP is considered to be a sensor for components of the type-3 secretion system and bacterial flagellin, which then leads to NLRC4 activation (Maier et al. 2002). The NLRP subgroup consists of 14 different receptors, called NLRP1-14. Their pyrin domains can transmit pyroptotic signals or are able to induce inflammatory responses (Platnich and Muruve 2019). The functions and ligands are very diverse and not all of them are fully characterized yet. What is common, that they build inflammasomes. These multiprotein complexes assemble in the cytosol after activation trough a MAMP or DAMP and lead to caspase activation and subsequently production of cytokines and induction of pyroptotic cell death (Broz and Dixit 2016). For example, NLRP1 is shown to respond to dsRNA, which finally leads to a cleaved IL-1β release and formation of GSDMD pores (Bauernfried et al. 2021). NLRC involves six receptors, called NLRC1 to 5 and NLRX1, but NLRC1 and NLRC2, the major receptors within this subgroup, are also called NOD1 and NOD2, respectively. These two receptors sense bacterial cell wall components but are also shown to sense RNA (Wicherska-Pawłowska, Wróbel, and Rybka 2021) and are of interest for this thesis.

The *NOD1* gene encodes for an intracellular scaffolding protein that consists of a CARD domain, a NACHT domain and multiple LRRs. Additionally, the NOD2 protein is structurally very similar to NOD1 but with a second CARD domain (Ogura et al. 2001). For a long time NOD1 and NOD2 were thought to only recognize peptidoglycan fragments form the bacterial cell wall of gram-positive and in smaller fractions also in gram-negative bacteria. Indeed, NOD1 is able to recognize the peptidoglycan γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP). In contrast, NOD2 can recognize muramyl dipeptide (MDP), another part of the peptidoglycan (Trindade and Chen 2020). But this MDP needs to be phosphorylated by N-acetylglucosamine kinase (NAGK) before NOD2 can bind to it (Stafford et al. 2022). However, further studies show NOD2 can sense additional ligands, like viral RNA. It is shown with overexpression *in vitro* and Nod2-deficient mice *in vivo* that ssRNA can activate NOD2, which then

interacts via the CARD and NBD domain with mitochondrial antiviral signaling (MAVS). This interaction resulted in TRAF3-dependent activation of IRF3 and production of IFNβ (Sabbah et al. 2009). Further studies demonstrated also the involvement of receptor-interacting serine/threonine-protein kinase 2 (RIPK2) directly downstream of NOD2 after sensing viral ssRNA (Lupfer et al. 2013). In contrast to NOD2, NOD1 cannot be activated by ssRNA but it was shown in a hepatocyte cell line that, *in vitro,* synthetic dsRNA or dsRNA from the hepatitis C virus produced RNA polymerase that induced NOD1 expression and moreover can directly interact with NOD1, which results in downstream signaling. Interestingly, this dsRNA and NOD1 interaction is LRR independent (Vegna et al. 2016). Furthermore, NOD1 and NOD2 are suggested to respond to DAMPs, such as ER stress induced by chemical reagents, like thapsigargin and dithiothreitol. This recognition led to a NOD1 and NOD2 partially dependent upregulation of IL-6 (Keestra-Gounder et al. 2016). ER stress inhibitors or absence of NOD1 and NOD2 could reverse the promoted inflammatory response from induced ER stress (Molinaro et al. 2019). In the inactive form NOD1 and NOD2 are in an autoinhibited status in which the domain of the LRR is folded onto the NACHT and CARD domain. This folding prevents oligomerization of the receptors and binding of signaling proteins downstream of NOD1 and NOD2 (Maekawa et al. 2016). Additionally, mutations that prevent the autoinhibited status and lead to oligomerization of NOD1 or deletion of the LRR domain result in NF-κB activation (Inohara et al. 2000).

Binding their ligands leads to an open conformation of NOD1 and NOD2. The proteins then self-oligomerize via the NACHT domain and can interact via a homotypic CARD-CARD interaction with RIPK2, which has a C- and N-terminal CARD domain (Kobayashi et al. 2002; Park et al. 2007). Additionally, these binding leads to Lysin63 (K63)-linked polyubiquitination within the kinase domain on Lysin209 of RIP2 mediated by several E3 ligases (Hasegawa et al. 2008). This polyubiquitination enables RIPK2 to recruit and activate the serine/threonine kinase TGFβ-activated kinase 1 (TAK1) and TAK1-binding proteins (TAB), which is a prerequisite for MAPK and NF-κB activation (Caruso et al. 2014). Another way of activation for NOD2 is a non-K63 ubiquitination via XIAP. This way results in recruiting linear ubiquitin chain assembly complex (LUBAC). LUBAC produces M1-linked ubiquitin chains, which facilitates along with K63-linked chains the activation of NF-κB (Damgaard et al. 2012). In contrast, deubiquitinases can downregulate the NOD1 and NOD2 signaling, which is also a mechanism to control and prevent overreaction and dysregulation of the NOD1 and NOD2 pathway (Hitotsumatsu et al. 2008; Fiil et al. 2013). The activation of this pathway leads to proinflammatory immune responses, like IL-8 release (Warner et al. 2014) and to type I interferon (IFN) signaling, which is involved in anti-viral responses (Seth, Sun, and Chen 2006). But it also leads to the activation of the adaptive immunity. It is shown that NOD2 activation can also regulate Th17 cell responses by programing dendritic cells to promote IL-17 production (van Beelen et al. 2007). Defects and dysregulation of NOD2 are associated to many diseases. For example, the highest known genetic risk factors that promote development of Crohn's disease is polymorphisms in NOD2 (Hugot et al. 2001) or the Blau syndrome (Wouters et al. 2014) or Yao syndrome, which formerly was called NOD2-associated autoinflammatory disease (Yao and Shen 2017).

1.3.3 Other PRRs

The TLRs and NLRs are the biggest groups of the PRRs but the RLRs are also part of it and are involved in the sensing of almost all viral infections (Wicherska-Pawłowska, Wróbel, and Rybka 2021). The RLRs consist of the three receptors, called retinoid acid inducible gene I (RIG-I), melanoma-differentiation-associated gene 5 (MDA5), and last but not least laboratory of genetics and physiology 2 (LGP2). All

three of them are located within the cytoplasm, but recent studies also demonstrated that RIG-I can be found in the cell nucleus (Liu et al. 2018). They consist of two N-terminal CARDs, a C-terminal regulatory domain and a conserved central DEAD box helicase/ATPase domain. They recognize dsRNA directly from dsRNA viruses or dsRNA as intermediate, generated during the replication of ssRNA viruses. RIG-I is recognizing short dsRNA of up to 1 kilobases (kb), which induces type-I IFN production. This can be even enhanced if the dsRNA contains a 5'-triphosphate. In contrast, MDA5 recognizes longer dsRNA of more than 2 kb, which induce type-I IFN production as well. Cutting of long dsRNA by specific dsRNA nucleases converts the dsRNA from a MDA5 to a RIG-I ligand, demonstrating that MDA5 is not able to recognize short dsRNA (Takeuchi and Akira 2010). LGP2 lacks the CARD domain and is thought to negatively regulate the other two RLRs RIG-I and MDA5 (Rothenfusser et al. 2005). In contrast to that, studies with LGP2 deficient mice demonstrated that LGP2 positively regulates type-I IFN production after RNA virus infections, recognized by RIG-I and MDA5 (Satoh et al. 2010). Binding of ligands to RLRs is mediated by their C-terminal domain, which has a large surface that forms a RNA binding loop (Cui et al. 2008). For the activation their central domain catalyzes ATP, which is an essential mechanism for type-I IFN production. Furthermore, the CARDs trigger a signaling cascade via direct interaction with MAVS that results in induction of antiviral responses (Kawai and Akira 2006). Thereby, the signaling pathways of MAVS and TRIF share downstream signaling molecules that lead to expression of interferon inducible genes (Wicherska-Pawłowska, Wróbel, and Rybka 2021).

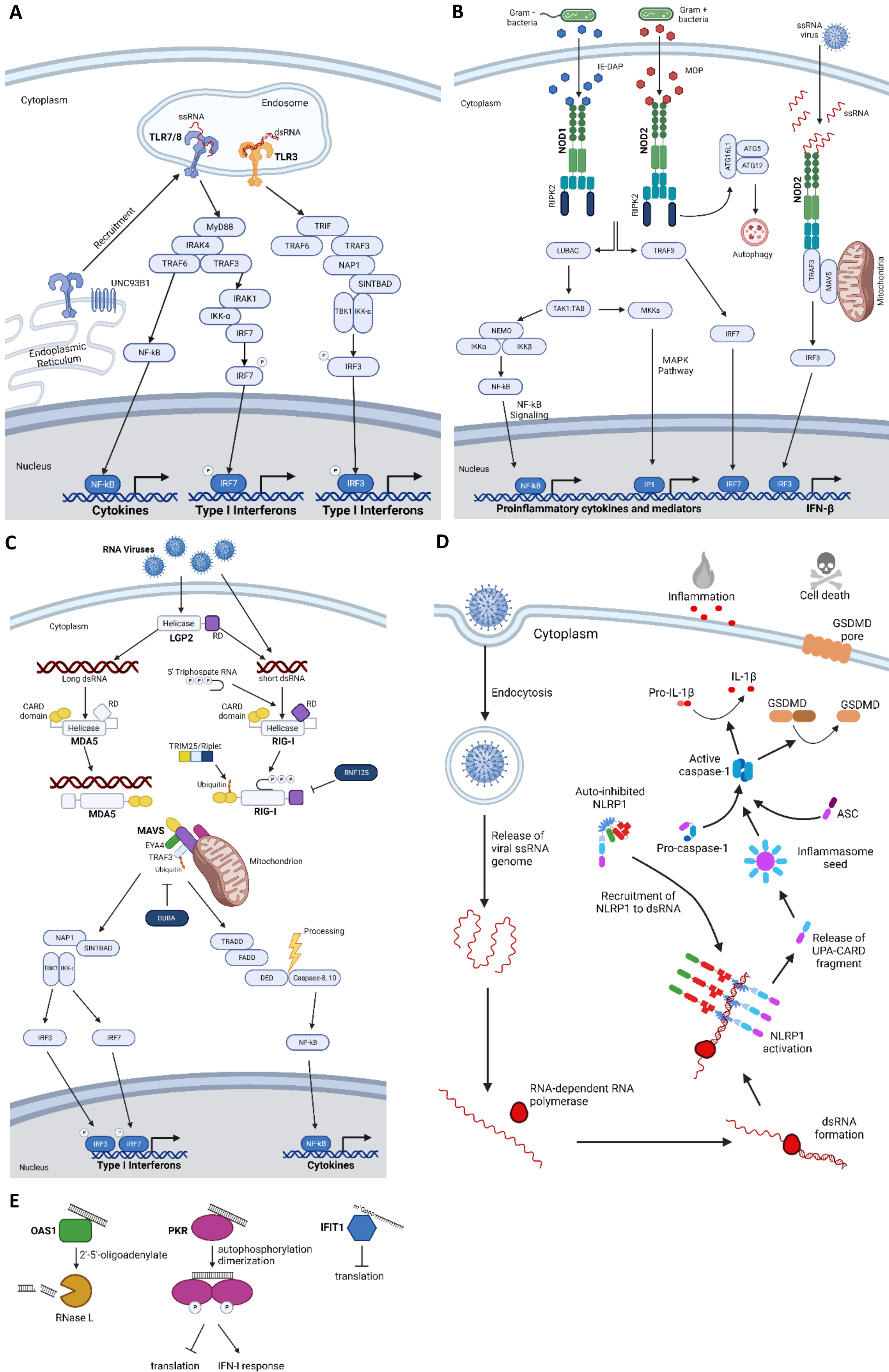
Activation of RLRs also induce the expression of alternative RNA sensing receptors. One of these receptors is 2'-5'-oligoadenylate synthetase 1 (OAS1), that generates 2'-5'-oligoadenylate, which then activates RNase L. This enzyme degrades dsRNA into shorter fragments that can be sensed by RIG-I to strengthen the sensing through this pathway. Another one is the protein kinase R (PKR), a serine/threonine protein kinase that after binding to dsRNA gets autophosphorylated and dimerized. This process results in type-I IFN response and inhibition of viral protein synthesis (Uehata and Takeuchi 2020). The third alternative RNA sensing receptor is interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) that senses 5'-triphosphate RNA and after activation blocks the viral RNA translation (Pichlmair et al. 2011).

The CLRs as another group of PRRs consists of three transmembrane proteins, called dectin-1, dectin-2, and macrophage-inducible C-type lectin (MINCLE) that are characterized by a carbohydrate-binding domain. As the name suggests CLRs bind carbohydrates from fungi, bacteria and viruses. They induce proinflammatory cytokine production but can also inhibit TLR-mediated immune complexes (Geijtenbeek and Gringhuis 2009). The ITAM coupled receptors dectin-1 and dectin-2 are both recognizing β-glucans from fungi (Robinson et al. 2009), while MINCLE also contributes to sense fungi infections but also recognizes endogenous proteins like spliceosome-associated protein 130 from necrotic host cells (Yamasaki et al. 2008). The activation of the receptors lead to downstream signaling mediated via the ITAM that results in MAP kinase and NF-κB activation and finally proinflammatory cytokine production (Kalia, Singh, and Kaur 2021).

There are also other PRRs that are responsible for sensing cytosolic DNA from various microbes in a TLR9 independent manner. These are cGAS, AIM2 and IFI16. Binding of DNA to cGAS leads to activation of the ER-membrane adaptor stimulator of interferon genes (STING) (Ishikawa and Barber 2008). The activation of STING leads to a conformational change and translocation that results in IRF3 and NF-κB activation and finally production of IFNs and proinflammatory cytokines, like IL-1β, IL-6 and TNF (Ishikawa, Ma, and Barber 2009). Activation of AIM2 by DNA leads to recruitment of apoptosis-associated speck-like protein containing a CARD (ASC) and subsequently to binding of caspase-1 and

formation of inflammasome (Li and Wu 2021). Similarly, IFI16 also plays a role in inflammasome activation and leads to pyroptosis by caspase-1 activation. Furthermore, IFI16 was shown to regulate type I IFN expression (Briard, Place, and Kanneganti 2020).

As RNA sensing is an important part of this work, an overview of the RNA sensing pathways is shown in figure 3.



A
Cytoplasm
Endosome
ssRNA
TLR7/8
dsRNA
TLR3
Recruitment
UNC93B1
Endoplasmic Reticulum
MyD88
IRAK4
TRAF6
TRAF3
IRAK1
IKK-α
IRF7
TRIF
TRAF6
TRAF3
NAP1
SINTBAD
TBK1
IKK-ε
IRF3
NF-kB
Nucleus
NF-kB
IRF7
IRF3
Cytokines
Type I Interferons
Type I Interferons
B
Gram - bacteria
Gram + bacteria
ssRNA virus
IE-DAP
MDP
Cytoplasm
NOD1
NOD2
RIPK2
ssRNA
ATG16L1
ATG5
ATG12
Autophagy
NOD2
TRAF3
MAVS
Mitochondria
LUBAC
TRAF3
TAK1:TAB
MKKs
NEMO
IKKα
IKKβ
IRF7
IRF3
NF-kB
MAPK Pathway
NF-kB Signaling
Nucleus
NF-kB
IP1
IRF7
IRF3
Proinflammatory cytokines and mediators
IFN-β
C
RNA Viruses
Cytoplasm
Helicase
LGP2
RD
Long dsRNA
5' Triphospate RNA
short dsRNA
CARD domain
RD
Helicase
MDA5
CARD domain
RD
Helicase
RIG-I
TRIM25/Riplet
Ubiquitin
RNF125
MDA5
RIG-I
MAVS
EYA4
TRAF3
Ubiquitin
Mitochondrion
DUBA
NAP1
SINTBAD
TBK1
IKK-ε
TRADD
FADD
Processing
DED
Caspase-8, 10
IRF3
IRF7
NF-kB
Nucleus
IRF3
IRF7
Type I Interferons
NF-kB
Cytokines
D
Inflammation
Cell death
GSDMD pore
Cytoplasm
Endocytosis
IL-1β
Pro-IL-1β
GSDMD
GSDMD
Active caspase-1
Auto-inhibited NLRP1
ASC
Pro-caspase-1
Inflammasome seed
Release of viral ssRNA genome
Recruitment of NLRP1 to dsRNA
Release of UPA-CARD fragment
NLRP1 activation
RNA-dependent RNA polymerase
dsRNA formation
E
OAS1
2'-5'-oligoadenylate
RNase L
PKR
autophosphorylation dimerization
P
P
translation
IFN-I response
IFIT1
translation

Figure 3: Different RNA sensing mechanisms in a cell

A: TLR3, TLR7 and TLR8 are localized in endosomes. TLR3 recognizes dsRNA, which leads to a TRIF dependent signal transduction, resulting in IRF3 phosphorylation and translocation into the nucleus, where it induces type I IFN genes. Trafficking of TLR7 and TLR8, which recognize ssRNA, via UNC93B1 into endosomes is induced after stimulation or virus infection. Activation leads to MyD88 dependent signal transduction through NFκB and IRF7, which are translocated into the nucleus to activate cytokine- or type-I IFN genes, respectively. Created with Biorender.com. Adapted from (Takeuchi and Akira 2010); **B**: NOD1 and NOD2 recognize different peptidoglycan ligands from bacteria via their LRR domain and recruit RIPK2 after activation, which starts a signaling cascade that results in NF-κB, MAPK pathway and IRF7 signaling. These results in expression of proinflammatory cytokine and mediator genes, as well as IFN-β. NOD2 is also able to recognize ssRNA from viruses which after activation lead either to signaling through RIPK2 (Lupfer et al. 2013) or through MAVS (Sabbah et al. 2009), which lead to IFN-β gene induction. Created with Biorender.com. Adapted from (Trindade and Chen 2020); **C**: LGP2 is a positive regulator of RIG-I and MDA5, which sense cytosolic short dsRNA with a 5'-triphosphate end or long dsRNA, respectively. After activation both interact with MAVS, which results in expression of type-1 IFN or cytokine genes via signaling of IRF3 and IRF7 or NFκB, respectively. Created with Biorender.com. Adapted from (Takeuchi and Akira 2010); **D**: ssRNA from a virus infection leads to dsRNA formation via RNA-dependent RNA polymerase, which recruits NLRP1 that forms inflammasome complexes after activation. These results in IL-1β maturation and release and pyroptosis induction via GSDMD. Created with Biorender.com. Adapted from (Bauernfried et al. 2021); **E**: RLRs induce expression of RNA receptors that mediate alternative RNA sensing. 2'-5'oligoadenylates, generated by OAS1, activate RNase L, that degrades dsRNA into smaller fragments that are sensed by RIG-I. The serin/threonine protein kinase PKR is autophosphorylated and dimerizes after dsRNA binding, which results in induction of IFN-I responses and inhibition of viral protein synthesis. RNA with capped 5'-triphosphate ends is sensed by IFIT1, which blocks translation of viral RNA. Created with Biorender.com. Adapted from (Uehata and Takeuchi 2020).

1.4 First line of defense – the skin

1.4.1 Architecture of the skin

The biggest organ of the body is the skin, a specialized barrier to protect the body against pathogens and water loss to maintain homeostasis. Furthermore, the skin is also involved in regulating blood pressure and body temperature, as well as its immunological functions to regulate immune responses. The skin can be divided into three layers: epidermis, dermis and hypodermis, which is also called subcutaneous tissues or subcutis (Benson 2012). An overview of the skin and its layers is shown in figure 4.

The epidermis is a stratified epithelium that consist mainly of keratinocytes, a highly specialized cell type of epithelial cells, which can be found only in the skin (Eckert and Rorke 1989). It is also equipped with sweat glands to regulate the body temperature and pilosebaceous follicles, which produce sebaceous excretions and the hair (Kanitakis 2002). The epidermis can be further divided into four subgroups, depending on the level of differentiation and unique biochemical and morphological features the keratinocytes have and that differ between the layers. The stratum basale, stratum granulosum, stratum lucidum and stratum corneum. The lowest layer is the stratum basale with basal keratinocytes that are attached by hemidesmosomes, a proteinaceous anchor to the underlying basement membrane at the dermal-epidermal junction to prevent moving of the basal layer or via desmosomes to each other (Burgeson and Christiano 1997). These basal keratinocytes are the only cells in the epidermis that can proliferate. This proliferation and the following migration to the skin surface is an essential mechanism to constantly renew the skin, whereas the normal turnover rate is 28 days (Wong et al. 2016). The stratum basale also contains other cells like Langerhans cells, Merkel

cells and melanocytes, which are responsible for the pigmentation of the skin (Nguyen and Soulika 2019). The keratinocytes undergo a process of maturation and differentiation as they migrate to the skin surface. The second layer of the epidermis is the stratum spinosum, the thickest layer of the epidermis. In this layer the keratinocytes start a process, called cornification, by starting to produce large amounts of keratohyalin. During this process the proliferation stops and differentiation of the keratinocyte is induced (Lee, Lee, and Wu 2017). Another step, which marks the transition from keratinocytes in the stratum basale to the stratum spinosum, is the switch in the expression of keratins. The basal keratinocytes mainly express keratin 5 and keratin 14, whereas the differentiating keratinocytes in the stratum spinosum mainly express keratin 1, keratin 2 and keratin 10, which can also be used as differentiation markers (Wang, Zieman, and Coulombe 2016). Furthermore, the epidermis itself has no blood vessels and therefore has no supply of nutrients. This leads to a poorer supply of nutrients the further they are from the basement membrane. This results in a changed morphology of the cells and a pyknosis (Fore 2006). Thereby, the initiation of the keratinocyte differentiation into late stages is a calcium-dependent process (Chieosilapatham et al. 2021). The next layer is the stratum granulosum, in which the keratinocytes have keratohyalin granules, filled with histidine- and cysteine-rich proteins that can bind keratin filaments together (Ovaere et al. 2009). Furthermore, the keratinocytes secrete lamellar granules that are filled with proteins and lipids into the extracellular space to form the hydrophobic lipid envelope, which is essential for the barrier properties especially to prevent water loss. Additionally, the cells lose during their differentiation process their nuclei and organelles (Fore 2006). The outer layer is called stratum corneum and consists of terminally differentiated keratinocytes, which are called corneocytes. These cells do not have a nuclei or any cytoplasmic organelles but are filled with highly cross-linked proteins, which give them high stability and lead to a polygonal squames form (Nguyen and Soulika 2019). It needs about two to four weeks until the cells from the stratum basale reach the stratum corneum under normal conditions but it can be accelerated after injuries or during diseases (Rittié 2016). Together with the lipids, secreted in the stratum granulosum, the corneocytes build the barrier that protects the body for example against invading pathogens and water loss (Wong et al. 2016). An injury that disrupts the stratum corneum or deeper layers leads to an initiation of a proinflammatory response to start a repair mechanism (Nickoloff and Naidu 1994). As a disruption of the barrier makes it easier for pathogens to penetrate, cytokines are released to initiate immune defense responses during the inflammatory response. Furthermore, the disruption leads also to an elevated proliferation rate of the keratinocytes in the basal layer, followed by a faster differentiation and migration to the skin surface. Additionally, the keratinocytes in the stratum granulosum release more lamellar granules to the extracellular space to restore the barrier and initiate the final differentiation into corneocytes to form more stratum corneum (Wang et al. 2018).

Beneath the epidermis and the dermal-epidermal junction is the dermis. The dermis consist to about 10% of cells, this includes Langerhans cells, macrophages, lymphocytes, eosinophils, mast cells and the dominant cell type of the dermis, fibroblasts. The other 90% are a connective tissue matrix that mainly consists of collagen along with elastic fibers and proteoglycans, which are all produced by the fibroblasts, and lymph and blood vessels (Fore 2006; Nguyen and Soulika 2019). Due to the blood vessels that are missing in the epidermis, the dermis is responsible for supply of the epidermis with nutrients through capillaries (Shirshin et al. 2017).

Underneath the dermis is the final layer of the skin, the subcutaneous tissues. This layer is rich in proteoglycans and glycosaminoglycans and has mucus-like properties (Guimberteau et al. 2010). The

layer also contains several cell types, including fibroblasts, macrophages and adipocytes. This tissue layer serves as an adipose tissue that can store fatty acids as an energy reservoir and thereby regulate lipid metabolism and glucose homeostasis. (Tran et al. 2008; Driskell et al. 2014).

For a long time, the skin was thought to be barrier and a sensory organ, but researchers could demonstrate that it is a highly active tissue in case of protein synthesis, regulating metabolism, production and secretion of signaling molecules, such as cytokines and that it has an essential role in the immune system by secreting supportive molecules, like antimicrobial peptides, but especially by activation and regulation of immune cells (Fore 2006; Nguyen and Soulika 2019).

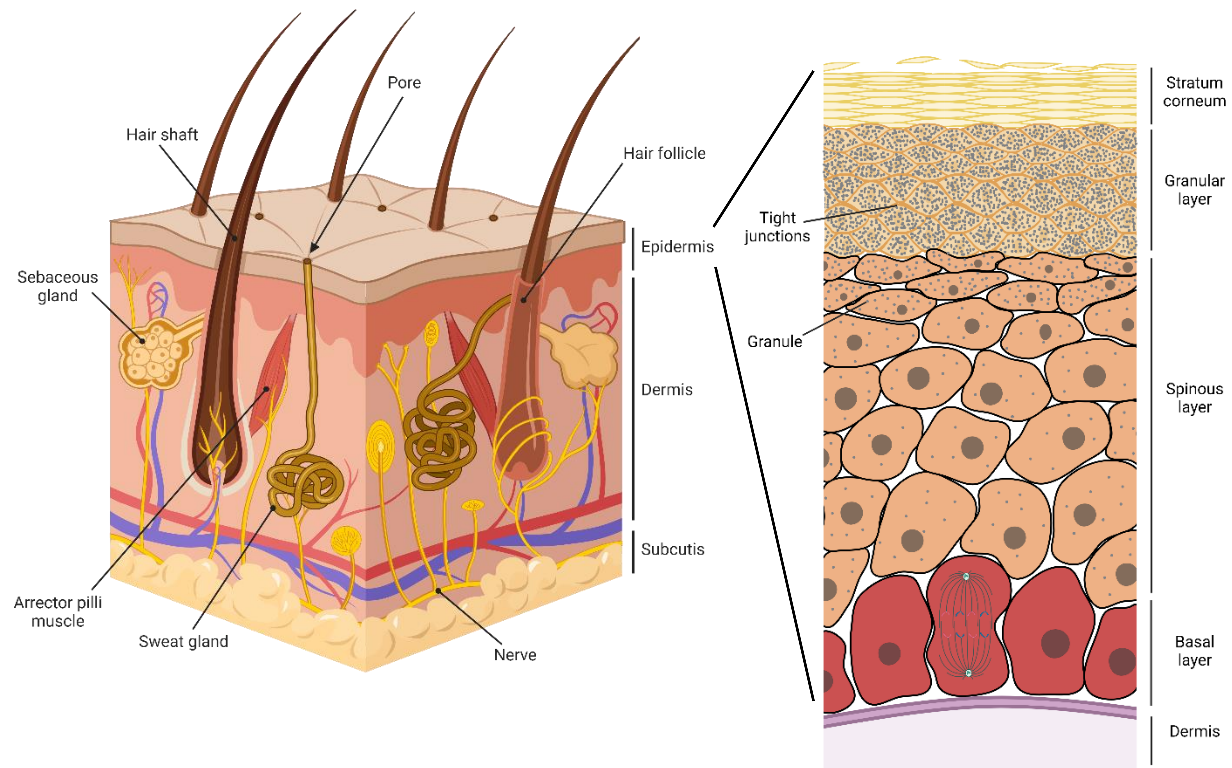


Figure 4: Anatomy of the skin
Structure of the skin with the subcutis as lowest layer, which is rich in proteoglycans and glycosaminoglycans. Over this layer is the dermis, which harbors among others nerves, sweat glands, hair follicle, sebaceous glands and blood vessels. It contains mainly connective tissue, like collagen, and immune cells and fibroblasts. The epidermis as uppermost layer is subdivided into four sublayers as shown on the right. The lowest of these layers is the basal layer, which contains proliferating keratinocytes. These keratinocytes migrate to the top and thereby differentiate. Dependent on the stage of differentiation the keratinocytes form the spinous layer, granular layer or when terminally differentiated stratum corneum. The terminally differentiated keratinocytes are also called corneocytes. Created with Biorender.com. Adapted from (Kobayashi, Ricardo-Gonzalez, and Moro 2020) and (Segre 2006)

1.4.2 Keratinocytes – immunological functions and interactions with immune cells

As predominant cell type in the skin, the keratinocytes are involved in many processes to maintain tissue homeostasis and body health, this includes immune responses. Keratinocytes are the first cell type in the skin to sense pathogens. They are therefore considered as sentinels that continuously monitor environmental changes and initiate immune responses, if necessary (Jiang et al. 2020). To recognize MAMPs and DAMPs they express a broad range of PRRs, this includes TLRs, NLRs, RLRs, CLRs and cytosolic DNA sensors, shown in table 2. The expression rate of the PRRs is varying and dependent

on the layer of the epidermis and with that on the differentiation status of the keratinocyte. For example, the expression of the RNA and DNA sensors TLR3, MDA5 and cGAS is higher in the basal layers, taking into account that in this layer antiviral responses are most abundant (Jiang et al. 2020). Thereby, the cytosolic DNA sensor cGAS is constitutively expressed and after activation can drive type-1 IFN production (Dombrowski et al. 2011).

Keratinocytes are able to express the cell surface TLRs 1, 2, 4, 5 and 6, as well as the endosomal TLRs 3, 7, 8 and 9 (Jiang et al. 2020), although the TLR7 and TLR8, which are important for sensing ssRNA, appear not to be constitutively expressed. TLR7 expression can be initiated either by former TLR3 activation with dsRNA, although it was not shown to be active (Kalali et al. 2008) or is expressed and functionally active after Ca^{2+}-induced differentiation, which occurs in the skin as they migrate to the skin surface (Li et al. 2013; Klymenko et al. 2017). Additionally, studies could show that the AMP LL37 is able to induce TLR7 and TLR8 expression (Miura et al. 2022). Thereby, TLR8 is functionally active and activation via LL37 in keratinocytes leads to IL-36γ production which then induces IL-17C production. These are both potent proinflammatory cytokines that are also associated with the autoinflammatory skin disease psoriasis (Miura et al. 2022). In addition to the ssRNA sensor TLR8, activation of TLR9 by methylated CpG as DNA ligand leads to the induction of CXCL9, CXCL10 and type I IFNs in keratinocytes (Miller and Modlin 2007). Moreover, activation of other TLRs in keratinocytes showed that binding of lipoproteins from *Staphylococcus aureus* to TLR2 homo- or heterodimers leads to AMP and proinflammatory cytokine production. Furthermore, in human keratinocytes the activation of TLRs leads predominantly to a Th1-type immune response and the induction of type-1 IFNs (Bitschar et al. 2017). But the activation of TLRs can also lead to an activation of a transcription factor, called nuclear factor erythroid 2–related factor 2 (NRF2). NRF2 is involved in several processes as it is essential for skin homeostasis, skin repair, stress responses and protection against ROS. Furthermore, NRF2 confers protection against severe sepsis and infection with bacterial pathogens such as *S. aureus*. Thus, it is suggested that in keratinocytes NRF2 inhibits NF-κB signaling to regulate and prevent excessive skin inflammations (Thimmulappa et al. 2016).

Additionally, it is also shown that keratinocytes express several NLRs. As they are in close contact with the environment and therefore often exposed to bacteria, NOD1 and NOD2 are important sensors for keratinocytes to sense bacterial peptidoglycan (Jiang et al. 2020) or in case of NOD2 additionally also ssRNA from viral infections (Sabbah et al. 2009). Their activation in keratinocytes leads to the production of proinflammatory cytokines like IL-8 (Jiang et al. 2020). Other NLRs that are expressed in keratinocytes and lead to the activation of inflammatory caspases are for example NLRP3. It is shown in keratinocytes that dsRNA mediated activation of the NLRP3 inflammasome leads to IL-1β and IL-18 secretion (Dai et al. 2017). The same could be shown for NLRP3 activation in keratinocytes with other activators like house dust mice, DNA damage induced by UVB and pesticides (Dai et al. 2011; Jang et al. 2015; Hasegawa, Nakashima, and Suzuki 2016). Although, these studies showed NLRP3 via microscopy or protein levels in keratinocytes, it has to be considered, that untypical NLRP3 activators were used and a NLRP3 effect could only be shown with NLRP3 siRNA or shRNA but never in a knockout. Even though keratinocytes seem to express NLRP3 their role within these cells is not completely clear. In contrast to the NLRP3 reports, it is suggested that NLRP1 is the predominant NLRP member in keratinocytes with its role in UVB sensing (Burian and Yazdi 2018) and the recently demonstrated recognition of viral dsRNA, which lead to IL-1β and IL-18 secretion (Bauernfried et al. 2021). But NLRP1 is not the only viral dsRNA sensor in keratinocytes that initiates anti-viral immune

responses. Keratinocytes also express TLR3, RIG-I and MDA5 that promote the activation of IRF3 and release of type-I IFNs after viral infections (Kalali et al. 2008).

The skin is not only confronted with pathogenic microbes, but also in constant contact with commensal bacteria. Thereby, it is shown that commensal bacteria directly modulate keratinocytes by releasing immune modulatory factors, that have either proinflammatory or tolerogenic properties (SanMiguel and Grice 2015). Researchers found that to prevent *S. aureus* skin colonization *S. epidermidis* secretes factors that act on keratinocytes to promote and initiate protective immune responses (Wanke et al. 2011).

Table 2: Expression of PRRs in keratinocytes

Adapted from Jiang et al. (Jiang et al. 2020)

PRR	Expression (induced by)	Reference
TLR1	constitutively	(Wanke et al. 2011)
TLR2	constitutively	(Meisgen et al. 2014)
TLR3	constitutively	(Lebre et al. 2007)
TLR4	constitutively	(Lebre et al. 2007)
TLR5	constitutively	(Lebre et al. 2007)
TLR6	constitutively	(Wanke et al. 2011)
TLR7	TLR3-activation Ca^{2+}-induced differentiation LL37	(Kalali et al. 2008) (Li et al. 2013; Klymenko et al. 2017) (Miura et al. 2022)
TLR8	LL37	(Miura et al. 2022)
TLR9	constitutively	(Lebre et al. 2007)
NOD1	constitutively	(Wanke et al. 2011)
NOD2	constitutively	(Voss et al. 2006)
NLRP1	constitutively	(Bauernfried et al. 2021)
NLRP3	constitutively	(Dai et al. 2017)
RIG-I	constitutively	(Kalali et al. 2008)
MDA5	constitutively	(Kalali et al. 2008)
Dectin-1	constitutively	(van den Berg et al. 2014)
cGAS	constitutively	(Beck et al. 2021)
AIM2	constitutively	(Dombrowski et al. 2011)

Keratinocytes are known as major producers of AMPs in the skin. Some of these are continuously expressed, some are produced only or in even higher amounts during inflammatory or infection responses. AMPs have antimicrobial activities against pathogens, like bacteria, enveloped viruses and fungi to either eliminate them or control their growth. They can also mediate chemotaxis to attract immune cells (Chambers and Vukmanovic-Stejic 2020). Upregulation of AMP expression is induced via signaling of proinflammatory cytokines, like TNF-α, IL-1β, IL-17 and IL-22 or directly by bacterial contact or during the epidermal differentiation and wound healing (Schittek 2011; Nguyen et al. 2020). For example, in keratinocytes IL-17A and IL-22, which are produced by Th17 cells, increase AMP expression (Clausen and Agner 2016). Whereas cytokines produced by Th2 cells, that are present in increased amounts in the skin of atopic dermatitis patients, inhibit AMP production (Schittek 2011). The main AMPs that are produced by keratinocytes are β-defensins, psoriasin, LL37 and RNase7. RNase7 is a potent antimicrobial ribonuclease that also can bind DNA and RNA for example from bacteria but also the host to form a complex just like LL37. Complexes of DNA and LL37 can be presented to cells to

activate immune responses and amplify the signaling in keratinocytes (Kopfnagel et al. 2020). RNase 7 is as well as LL37 expressed constitutively and after keratinocyte activation their expression is highly upregulated (Harder and Schröder 2002; Chen et al. 2013).

Keratinocytes are important cytokine producers to mediate pro- and anti-inflammatory responses. This includes TNF, IL-6, IL-10, IL-8, several other CXCLs, several members of the IL-1 family and more that attract and activate leucocytes (Jiang et al. 2020; Chambers and Vukmanovic-Stejic 2020). The cytokines of the IL-1 family are crucial for the barrier functions of the skin and are secreted by keratinocytes after PRR activation (Bitschar et al. 2017). Interestingly, proteases from recruited PMNs can process IL-36 cytokines, members of the IL-1 cytokine family and secreted by keratinocytes, for activation, which could drive an escalation of the inflammation (Henry et al. 2016). The secretion of CC-chemokine ligand (CCL) 20 by keratinocytes lead to an attraction and migration of Treg cells and precursors of Langerhans cells into the skin (Bitschar et al. 2017). In contrast, secretion of CXCL9, CXCL10 and CXCL11 by keratinocytes leads to the attraction of different T-cell subtypes into the skin (Nestle et al. 2009). Furthermore, the skin keratinocytes are one of the major cellular sources for the important T-cell growth factor IL-15 (Schittek 2011). But keratinocytes do not only attract T-cells. After IFN-γ stimulation the keratinocytes express MHC class II molecules on the cell surface. With these MHC molecules it is indicated that they can serve as an antigen-presenting cell to activate $CD4^{+}$ and $CD8^{+}$ T-cells (Black et al. 2007), but only the expression could be shown, not the interaction. Furthermore, in keratinocytes these MHC molecules are also involved in the regulation of immune responses to commensal microbes. Expression of the intrinsic MHC class II molecules were shown to control the immune responses that are commensal induced by Th1, but not by Th17, demonstrating the crucial role that keratinocytes play in regulating the communication between host and microbiota (Tamoutounour et al. 2019). In several inflammatory skin diseases the epidermis of patients shows many activated proinflammatory signaling pathways, which demonstrates that the regulation of these keratinocyte-intrinsic pathways are essential for skin and immune homeostasis (Pasparakis, Haase, and Nestle 2014).

So far it is known that keratinocytes can attract immune cells with chemoattractants or that they can be activated by sensing cytokines from other keratinocytes and immune cells with their cytokine receptors to contribute to immune responses by secreting further cytokines. But direct interactions of keratinocytes with immune cells remain unclear and need to be further elucidated.

1.5 Psoriasis, a common inflammatory skin disease

1.5.1 Characteristics of psoriasis

The process of proliferation and differentiation of keratinocytes in the skin is highly regulated, but altered processes can be observed in several skin pathologies, such as psoriasis. About 2-3% of the worldwide population suffer from this common inflammatory skin disorder (Ortiz-Lopez, Choudhary, and Bollag 2022). There are several clinical subtypes of this autoimmune disease. The most common type (about 90% of all psoriasis cases) is the chronic plaque type, also called psoriasis vulgaris. The plaques are erythematous, itchy, sharply demarcated and covered in silvery scales. They can occur in a scattered pattern or cover large parts of the skin and even coalesce. Typical locations of the plaques include the scalp and trunk, as well as extensor surfaces of the limbs. Another form is the inverse psoriasis, which affects mainly skin folds and body bends. It is characterized by plaques and patches that are erosive erythematous (Rendon and Schäkel 2019). Guttate psoriasis, another type, mainly affects children and adolescents and these patients have a predisposition for psoriasis vulgaris, as

about one-third develop it during their adult life (Ko et al. 2010). It is often triggered by infections of the tonsils with group-A streptococcal. The fourth type is called pustular psoriasis, which usually occurs as multiple sterile pustules, that are able to coalesce, all over the body or in a localized area of the skin (Rendon and Schäkel 2019).

Psoriasis is a complex disease that can be triggered by genetic, environmental and immunological factors but the exact cause remains unknown (Dopytalska et al. 2021). These triggers can vary between different patients, but the most common ones are physical trauma, injury, stress, streptococcal infection, medications and genetic components (Ran, Cai, and Zhang 2019; Ortiz-Lopez, Choudhary, and Bollag 2022). Psoriasis is characterized by the hallmark of sustained inflammation together with uncontrolled keratinocyte hyperproliferation and aberrantly differentiation (Tian and Lai 2022). The inflammation is driven by a broad range of cytokines, of which TNF-α, IL-8, IL-12, IL-23, several IL-17 family members (e.g. IL-17A, IL-17C and IL-17F) and several IL-36 family members (e.g. IL-36α and IL-36γ) are the most important ones (Johnston et al. 2013; Baliwag, Barnes, and Johnston 2015; Benhadou, Mintoff, and Del Marmol 2019; de Alcantara, Reiche, and Simão 2021). These cytokines are produced by many different cell types, but the most prominent producers in psoriasis are the keratinocytes, Th1 and Th17 cells (Baliwag, Barnes, and Johnston 2015). Furthermore, for plaque psoriasis the TNF-α–IL-23–Th17 inflammatory pathway is characteristic and the IL-17 cytokine family, composed of IL-17A to F, are key regulators of the inflammation (Matsuzaki and Umemura 2018).

In the histology of psoriatic plaques, the skin shows an epidermal hyperplasia, also called acanthosis, which is the thickening of viable skin layers, hyperkeratosis, characterized by thickening of the cornified layer, parakeratosis, characterized by the fact that the cells of the stratum corneum still have their cell nuclei, and an excessive immune cell infiltration, composed mainly of macrophages, dermal dendritic cells, T-cells and PMNs (Boehncke and Schön 2015; Rendon and Schäkel 2019). It can happen that the immune cells accumulate in the epidermis to form subcorneal microabscesses, also called Munro's microabscesses (Boehncke and Schön 2015). As the cause and the first initiator of psoriasis remains unknown, researchers suggested different theories about the etiology of this disease, but the most popular thesis is the feed-forward mechanism. Crosstalk between immune cells and keratinocytes initiate, shape and maintain an inflammatory milieu that results in the uncontrolled hyperproliferation and aberrantly differentiation of keratinocytes and sustained inflammation in the psoriatic plaques (Tian and Lai 2022).

The diagnosis of psoriasis is mainly based on clinical findings, rather than skin biopsies. Disease severity can be quantified by the psoriasis area and severity index (PASI) that takes thickness of the skin, erythema, scaling and the area covered by plaques into account (Schmitt and Wozel 2005). There is also a broad range of comorbidities that are associated with psoriasis. The most aberrant one is psoriatic arthritis, that about 40% of the patients with psoriasis develop (Henes et al. 2014), but also other comorbidities are described, this includes metabolic syndrome, cardiovascular disorders, type-II diabetes, Crohn's disease, non-alcoholic fatty liver disease, lymphoma, coronary plaques, depression and anxiety (Griffiths and Barker 2007; Ludwig et al. 2007; Gerdes, Mrowietz, and Boehncke 2016; Rendon and Schäkel 2019).

The treatment of psoriasis depends on the severity and the comorbidities. Therefore, the patients are divided into two groups according to their severity as assessed by the PASI score (Mrowietz et al. 2011). Around 70-80% of psoriasis patients have the mild psoriasis, which is treated with topical therapies, like vitamin D analogues and glucocorticoids, and/or phototherapy. For the moderate-to-severe

psoriasis patients a systemic drug in combination with phototherapy is used for treatment. Traditional systemic treatment drugs are cyclosporin A, methotrexate and retinoids (Boehncke and Schön 2015). But meanwhile more specific and efficient drugs are available for systemic treatments. These are monoclonal antibodies and fusion proteins targeting mainly two crucial pathways of psoriasis development: TNF-α signaling and the IL-23 - Th17 axis. They usually inhibit cytokines like TNF-α, IL-12, IL-23 or IL-17A to prevent further responses by blocking their binding to respective receptors or inhibit their production. But these drugs are only able to decrease the plaque number and size but are not enough to conquer psoriasis (Rendon and Schäkel 2019). This means to find better drugs and maybe even conquer psoriasis, the disease and its molecular initiators and pathways needs to be further investigated.

1.5.2 Keratinocytes in psoriasis

Psoriasis was considered until the 1970s as keratinocyte dysfunction disease (Voorhees 1977). This has changed, as now it is known to be an autoimmune disease triggered by a dysregulated response and interplay of keratinocytes and immune cells. Thereby, keratinocytes have been shown to play an important role in the initiation, pathogenesis and maintenance of psoriasis. The keratinocytes produce cytokines, chemokines and AMPs to attract and activate immune cells, which then also release cytokines that activate keratinocytes and lead to the hyperproliferation of them. This feedback loop amplifies the response and increases complexity and severity of psoriasis. Therefore, the secreted products of keratinocytes are indispensable as they reinforce continuous immune cell attraction and activation into the skin to fuel the inflammatory process during psoriasis (Ortiz-Lopez, Choudhary, and Bollag 2022). The Koebner phenomenon also suggests a crucial role of keratinocytes in psoriasis, as it describes the fact that in psoriasis patients an injury of the skin often leads to formation of psoriatic plaques at the damaged area (Ni and Lai 2020). Furthermore, it may also explains why psoriatic plaques are often developed at sites of mechanical stress like knees and elbows (Qiao et al. 2019).

Keratinocytes from psoriasis patients show intrinsic differences to normal keratinocytes. There is the hyperproliferation and aberrant differentiation, which lead to the fact that these keratinocytes only need 4-5 days to migrate through the epidermis (Lowes, Suarez-Farinas, and Krueger 2014), whereas in healthy skin keratinocytes need about 28-37 days (Weinstein, McCullough, and Ross 1984). Additionally, the aberrant differentiation can cause dysfunctions in the epidermal barrier, which can trigger infections and further inflammation (Ye et al. 2014). This abnormal differentiation is due to several factors. As mentioned, the differentiation is a calcium-controlled process, as low levels support proliferation and higher concentrations support differentiation. But the calcium metabolism is altered in several ways. Usually, the calcium is sensed by a G-protein-coupled receptor, that after binding of calcium induces a phosphoinositide turnover, which results in keratinocyte differentiation. In psoriatic skin the calcium gradient is dysregulated, and therefore the calcium sensor cannot induce the differentiation (Tu and Bikle 2013). Furthermore, psoriatic keratinocytes overexpress calcium-binding proteins, like S100A7, S100A8 and S100A9 (Saintigny et al. 1992). These S100 proteins are regulators of extra- and intracellular pathways, that not only regulate the proliferation and differentiation of keratinocytes but also death and immune responses (Benhadou, Mintoff, and Del Marmol 2019).

Keratinocytes are the main producers of AMPs in the skin. This could be another way to initiate or maintain psoriasis inflammation. Many AMPs do not only act on microorganisms but also act as DAMPs that alone or by forming a complex with for example nucleic acids, like LL37 does, activate TLRs and

lead to cytokine release. Most of the AMPs, like LL37, β-defensins and S100A proteins, produced by keratinocytes are highly upregulated in psoriatic skin. For example, LL37 binds DNA and RNA either from infiltrating microbes or endogenous from damaged cells and activate via TLR7 and TLR8 or TLR9 cells like PMNs, plasmacytoid dendritic cells or keratinocytes (Herster et al. 2020; Ortiz-Lopez, Choudhary, and Bollag 2022). Additionally, in two-thirds of psoriasis patients that have a moderate-to-severe disease, autoantigens against LL37 recognized by T-cells are found (Lande et al. 2014). This means that the production of a frequent autoantigen in psoriasis secreted by keratinocytes is highly upregulated.

It is further described that keratinocytes have the ability to serve as antigen-presenting cell and activate immune cells by expression of HLA antigens. This expression of class I and class II HLA antigens is induced by stimulation and elevated in psoriasis skin but the impact on psoriasis pathogenesis remains unclear (Benhadou, Mintoff, and Del Marmol 2019).

As mentioned, keratinocytes are a key producer of cytokines in the skin by expressing a broad range of cytokines. IL-17A was long thought to be the most abundant IL-17 cytokine in psoriasis, produced mainly by Th17 cells (Blauvelt and Chiricozzi 2018) but it is indicated that IL-17C is even stronger expressed. Furthermore, IL-17C overexpression in mice keratinocytes results in the development of a psoriasis-form skin phenotype (Johnston et al. 2013). This could suggest that IL-17C plays also a key role in the inflammation during psoriasis. Additionally, the main producers of IL-17C in lesional skin are keratinocytes (Baliwag, Barnes, and Johnston 2015).

The effects of keratinocytes in the pathogenesis of psoriasis are also shown in mouse models. Knockout of the IL-6 receptor or IL-17A receptor in mouse keratinocytes inhibits the psoriatic skin phenotype induced by imiquimod (Moos et al. 2019; Ravipati et al. 2022). In contrast, knockout of the IL-17A receptor in T-cells or myeloid cells has no impact on the imiquimod induced psoriasis phenotype. Furthermore, the migration of PMNs into the skin, but not of monocytes, depends on the IL-17 receptor of keratinocytes in the imiquimod induced psoriasis model of mice (Moos et al. 2019). Based on the correlation of reduced PMN influx and decreased psoriatic lesion development and supported by further studies it is accepted that also PMNs have a key role in psoriasis (Nakabo, Romo-Tena, and Kaplan 2022).

Taken together this demonstrates the crucial role of keratinocytes in the pathogenesis of psoriasis. It should further be considered that around 90% of the epidermis are keratinocytes, which in psoriatic lesions even have highly elevated numbers due to the hyperproliferation. Even if each keratinocyte only secretes low amounts of cytokines, chemokines and AMPs the enormous number of keratinocytes could result in significant release (Ortiz-Lopez, Choudhary, and Bollag 2022). Thus, the effects of keratinocytes should not be underestimated and demonstrates their key role in psoriasis.

1.5.3 Other immunological processes in psoriasis

Besides keratinocytes, many other immune cells are involved in the pathogenesis of psoriasis. Activation of plasmacytoid dendritic cells (pDCs) is described as a crucial initiator of psoriatic plaque development. A key activator *in vivo* for pDCs is LL37 bound to RNA or DNA to activate TLR7 and TLR8 or TLR9, respectively. This leads to proinflammatory responses like type I IFN production, which then result in maturation and activation of myeloid dendritic cells (mDCs) (Lande et al. 2007; Ganguly et al. 2009). mDCs can also be activated in a pDC independent way by CCL20, secreted by keratinocytes after

microbiota, trauma or drug responses (Kennedy-Crispin et al. 2012). Activated mDCs secrete IL-12 and IL-23, cytokines that are potent inducers of inflammation and elevated in psoriatic lesions (Ortiz-Lopez, Choudhary, and Bollag 2022). Furthermore, mDCs also produce TNF-α, which can activate further mDCs and Langerhans cells, and the enzyme inducible nitric oxide synthetase, which are all mediators of an inflammation (Lowes et al. 2005). IL-12 leads to the differentiation of naïve $CD4^+$ T-cells into Th1 cells, that secrete IFN-γ (Teng et al. 2015). IL-23 can bind to Th17 cells, which leads to activation and results in IL-17A and IL-17F secretion (Zaba et al. 2009; Malakouti et al. 2015), as well as IL-22, IL-23, IFN-γ and TNF-α release (Volpe et al. 2008; Benhadou, Mintoff, and Del Marmol 2019). The IL-17A and IL-17F cytokines activate keratinocytes, that subsequently secrete a broad range of proinflammatory cytokines, including IL-8, CXCL2, CXCL3, CXCL9, CXCL10, CXCL11 and CXCL20. This cytokine release culminates in the migration of further T-cells into the skin, creating a feed-forward loop that amplifies the signaling (Lowes et al. 2013). This process is called the IL-12/IL-23 and IL-17 axis, which is a hallmark of psoriasis inflammation.

Another abundant immune cell type in psoriatic plaques are PMNs. The presence of PMNs in psoriatic plaques and in Munro's microabscesses, which are mainly filled by PMNs, serves as another histopathological hallmark of psoriasis. Furthermore, granular components, oxidative stress and NETs from PMNs are associated with initiation, maintenance and severity of psoriasis. Comparison of PMNs of healthy individuals and psoriasis patients, showed that psoriatic PMNs have elevated NOX2 and MPO activities and produce more ROS (Chiang et al. 2019). This ROS overproduction is due to T-cells and keratinocytes that prime PMNs by cytokines and chemokines within the psoriatic lesions, which lead to augmented respiratory bursts (Guérard et al. 2013; Vogt et al. 2018). Additionally, the increased MPO correlates with psoriasis severity (Dilek et al. 2016). PMNs can also activate IL-36 by cleaving pro-IL-36, which is upregulated in psoriatic plaques by their serine proteinases from the PMN granules, like cathepsin G, proteinase 3 and NE, and thereby further escalate the inflammation within the psoriatic lesion (Henry et al. 2016). NETs are also associated with psoriasis. Their role in psoriasis will be explained further in the following chapter, as they are of special interest for this work.

1.5.4. NETs in psoriasis

NETs are associated with the severity of psoriasis, as they are associated to many immune responses that participate in the initiation and maintenance of psoriasis (Lee et al. 2017). They are highly abundant in psoriatic lesions. This is due to the fact that the psoriatic PMNs are preactivated and lead to an elevated NET formation in the psoriasis plaques (Schön, Broekaert, and Erpenbeck 2017; Herster et al. 2020). In addition, NETs are reported to mediate the release of inflammatory cytokines through crosstalk of TLR4 and the IL-36 receptor (Shao et al. 2019). The molecules that are secreted via granules or during the NET formation contribute to immune responses and therefore also to psoriasis, including keratinocyte proliferation, T-cell imbalance, auto-antigen formation and angiogenesis. For example, the LL37 can bind to the chromatin of NETs in psoriatic plaques to form an immune-modulatory complex that activates pDCs and lead to release of IFN-α and IFN-β and other proinflammatory cytokines, which then activate mDCs and the whole signaling cascade, as described before. The Th17 activation and the IL-17 release result in activation of keratinocytes and PMNs via their IL-17 receptor, which amplifies the immune response and strengthens the feed forward loop. Thereby, the activation of keratinocytes also leads to release of CXCL1, CXCL2 and IL-8, which are shown to attract PMNs (Chiang et al. 2019). Besides keratinocytes and T-cells, PMNs have been reported to be the other major cellular source of IL-17 cytokines via the formation of NETs within psoriatic lesions (Blauvelt and

Chiricozzi 2018). Furthermore, a crosstalk between PMNs and IL-17 expressing T-cells is shown, as the cytokines of these T-cells are shown to promote not only recruitment but also development and longer lifespans of PMNs. Additionally, experimental models demonstrated that NETs induce Th17 generation from peripheral blood mononuclear cells, that requires cell-cell contact and monocytes (Lambert et al. 2019; Chiang et al. 2019). Moreover, mDCs can readily sense neutrophilic antigens within the NETs, which allows T-cells to get directly primed (Tillack et al. 2012; Sangaletti et al. 2012). Besides their antimicrobial and immune modulatory properties, especially excessive NET formation leads to tissue damage. Nearby cells, like keratinocytes in these psoriatic lesions, get damaged by the released molecules, which lead to injuries and the release of more DAMPs, resulting in an amplified proinflammatory signaling (Hidalgo et al. 2022).

Taken together, PMNs are, besides keratinocytes and T-cells, one of the key cell types that contribute to the autoimmune skin disease psoriasis. But so far only a direct interaction of PMNs and T-cells is shown. Until now it remains unknown if the most abundant cell type, the keratinocytes, and PMNs only communicate via cytokines or if there is also direct interaction.

1.6 Aim of this study

The main research on the functions and influence of different NET components focused on DNA or proteins. Nothing was reported about RNA in NETs, until recently the NET-associated RNA, short naRNA, was described and suggested to fuel inflammations (Herster et al. 2020). As NETs are highly abundant in the autoimmune skin disease psoriasis, it was hypothesized that naRNA could fuel immune responses within psoriatic lesions.

The aims of this study were to examine if naRNA has an effect on innate immune cells that may be abundant within psoriatic lesions, for example macrophages. Furthermore, as keratinocytes are the main cell type in the skin and are key contributors to psoriasis, a special focus was to determine if NETs generally or naRNA in particular have a direct impact on keratinocytes. Additionally, it was aimed to explore through which receptors naRNA is sensed in macrophages and keratinocytes. Moreover, we sought to investigate, whether an activation of keratinocytes by naRNA could contribute to escalation of inflammation within psoriatic lesions. Collectively, these investigations were aimed to determine if naRNA could be a driver of psoriasis inflammation.

2. Material and Methods

2.1 Materials

2.1.1 Consumables

Table 3: Consumables

Consumable	Company	Product number
6-well plate	Greiner Bio-One	657160
48-well plate	Greiner Bio-One	662950
384-well plates MicroAmp	Life Technologie GmbH	4309849
0.5 ml tube	Greiner Bio-One	667201
1.5 ml tube	Greiner Bio-One	616201
2 ml tube	Greiner Bio-One	623201
15 ml tube	Greiner Bio-One	188271
50 ml tube	Greiner Bio-One	227270
Adhesive film for microplates (PE-Film)	VWR	60941-062
Adhesive film for microplates (aluminium-film)	VWR	391-1275
EDTA blood collection tubes (S-Monovette)	Sarstedt	04.1931.010
F-bottom 96-well plate	Greiner Bio-One	655101
Half-area 96-well plate	Greiner Bio-One	675061
Nitrocellulose Membrane 0.45 µm	Bio-Rad	1620094
Poly-L-lysine coated glass coverslips	Electron Microscopy Sciences	72292-04
TPP Cryotubes	Sigma Aldrich	Z760951
U-bottom 96-well plate	Greiner Bio-One	650101
V-bottom 96-well plate	Greiner Bio-One	651101

2.1.2 Chemicals and reagents

Table 4: Chemicals and reagents

Chemical / Reagent	Company	Product number
Ampuwa	Fresenius Kabi	1833
Bovine Serum Albumin	Biomol	01400.100
$CaCl_2$	Carl Roth	CN93.1
Chemiluminescent Substrate; 500 mL kit	Thermo Fisher Scientific	10743105
CnT-07 medium	CELLnTEC	CnT-07
DOTAP	Roth	L787.2
EDTA (pH = 8)	Thermo Fisher Scientific	15575020
β-estradiol	Sigma Aldrich	E2758
Ethanol	VWR	20821.330
FCS (heat inactivated, sterile filtered)	Th. Geyer	11682258
FCS, iron supplemented (heat inactivated, sterile filtered)	Sigma Aldrich	C8056-500ML
Fixation buffer	BioLegend	420801
HEPES	Sigma Aldrich	H0887
Histopaque/ Ficoll 1.077 g/mL	Sigma Aldrich	10771
hEGF	Sigma Aldrich	E9644-.2MG
hIL-3 (recombinant)	Peprotech	200-03

HiPerFect Transfection Reagent	Qiagen	301705
hM-CSF (recombinant)	Peprotech	300-25
Hydrocortison	Sigma Aldrich	H0888-5G
Keratinocyte Basal Medium 2	PromoCell	C-20211
Keratinocyte Growth Medium 2	PromoCell	C-20111
L-glutamine	Gibco	25030081
Human Fibroblast Expansion Basal Medium (Medium 106)	Gibco	M106500
Medium 199	Gibco	11150059
Methanol	Honeywell	32213-2.5L
2-Mercaptoethanol	Gibco	21985023
Milk powder	Carl Roth	T145.3
M-PER™ Mammalian Protein Extraction Reagent	Thermo Fisher Scientific	78501
NuPAGE™ Transfer Buffer (20X)	Thermo Fisher Scientific	NP00061
PBS	Thermo Fisher Scientific	14190-169
Pencicillin/Streptomycin	Gibco	15140122
Poly-L-lysine coated glass coverslips	Electron Microscopy Sciences	72292-04
ProLong™ Diamond Antifade Mountant	Thermo Fisher Scientific	P36961
Pooled human serum	Transfusion medicine Tübingen	-
RNase/DNase free water	Thermo Fisher Scientific	10977049
RPMI 1640 culture medium	Sigma Aldrich	R8758
RPMI 1640, very low endotoxins	PAN Biotech	P04-18525
Saponin	Applichem	A4518.0100
Sodium pyruvate	Gibco	11360070
Sulfuric acid	Carl Roth	9316.1
TaqMan™ Universal Mastermix II	Thermo Fisher Scientific	4440040
TWEEN20	Sigma Aldrich	P7949-500ML

2.1.3 Commercial kits

Table 5: Commercial Kits

Kit	Company	Product number
ELISA MAX™ Deluxe Set Human IL-8	BioLegend	431504
High-Capacity RNA-to-cDNA-Kit	Thermo Fisher Scientific	4387406
NucleoSpin NSP isolation Kit	Macherey-Nagel	740971.50
RNeasy Mini QIAcube Kit	Qiagen	74116

2.1.4 Buffers and media

Table 6: Buffers and media

Buffer / media	Components
BlaER1 culture medium	RPMI 1640, very low endotoxins + 10% FCS + 1% Sodium pyruvate + 1% HEPES + 1% Penicillin / Streptomycin
BlaER1 transdifferentiation medium	RPMI 1640, very low endotoxins + 10% FCS + 1% Sodium pyruvate + 1% HEPES

	+ 1% Penicillin / Streptomycin + 10 ng/mL hIL-3 + 10 ng/mL hM-CSF + 150 nM β-estradiol
IF blocking buffer	PBS (1x) + 0.1% heat inactivated DEPC + 0.1% Saponin + 10 U/µL RNase inhibitor + 10% Normal Chicken Serum
ELISA wash buffer	1 L 10x PBS + 5 mL Tween20 Fill up to 10 mL with Milli-Q water
ELISA stop solution	11 mL sulfuric acid + 189 mL Milli-Q water
10x Erythrocyte lysis buffer (ACK)	1.54 M NH_4Cl + 100 mM $KHCO_3$ + 1 mM EDTA, pH = 8 dissolved in Ampuwa water, pH adjusted to 7.3 and sterile filtered (0.22 µm) dilute with sterile H_2O to 1x
NHEK stimulation medium	Keratinocyte Basal Medium 2 + 1,7 mM $CaCl_2$
PMN culture medium	RPMI (Sigma) + 10% FCS
R2F/TERT-1 medium	1:1 mixture of M106 and M199 + 15 % FCS with iron supplemented + 10 ng/ml hEGF + 0.4 µg/ml hydrocortisone + 1% Penicillin / Streptomycin
THP-1 culture medium	RPMI 1640 + 10% FCS + 1% L-glutamine + 1% Penicillin / Streptomycin

2.1.5 TLR ligands

Table 7: TLR ligands

Component	Company	Product number	Dissolved in
DOTAP	Carl Roth	L787.2	-
Ionomycin	Sigma Aldrich	I0634-1MG	H_2O
LL37	Invivogen	tlrl-l37	H_2O
LPS-EK (ultrapure)	Invivogen	tlrl-peklps	H_2O
Muramyldipeptide (MDP)	Invivogen	tlrl-mdp	H_2O
Pam_2CSK_4	Invivogen	tlrl-pm2s-1	H_2O
PMA	Invivogen	tlrl-pma	H_2O
Poly(I:C)	Invivogen	tlrl-picw	H_2O
R848	Invivogen	tlrl-r848	H_2O
Talabostat mesylate (Val-boroPro, VbP)	Biozol	APE-B3941-10MG	DMSO
TL8-506	Invivogen	tlrl-tl8506	H_2O
Tri-DAP	Invivogen	tlrl-tdap	H_2O

2.1.6 Nucleic acids

Table 8: Nucleic acids

Name	Sequence	Backbone	Company
CpG PTO 2006	5’TsCsGsTsCsGsTsTsTsTsGsTsCsGsTsTsTsTsGsTsCsGsTsT3’	Phosphorothioate	TIB
ssRNA40	5’GsCsCsCsGsUsCsUsGsUsUsGsUsGsUsGsAsCsUsC3’	Phosphorothioate	Eurogentec

“s” signifies phosphorothioate linkage.

2.1.7 Nucleic acid digesting enzymes

Table 9: Nucleic acid digesting enzymes

Enzyme	Company	Product number
DNase I	Thermo Fisher Scientific	EN0521
RNase A	Thermo Fisher Scientific	EN0531

2.1.8 Inhibitors

Table 10: Inhibitors

Component	Company	Product number
CU-CPT9a	Invivogen	inh-cc9a
GSK583	Sigma Aldrich	SML1960-5MG
RNase inhibitor	Promega	N2615

2.1.9 siRNAs

Table 11: siRNAs

siRNA	GeneGlobe ID
Hs_MYD88_5 FlexiTube siRNA (MyD88)	SI00300909
Hs_CARD15_3 FlexiTube siRNA (NOD2)	SI00133049
Hs_TICAM1_2 FlexiTube siRNA (TRIF)	SI03069332|S1
Hs_VISA_1 FlexiTube siRNA (MAVS)	SI04190368
Hs_NALP1_5 FlexiTube siRNA (NLRP1)	SI02641086
Hs_RNASE7_7 FlexiTube siRNA (RNase7)	SI04348253
AllStars Negative Control siRNA	1027281

2.1.10 TaqMan™ Gene Expression Assays

Table 12: TaqMan™ Gene Expression Assays

Gene	Assay number
CAMP (LL37)	Hs00189038_m1
CXCL8	Hs00174103_m1
IL17C	Hs00171163_m1
IL36G	Hs00219742_m1
NOD2	Hs01550753_m1
RNASE7	Hs00922963_s1
TBP	HS00427620_m1

2.1.11 Antibodies and dyes

Table 13: Antibodies and dyes

Antibody	Fluorophore /Conjugate	Species	Isotype	Company	Product number
Anti-β-actin	-	mouse	IgG1	Sigma Aldrich	A5441-100UL
Anti-rRNA (Y10b) Alexa Fluor® 647	AF647	mouse	IgG_{2a} κ	Santa Cruz Biotechnology	sc-33678 AF647
Anti-mouse IgG	AF647	chicken	IgY	Thermo Fisher Scientific	A-21463
Anti-MyD88 (4D6)	-	mouse	IgG1 κ	Thermo Fisher Scientific	MA5-16231
Anti-mouse Cy3	Cy3	goat	IgG	Jackson ImmunoResearch	115-165-146
Anti-hCD11b	APC	mouse	IgG1 κ	BioLegend	301310
Anti-hCD14	PE	mouse	IgG1 κ	ImmunoTools	21620144
Anti-hCD15	PE	mouse	IgG1 κ	BioLegend	323006
Anti-hCD19	BV421	mouse	IgG1 κ	BioLegend	302234
Anti-hCD62L	BV421	mouse	IgG1 κ	BioLegend	30482
Anti-hCD66b	FITC	mouse	IgG1 κ	BioLegend	305103
Anti-rabbit IgG-HRP	Peroxidase	goat	-	Vector laboratories Inc.	PI-1000
Anti-mouse IgG-HRP	Peroxidase	goat	-	Promega	W402B
Hoechst 33342	-	-	-	Thermo Fisher Scientific	H21492
Isotype control	APC	mouse	IgG1 κ	BioLegend	400120
Isotype control	PE	mouse	IgG1 κ	eBioscience	12471442
Isotype control	FITC	muse	IgM	BioLegend	401605
Isotype control	BV421	mouse	IgG1 κ	BioLegend	400157
SYTO RNAselect Green fluorescent dye	-	-	-	Thermo Fisher Scientific	S32703

2.1.12 Equipment

Table 14: Equipment

Equipment	Company
Axioplan microscope	Zeiss
BD FACS CANTO™ II	BD Bioscience
Blotcycler	Biozym
Confocal microscope LSM800	Zeiss
Nanodrop™ 1000	Thermo Fisher Scientific
Neubauer counting chamber	Brand GmbH & Co KG
Odyssey Fc Imager	LI-COR Biosciences
PEQLAB peqSTAR 2X	Peqlab Biotechnologie GmbH
QIAcube Connect	Qiagen
QuantStudio 7 Flex System	Thermo Fisher Scientific

2.1.13 Software

Table 15: Software

Software	Company	Version
MS office pack	Microsoft	2019
FACSDiva	BD Bioscience	Version 6
FlowJo	FlowJo LLC	Version 10.01
ImageJ	NIH	Version 1.54c
Prism	GraphPad	Version 8.2
QuantStudio Real-Time-PCR software	Thermo Fisher Scientific	Version 1.3
Zen Blue	Zeiss	Version 3

2.2 Cell biology methods

2.2.1 Study participants and human blood acquisition

Healthy donors, which provided blood for this study, gave their written informed consent prior to participation. Approval for the use of biological materials for this project was provided by the local ethics committee of the Medical Faculty of Tübingen University in accordance with the principles defined in the declaration of Helsinki and the currently applicable laws and regulations.

2.2.2 Neutrophil isolation

Isolation of neutrophils was done by the PhD student Francesca Bork from the Weber laboratory at the Department of Immunology, University of Tübingen. Blood from healthy donors was drawn into 9 mL EDTA blood collection tubes for subsequent isolation of neutrophils. EDTA-anticoagulated whole blood was diluted 1:2 with sterile PBS and then slowly loaded onto 20 mL Ficoll with a density of 1.077 g/mL in a 50 mL tube. The blood was centrifuged at 509 x *g* at room temperature (RT) for 25 min without acceleration and brake to achieve a density gradient separation. Next, all layers above the lowest layer, the erythrocyte-granulocyte layer, were removed by a vacuum pump. Afterwards the tube was slowly and completely filled with 1x ACK buffer and incubated on a roller shaker for 20 min at 4 °C to lyse and thereby remove erythrocytes. This was followed by another centrifugation step with the same settings for 10 min and the removal of the supernatant by a vacuum pump. To remove the remaining erythrocytes, another erythrocyte lysis step was performed by slowly adding 25 mL 1x ACK and incubation on a roller shaker for 10 min at 4 °C, followed by another centrifugation step with the same settings for 5 min. The supernatant was removed by a vacuum pump and the PMNs were carefully resuspended in PMN culture medium to a density of 1.6 x 10^6 cells/mL for seeding in 24-well plates or 5 x 10^6 cells/mL for seeding in 10 cm uncoated dishes. Furthermore, the purity and activation status of each isolation was tested via flow cytometry (described in 2.3.2).

2.2.3 Preparation of NET-content

Preparation of NET-content was done by the PhD student Francesca Bork from the Weber laboratory at the Department of Immunology, University of Tübingen. To create NET content 8 ml of a 5 x 10^6 cells/mL suspension of freshly isolated PMNs were seeded in a 10 cm uncoated dish. For the PMA NETs the cells were stimulated with 600 nM PMA for 4 h at 37 °C and 5% CO_2, whereas for the Mock control the cells were left unstimulated. Afterwards the dish was carefully washed three times with 5 mL PBS to remove the stimulus, released cytokines and unstimulated PMNs, which did not attach to the dish. To protect the naRNA from degradation, 10 U/µL RNase inhibitor were added during NET preparation, when indicated as “Mock NETs + RNase inhibitor” or “PMA NETs + RNase inhibitor”. To create digested

NET-contents, the NETs were incubated with either 100 µg/mL RNase A or 10 U/mL DNase I for 20 min at 37 °C. NET content was stored at -80 °C until it was used for stimulation.

2.2.4 Complex preparation of ssRNA+LL37 or Poly(I:C)/CpG+DOTAP

To form ssRNA+LL37 complexes for a stimulation of cells in 125 µL, 5.8 µM ssRNA40 (~ 34.4 µg/mL) and 5 µg LL37 were mixed and incubated for 1 h at RT. To form complexes of Poly(I:C) with DOTAP the indicated concentration of Poly(I:C) was generated by diluting it 1:5 with DOTAP. To form complexes of CpG with DOTAP the indicated concentration of CpG was generated by diluting it 1:10 with DOTAP. Afterwards, the mixture was incubated for 10 min before stimulating the cells.

2.2.5 BlaER1

BlaER1 cells (WT, *Unc93b*$^{-/-}$ and *Tlr8*$^{-/-}$) were a kind gift from Holger Heine, Borstel, Germany (Vierbuchen et al. 2017). BlaER1 cells, derived from malignant human B cells, were cultured in BlaER1 culture medium. For differentiation into macrophage-like cells, 2 mL/well with 0.5 x 10^6 cells/mL in BlaER1 transdifferentiation medium (culture medium supplemented with 10 ng/mL hM-CSF, 150 nM β-estradiol and 10 ng/mL hIL-3) were seeded in a 6-well plate and incubated at 37 °C and 5% CO_2. On day two and five, 2 mL of fresh BlaER1 transdifferentiation medium were added to each well. 7 days after starting the differentiation, undifferentiated cells, which were floating, were removed first, and then differentiated cells, which were attached to the plate, were removed by flushing with BlaER1 culture medium. 5 x 10^4 cells/well were reseeded in a 96-well plate and rested for 1 h at 37 °C and 5% CO_2. BlaER1 cell differentiation was verified via flow cytometry (see 2.3.3). Next, BlaER1 cells were stimulated in a final volume of 125 µL/well for 18 h at 37 °C and 5% CO_2. The stimulants were: 0.1 µg/mL LPS, 5 µg/mL R848, 100 ng/mL TL8, ssRNA+LL37 complex (see 2.2.4) and Mock or PMA NETs with or without RNase inhibitor in the indicated dilutions. After 18 h stimulation, the plate was centrifuged for 5 min at 500 x *g* and the supernatant was transferred into a new 96-well plate and stored at -80 °C until ELISA for IL-8 was performed (see 2.3.1).

2.2.6 THP-1

THP-1 cells (WT, Tlr7$^{-/-}$ and *Tlr8*$^{-/-}$) were a kind gift from Thomas Zillinger, Bonn, Germany (Coch et al. 2019). THP-1 cells, derived from a human leukemia monocytic cell line (Chanput, Mes, and Wichers 2014), were cultured in THP-1 culture medium. For differentiation into macrophage-like cells, 5 x 10^4 cells/well were seeded in a 96-well plate and differentiation was induced by treatment with 300 ng/mL PMA for 16 h at 37 °C and 5% CO_2. The cells were then washed three times with PBS and rested for 48 h in 200 µL fresh culture medium at 37 °C and 5% CO_2. Afterwards, the medium was carefully removed and replaced by fresh culture medium containing 200 U/mL IFN-γ and incubated for another 6 h at 37 °C and 5% CO_2. Next, the THP-1 cells were carefully washed again three times with PBS and stimulated in a final volume of 125 µL/well with THP-1 culture medium for 18 h at 37 °C and 5% CO_2. The stimulants were: 0.1 µg/mL LPS, 25 µg/mL PMA + 0.375 µg/mL Ionomycin, 5 µg/mL R848, 100 ng/mL TL8, ssRNA+LL37 complex (see 2.2.4) and Mock / PMA NETs with or without RNase inhibitor in the indicated dilutions. After 18 h stimulation, the plate was centrifuged for 5 min at 500 x *g* and the supernatant was transferred into a new 96-well plate and stored at -80 °C until ELISA for IL-8 was performed (see 2.3.1).

2.2.7 N/TERT-1

N/TERT-1 cells were a kind gift from Prof. James Rheinwald (Dickson et al. 2000). N/TERT-1 cells are immortalized keratinocytes which were immortalized by expression of hTERT and loss of the pRB/p16^{INK4a} cell cycle control mechanism. The cells were cultured in CnT-07 medium until passage 10

and then replaced. For stimulation 2 x 10^4 cells were seeded in a 96-well plate and incubated for 48 h at 37 °C and 5% CO_2 until they were confluent. Afterwards, the medium was replaced by fresh medium and the N/TERT-1 cells stimulated in a final volume of 125 µL/well for 24 h at 37 °C and 5% CO_2. The stimulants were: 25 µg/mL PMA + 0.375 µg/mL Ionomycin, 200 ng/mL TL8, ssRNA+LL37 complex (see 2.2.4) and Mock / PMA NETs with or without RNase inhibitor in the indicated dilutions. After stimulation, the plate was centrifuged for 5 min at 400 x *g* and the supernatant was transferred into a new 96-well plate and stored at -80 °C until ELISA for IL-8 was performed (see 2.3.1).

2.2.8 NHEK

Normal human epidermal keratinocytes (NHEK), from either a single juvenile donor (PromoCell, C-12002) or pooled juvenile donors (PromoCell, C-12005) were cultured in Keratinocyte Growth Medium 2 until passage 6. For stimulation 2 x 10^4 cells/well were seeded in a 96-well plate or 2.5 x 10^5 cells/well were seeded in a 6-well plate and incubated for 48 h at 37 °C and 5% CO_2 until they were confluent. To induce differentiation, the culture medium was replaced by NHEK stimulation medium, which contains 1.7 mM $CaCl_2$. NHEK cells were stimulated by adding stimuli in a final volume of 125 µL/well or 750 µL/well for the 96-well plate or 6-well plate, respectively, for 24 h at 37 °C and 5% CO_2. The stimulants were: 2.54 µg/mL PAM_2CSK_4, 25 µg/mL PMA + 0.375 µg/mL Ionomycin, 200 ng/mL TL8, 20 µg/mL R848, 20 µg/mL MDP, 1 µM VbP, 5.8 µM ssRNA40 (~ 34.4 µg/mL), ssRNA+LL37 complex (see 2.2.4), 50 µg/mL Poly(I:C), 50 µg/mL Poly(I:C)+DOTAP (see 2.2.4), 5 µM CpG+DOTAP (see 2.2.4) and Mock or PMA NETs with or without RNase inhibitor in the indicated dilutions. After stimulation, the plate was centrifuged for 5 min at 400 x *g* and the supernatant was transferred into a new 96-well plate or 2 mL tubes and stored at -80 °C until ELISA for IL-8 was performed (see 2.3.1). For RNA isolation the cells of a 6-well plate were lysed by adding 350 µL/well RLT buffer + β-mercapthoethanol (1:100). The lysate was transferred to a 2 mL tube and stored at -80 °C until RNA isolation was performed to check for mRNA levels of genes of interest (see 2.2.12).

2.2.9 siRNA transfection

To create a gene knockdown in keratinocytes with either N/TERT-1 or NHEK cells, 2.5 x 10^5 cells/well were seeded in a 6-well plate and incubated for 24 h at 37 °C and 5% CO_2. The plate was carefully washed twice with PBS and then 1.74 mL basal medium (for N/TERT-1: CnT-07 without supplements; for NHEK: Keratinocyte Basal Medium 2) was added to each well. The siRNA transfection complex was prepared by mixing per well 250 µL of basal medium (for N/TERT-1: CnT-07 without supplements; for NHEK: Keratinocyte Basal Medium 2) with 12 µL HiPerFect transfection reagent and the amount of siRNA for the final concentration (recommended by the company or tested for a sufficient knockdown effect): 3.5 nM for MyD88 and TRIF and 10 nM for NOD2, MAVS and NLRP1. This mixture was incubated for 15 min at RT and then slowly added to the well, while the plate was carefully rotated. The cells were incubated for 16 h at 37 °C and 5% CO_2 and then washed three time with PBS. Afterwards, the cells were either rested in the 6-well plates by adding 2 mL of culture medium and incubated for ~30 h at 37 °C and 5% CO_2. Alternatively, the cells were detached by adding trypsin for 5 min, followed by adding culture media to stop the reaction and centrifugation at 400 x *g* for 5 min. The supernatant was removed, the cells were resuspended in culture medium and 4 x 10^4 cells/well were reseeded in a 96-well plate and incubated for ~30 h at 37 °C and 5% CO_2. For stimulation the wells were washed once with PBS and stimulated for 24 h in a final volume of 125 µL/well or 750 µL/well for the 96-well plate or 6-well plate, respectively. For N/TERT-1 stimulants see 2.2.7 or for NHEK stimulants see 2.2.8. After stimulation, the plate was centrifuged for 5 min at 400 x *g* and the supernatant was transferred into a new 96-well plate or 2 mL tubes and stored at -80 °C until ELISA for IL-8 was performed (see 2.3.1). For

immunoblot the cells of a 6-well plate were lysed by adding 100 µL M-PER mammalian protein extraction reagent (Thermo Fisher Scientific, see 2.1.2) per 1 x 10^6 cells and incubation for 20 min on ice, followed by a 5 min centrifugation at 16000 x *g*. The cell lysate was stored at -80 °C until immunoblot was performed (see 2.3.6). For RNA isolation the cells of a 6-well plate were lysed by adding 350 µL/well RLT buffer + β-mercapthoethanol (1:100). The lysate was transferred to a 2 mL tube and stored at -80 °C until RNA isolation was performed to check for mRNA levels of genes of interest (see 2.2.12).

2.2.10 3D human skin equivalents

The 3D human skin equivalents (HSEs) were created and stimulated by Jasmin Scheurer and Birgit Sauer from the laboratory of Birgit Schittek, Division of Dermatooncology, Department of Dermatology, University Hospital Tübingen. The skin equivalents were created as described in Bitschar et al. 2020. In brief, primary fibroblast were seeded on collagen and incubated for five days in fibroblast medium, followed by seeding of NHEKs on top of the fibroblast and incubation for a further 12 days in keratinocyte medium with medium changes every second day. Then a so-called airlifting step was performed: the medium level was adjusted to be as high as the skin construct so that the uppermost layers were in constant contact with air. This was maintained for 10 consecutive days. HSEs prepared in parallel were stimulated for 24 h with 25 µL/well NET-content of Mock or PMA NETs with or without RNase inhibitor added to the top of the constructs. After the stimulation the media that surrounded the HSE was transferred into 2 mL tubes and stored at -80 °C until ELISA for IL-8 was performed (see 2.3.1). The cells were either embedded in paraffin and stained with H&E to verify the correct growth of the 3D human skin equivalents or 350 µL/well RLT buffer + β-mercapthoethanol (1:100) were added to lyse the cells and stored at -80 °C until RNA isolation was performed to check for the mRNA levels of genes of interest (see 2.2.12).

2.2.11 Isolation and stimulation of mouse keratinocytes

Isolation and stimulation of mouse keratinocytes was done by the PhD student Berenice Fischer from the laboratory of Daniela Kramer at the Department of Dermatology, University Medical Centre of Johannes Gutenberg University Mainz. The mice and reagents for the isolation of the keratinocytes were kindly provided by the laboratory of Daniela Kramer, Department of Dermatology, University Medical Centre of Johannes Gutenberg University Mainz. The keratinocytes were isolated from 8-to 10-week-old C57BL/6 WT or NOD2$^{-/-}$ mice as described in Lorscheid et al. 2019. In brief, after cervical dislocation the tail was cut off and the skin was separated from the muscle. After overnight incubation of the skin at 4 °C in keratinocyte-SFM medium, containing 50 µg/mL dispase, dermis and epidermis were separated. The epidermis was further incubated in trypsin for 15 min at RT. Afterwards, the trypsin reaction was stopped with RPMI 1640 medium with 10% FCS and then a single-cell suspension of keratinocytes was gently washed out. A 100-µm cell strainer was used and the keratinocytes then collected in a 50 mL tube. In the next step, the keratinocytes were centrifuged at 180 x *g* for 5 min and afterwards resuspended in keratinocyte-SFM medium supplemented with $CaCl_2$ to a final concentration of 0.05 M. For seeding the cells were carefully applyed on a collagen type I–coated plate and stimulated when they were confluent. The stimulation was performed with Mock or PMA NETs with RNase inhibitor or 5.8 µM ssRNA40 (~34.4 µg/mL) for the indicated times. After stimulation RNA was isolated with QIAzol (79309, QIAGEN) according to the manufacturer's instructions, while contaminating DNA was removed by digestion with DNase I. Afterwards, RNA was transcribed into cDNA by using Revert Aid reverse transcriptase (EP0441, Thermo Fisher Scientific) and random hexamer primers (SO142, Thermo Fisher Scientific) for 1 h at 42 °C. In the next step, real-time PCR was

performed with the Green master mix from Genaxxon (M3023) and the indicated primers on a Light Cycler 480 II system (Roche).

2.2.12 RNA isolation and RT-qPCR of NHEKs and HSEs

To analyze the response of NHEKs from 2D culture or of HSEs to stimuli on transcript level, total RNA was isolated and mRNA levels of genes of interest were quantified by quantitative RT-qPCR. To isolate the total RNA a QIAcube Connect instrument was used together with the RNeasy Mini QIAcube Kit according to the manufacturer's protocol for animal tissue and cells with an DNA digestion step. After RNA isolation, the RNA concentration was measured using a Nanodrop 1000 instrument and equal amounts of RNA for all conditions were used to transcribe RNA into cDNA by using the High-Capacity RNA-to-cDNA-Kit according to the manufacturer's protocol. In brief, 500 ng RNA were filled up to 10 µl with RNase/DNase free water (Thermo Fisher Scientific, see 2.1.2) and 10 µl RT buffer and 1 µl Reverse Transkriptase was added. In the next step, the mix was incubated in the PEQLAB peqSTAR 2X instrument for 1 h at 37 °C, followed by 5 min at 95 °C and then cooled to 4 °C. Afterwards, the cDNA was diluted 1:10 with RNase/DNase free water. To quantify the mRNA levels, the TaqMan™ system was used. Here, a master mix for each gene of interest was prepared by mixing TaqMan™ Universal Mastermix II and the TaqMan™ gene expression assay for each gene of interest and a housekeeping gene (TBP) (see 2.1.10). Afterwards, 4.5 µL of the cDNA and 5.5 µL of the master mix were added into triplicate wells of a 386-well plate. The RT-qPCR was performed with the QuantStudio 7 Flex System and analyzed with the related QuantStudio Real-Time-PCR software.

2.3 Immunochemical methods

2.3.1 Enzyme-linked Immunosorbent Assay

Enzyme-linked Immunosorbent Assay (ELISA) was used to determine the concentration of released IL-8. The Biolegend ELISA MAX™ Standard Set Human IL-8 Kit was used according to the manufacturer's protocol. Half area 96-well plates were used to reduce the amounts of reagents and supernatant by 50% of what is described in the manufacturer's protocol. In brief, half area 96-well plates were coated overnight with a coating antibody in coating buffer. After three washes with ELISA wash buffer, non-specific binding was blocked by incubation with Assay Diluent A (ADA) from the kit for 1 h at RT on a shaker. After three further washes with ELISA wash buffer, 40 µl supernatant or the standards (range 8-1000 in pg) were added in triplicates and incubated for 2 h at RT on a shaker. After another washing step, detection antibody in ADA was added and incubated for 1 h at RT on a shaker, followed by another washing step. HRP-conjugate was added and incubated for 30 min at RT on a shaker. After a final washing step, the plate was incubated with substrate at RT in the dark without shaking for 15 min and the reaction was stopped by adding ELISA stop solution. The absorption was measured at 450 nm with a standard plate reader.

2.3.2 Neutrophil purity and pre-activation check via FACS

Neutrophil purity and pre-activation check was done by the PhD student Francesca Bork from the Weber laboratory at the Department of Immunology, University of Tübingen. To determine if purified PMNs were of sufficient purity and quality, the purity and activation status were measured by fluorescence activated cell sorting (FACS) as described by Herster et al. 2020. In brief, 2 x10^5 cells/well were seeded in a U-bottom 96-well plate, centrifuged for 5 min at 448 x *g* and the supernatant was removed. The cells were resuspended in 50 µL/well pooled human serum diluted 1:10 with PBS for blocking for 15 min at 4°C. After a washing step with 50 µL/well PBS and a centrifugation step as before,

50 µL/well antibody solution (each antibody diluted 1:100 in PBS) was added and incubated for 20 min at RT in the dark. Three antibody panels were used to test the purity and activation status: (i) anti-CD15-PE, anti-CD66b-FITC, and anti-CD62L-BV421, (ii) anti-CD14-PE and anti-CD66b-FITC and (iii) isotype controls for PE-, FITC-, and BV421. After staining, another washing step with PBS was performed, the cells centrifuged as before, the supernatant discarded, and the cells were fixed in 100 µL/well fixation buffer (Biolegend; see 2.1.2) for 10 min at RT in the dark. The cells were washed once more with PBS and were resuspended in 300 µL/well PBS. In the final step, the cells were measured on a BD FACS Canto II instrument. The analysis was done with FlowJo.

2.3.3 FACS for differentiation check of BlaER1 cells

The differentiation status of the BlaER1 cells was measured via FACS to determine if the cells were fully differentiated into macrophage-like cells as described in Herster et al. 2020. In brief, 5 x 10^4 cells/well of differentiated and undifferentiated cells from the same original culture (for comparison) were seeded in a U-bottom 96-well plate, centrifuged for 5 min at 448 x *g* and supernatant was removed. The cells were resuspended in 50 µL/well pooled human serum diluted 1:10 with PBS for blocking (blocking buffer) for 15 min at 4 °C. After a washing step with 100 µL/well PBS and a centrifugation step as before, 50 µL/well antibody solution was added and incubated for 20 min at RT in the dark. For the antibody solution each antibody was diluted 1:100 in blocking buffer, except anti-CD19-BV421, which was diluted 1:500. The antibody solutions were: anti-CD14-PE, anti-CD11b-APC and anti-CD19-BV421 and the isotype control for PE, APC and BV421. After staining, another washing step as before was performed and the BlaER1 cells were fixed in 100 µL/well fixation buffer (Biolegend; see 2.1.2) for 10 min at RT in the dark. The cells ware washed once more as before and were resuspended in 300 µL/well PBS. Finally, the cells were measured on a BD FACS Canto II instrument. The analysis was done with FlowJo.

2.3.4 Immunofluorescence microscopy of fixed PMNs

Immunofluorescence microscopy was done by the PhD student Francesca Bork from the Weber laboratory at the Department of Immunology, University of Tübingen. Immunofluorescence (IF) microscopy was performed to visualize the response of PMNs and the formation of NETs. Therefore, 500 µL of 1.6 x 10^6 cells/mL of freshly isolated PMNs (see 2.2.2) were seeded on a pre-coated poly-L-lysine-coated glass coverslip (Electron Microscopy Sciences, see 2.1.2) in a 24-well plate and rested for 30 min at 37 °C and 5% CO_2. The PMNs were treated with the indicated stimuli for 3 h, as described and adapted from Brinkmann et al. 2004. In the next step, the PMNs were washed three times with 500 µL PBS/well and then fixed with 250 µL/well fixation buffer (Biolegend; see 2.1.2) for 10 min at RT in the dark. After another washing step, 200 µL/well IF blocking buffer (see 2.1.4) was added for 2 h at RT in the dark. After blocking, 200 µL/well of anti-rRNA Y10b-AF647 (diluted 1:50 in blocking buffer) was added for 2 h at RT with slow and gentle agitation of the plate. After three additional washings with 5 min incubation between each washing, 1 µg/mL Hoechst 33342 was added for 5 min at RT in the dark to stain nuclear DNA. Next, the coverslips were carefully removed from the wells and mounted on glass slides with 4 µL/coverslip ProLong™ Diamond Antifade Mountant solution and then left to dry at RT in the dark overnight. The slides were stored at 4 °C in the dark until analyzed by confocal microscopy on a Zeiss LSM800 Confocal microscope. The images were analyzed via ImageJ and Zen Blue3.

2.3.5 Bradford assay

A Bradford assay was performed to determine the total protein concentration of lysed cell samples after knockdown via siRNA. First a dilution series with bovine serum albumin (BSA) in RNase/DNase-free water (Thermo Fisher Scientific, see 2.1.2) was prepared as a standard, starting at 2 mg/mL and with subsequent 2-fold dilutions. Afterwards, 5 µL/well of each standard or sample were loaded as triplicates in a 96-well plate and 250 µL Bradford reagent was subsequently added. After 15 min of incubation at RT, the absorbance at 595 nm was measured. The protein concentration was calculated using a XY-point of the generated standard curve computed using Prism GraphPad software (see 2.1.13).

2.3.6 Immunoblot

Immunoblot analysis was used to determine if MyD88 protein levels were decreased after siRNA knockdown. After the protein concentration had been determined by Bradford assay (see 2.3.5) the protein concentrations in the lysates (see 2.2.9) were adjusted to 50 µg. Subsequently, identical amounts were mixed with 10x reducing agent and 4x LDS loading buffer and boiled for 5 min at 90 °C. The protein mix or a protein ladder were loaded on a 12% Tris-glycine gel. An electric field of 120 V was applied for 90 min to separate the proteins. Afterwards, the proteins were blotted onto a 0.45 µm nitrocellulose membrane (Bio-Rad, see 2.1.1) by arranging the gel on top of the membrane and between 5 mm filter papers soaked in NuPAGE transfer buffer (Thermo Fisher Scientific, see 2.1.2). The transfer stack was fixed in a semi-dry blotting chamber and 25 V were applied for 35 min by a Blotcycler (Biozym, see 2.1.12). Next, the membrane was blocked to prevent non-specific binding by incubation in 5% non-fat milk powder in PBS for 1 h on a shaker. After blocking, the primary antibody anti-MyD88 (4D6), diluted 1:1000 in blocking buffer, or an anti-β-actin antibody, diluted 1:7000 in blocking buffer, was added over night at 4 °C on a shaker. Afterwards, the membrane was washed three times with PBS-T (PBS with 0.01% Tween20) and incubated in PBS-T for 5 min on a shaker between each washing step. Then horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG-HRP or anti-mouse IgG-HRP, see 2.1.11) were added in a 1:10000 dilution in blocking buffer for 1 h at RT on a shaker. After a final washing with PBS-T, HRP enhanced chemiluminescence substrate (Thermo Fisher Scientific, see 2.1.2) was added to the membrane and the membrane was imaged on a Li-Cor Odyssey Fc Imager.

2.4 Analysis software and statistics

Microscopy and FACS data were, as indicated above, processed by ImageJ and ZenBlue3 or FlowJo software, respectively. All further experimental data was analyzed and visualized with Excel 2019 and GraphPad Prism 8. For statistical analysis GraphPad Prism was used, first testing normal distribution using the Shapiro–Wilk test. For normally distributed data, Student's t or ANOVA tests were used. Mann–Whitney U or non-parametric ANOVA tests were used for not normally distributed data. Multiple testing was corrected by the post-hoc test recommended by Prism. All tests are stated in the appropriate figure legends and typically refer to comparisons between different stimuli compared to an unstimulated condition unless indicated otherwise with a line. For statistical significance, a p-value ($\alpha = 0.05$) of $p < 0.05$ was considered significant and shown with * even if lower than 0.05.

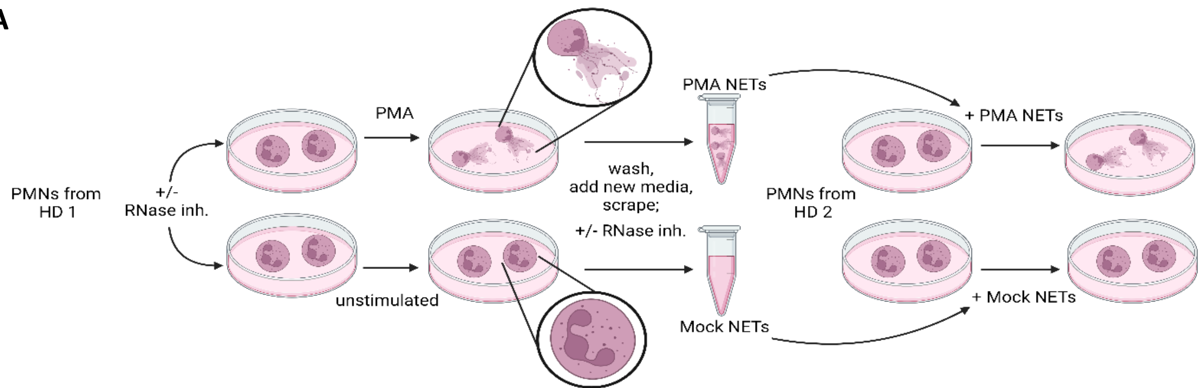
3. Results

The mechanism of forming NETs were first described in 2004 (Brinkmann et al. 2004). Besides their well-studied components, like chromatin and proteins from inside the PMN granules, NET-associated RNA (naRNA), a novel component of NETs, first described in previous work by our group (Herster et al. 2020), is part of the NETs. Due to relatively recent discovery, naRNA and its effects have not yet been further characterized.

It is described that NETs are able to activate macrophages (Papayannopoulos 2018; Josefs et al. 2020) and skin residue cells, like keratinocytes (Takahashi and Yamasaki 2020; Shao et al. 2019) and fibroblasts (Chrysanthopoulou et al. 2014; Khandpur et al. 2013). But which component of NETs and hence which role naRNA plays in these responses remains unclear. To further determine the effects of naRNA especially on cells like macrophages and keratinocytes has been the aim of this work. In order to understand the experimental data regarding this aim, a brief summary of what other information has become available about the immunostimulatory properties of naRNA is first given.

3.1 naRNA is a novel NET component and DAMP, sensed by TLR8 in neutrophils

It could be shown that naRNA is a common NET component and part of all NETs, independent of the stimulus and pathway that induced the NET-formation (Bork et al. 2022). Further, it is described that NETs can induce the formation of more NETs from naïve PMNs (Herster et al. 2020) but the involvement of naRNA as the immunostimulatory component responsible for this effect was only hypothesized. Therefore, the dependence on naRNA and a specific RNA receptor was first established by a colleague in our lab: As synthetic or bacterial RNA complexed to LL37 is mainly sensed via TLR8 in PMNs, naïve PMNs were incubated for 30 min with CU-CPT9a, a TLR8 inhibitor that stabilizes the receptor in its resting state, restricting TLR8 activation (Zhang et al. 2018). The PMNs were stimulated with PMA as positive control, ssRNA in complex with LL37 as TLR8 control (Herster et al. 2020) or NET-content in a 1:100 dilution. The preparation of NET-content for stimulation is shown in figure 5A. Although the CU-CPT9a inhibitor was present, it did not affect the ability of PMNs to form NETs in general as after stimulation with PMA formation occurred. In contrast, the NET induction observed for ssRNA + LL37 and PMA NETs in the absence of CU-CPT9a was abolished by the TLR8 inhibitor (Figure 5B). Moreover, the RNA receptor dependence was demonstrated genetically in mouse bone-marrow-derived neutrophils (BM-PMN) that are deficient for the TLR8-equivalent receptor Tlr13. WT and TLR13$^{-/-}$ BM-PMNs were stimulated with PMA, which lead to NET formation (Figure 5C), but stimulation with PMA NETs only lead to NET formation in the BM-PMNs from the WT mice but not in the TLR13$^{-/-}$. These and additional results not shown here (Bork et al. 2022) demonstrate that naRNA is involved in the self-amplification of NETs and indicate that naRNA is recognized by PMNs through TLR8/Tlr13 signaling. The questions arising from the observation of a potent RNA-mediated response in PMN were whether, and by which receptors, macrophages and tissue cells like keratinocytes responded to naRNA.


A
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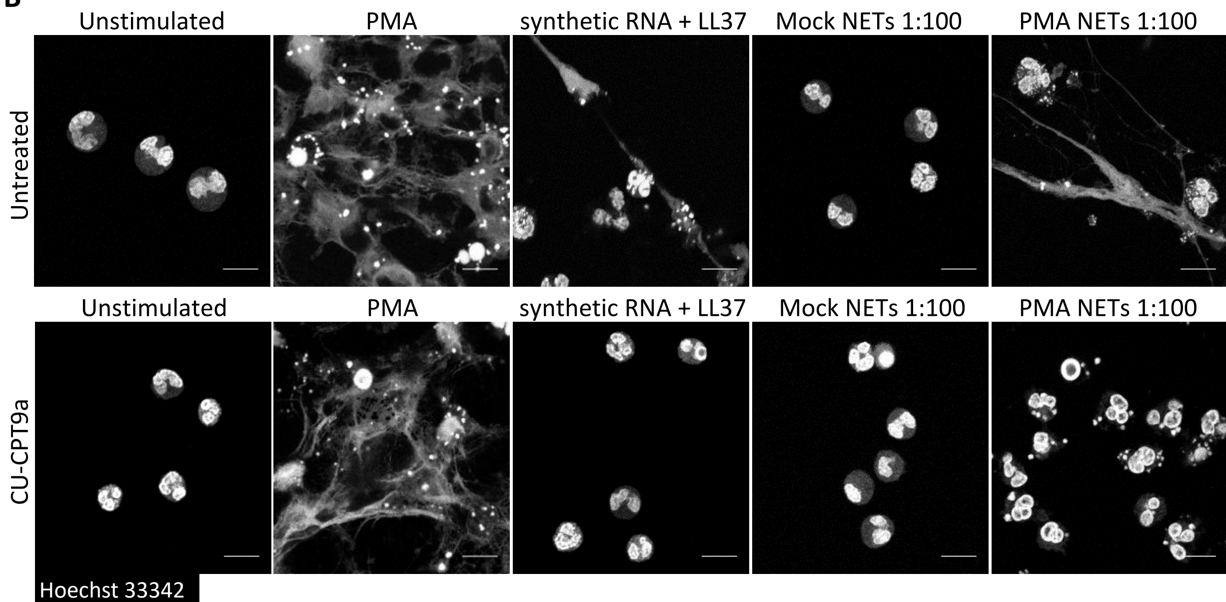
B
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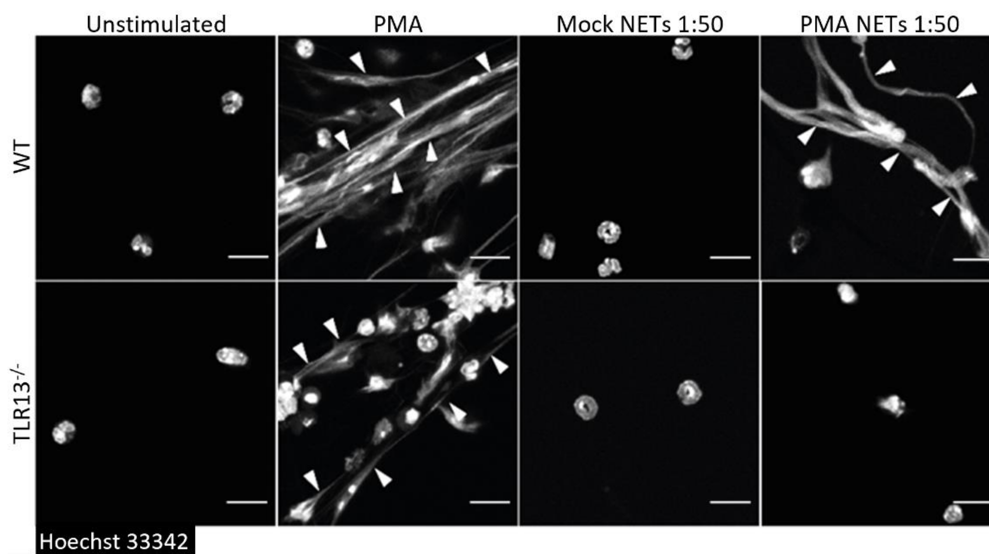

C murine BM-PMNs
Unstimulated
PMA
Mock NETs 1:50
PMA NETs 1:50
WT
TLR13-/-
Hoechst 33342

Figure 5: naRNA is an abundant NET component that is sensed by TLR8 in PMNs

(**A**) Procedure of NET content preparation from healthy donors (HD) used throughout this study. Freshly isolated PMNs were seeded on an uncoated petri dish and subsequently stimulated for 4 h with PMA (600 nM). Afterwards, supernatant was removed, and cells were washed three times with PBS, as only PMNs that formed NETs adhere to the dish and unstimulated PMNs, as well as residual PMA and secreted proteins, like cytokines and chemokines, were removed. In the following step, fresh medium was added and cells/NETs were scraped from the plate and transferred into a new tube. This NET content was stored at -80 °C until further stimulation of naïve PMNs from another healthy donor or other cell types like macrophages, keratinocytes or fibroblasts. If indicated, RNase inhibitor (10 U/µL) was added to protect naRNA from degradation during the 4 h stimulation step and storage. (**B**) PMNs from healthy donor were stimulated for 3 h with PMA (600 nM), ssRNA+LL37 or Mock/PMA NETs (1:100 dilution) with or without TLR8 inhibitor CU-CPT9a (100 nM). PMNs were stained for DNA/NETs with Hoechst33342 (white) and imaged with confocal microscopy (representative images of n = 3, scale bar = 10 µm). (**C**) Primary C57BL/6 WT or Tlr13$^{-/-}$ murine bone-marrow-derived neutrophils (BM-PMN) were stimulated for 16 h with PMA (600 nM) or Mock/PMA NETs (1:50 dilution). BM-PMNs were stained for DNA/NETs with Hoechst33342 (white) and imaged with confocal microscopy (representative images of n = 3, scale bar = 10 µm). Experiments with PMNs were performed by Francesca Bork, PhD student from the Weber laboratory at the Department of Immunology, University of Tübingen.

3.2 Macrophages sense naRNA in a TLR8-dependent immune reaction

Human macrophages are reported to migrate in peripheral tissue to sites of PMN activation (Delavary et al. 2011) and furthermore to sense foreign RNA through TLR8 (Ishii et al. 2014; Heil et al. 2004). We hypothesized that macrophages directly sense NETs via naRNA and that naRNA is recognized through TLR8. To asses this, we used genetically modified macrophage-like BlaER1 cell lines (Vierbuchen et al. 2017), which were provided by Holger Heine, Borstel, Germany. The BlaER1 cell lines were either WT or lacking functional *TLR8* (*TLR8*$^{-/-}$) or *UNC93B1* (*UNC93B1*$^{-/-}$) genes to probe the effect of NETs in dependence of TLR8 and/or endosomal signaling.

The three BlaER1 cell lines WT, *TLR8*$^{-/-}$ and *UNC93B1*$^{-/-}$ were treated for 18 h either with LPS as TLR4 ligand (positive control), R848 as TLR7 and 8 ligand, TL8-506 (from now on referred to as TL8) as specific TLR8 ligand, ssRNA + LL37 in complex as TLR7 or 8 ligand and different NET contents (Mock or PMA NETs with or without RNase inhibitor) in different dilutions. As readout for a reaction, IL-8 release was measured using a cytokine ELISA. Figure 6A shows that all cell lines did not respond to Mock NETs with or without RNase inhibitor. Furthermore, the WT cells responded clearly to all other ligands and also dose-dependently to NET-content. Interestingly, NET-content with stabilized naRNA (PMA NETs + RNase inhibitor) led to a higher IL-8 release compared to PMA NETs without RNase inhibitor. Conversely, the *UNC93B1*$^{-/-}$ and *TLR8*$^{-/-}$, showed no response to the control ligands TL8 and ssRNA + LL37. Furthermore, the *UNC93B1*$^{-/-}$ have a reduced IL-8 release at all dilutions for the PMA NETs with RNase inhibitor and for 1:20 and 1:50 the release was even significantly reduced. Additionally, not only the *UNC93B1*$^{-/-}$, but also the *TLR8*$^{-/-}$ showed decreased IL-8 secretion after stimulation with PMA NETs with RNase inhibitor at all dilutions. These results demonstrate that on the one hand naRNA leads to a highly increased response in form of IL-8 release and that on the other hand TLR8 is essential for naRNA recognition in BlaER1 cells.

As a next step and in order to investigate the precise involvement of TLR7, THP-1 cells with individual TLR7- and TLR8-editing (Coch et al. 2019), provided by Thomas Zillinger, Bonn were differentiated into macrophage-like cells and stimulated as before. Their response was measured via IL-8 cytokine ELISA and for comparison the IL-8 release of each cell line (WT, *TLR8*$^{-/-}$ and *TLR7*$^{-/-}$) was normalized to the PMA + Ionomycin control ligand as 100% (Figure 6B). In the WT cells, similarly to the results obtained

for BlaER1 macrophages, naRNA stabilized NET-content (PMA NETs + RNase inhibitor) led to a greater IL-8 release compared to the NET-content without RNase inhibitor. Furthermore, the *TLR8*$^{-/-}$ cell line had a significant reduced IL-8 release with PMA NETs + RNase inhibitor compared to the WT cells. Interestingly, *TLR7*$^{-/-}$ cells also showed a greatly reduced response, indicating that both TLR7 and TLR8 are able to sense naRNA. These results verify that TLR8 is essential for the sensing of naRNA in macrophages and that naRNA plays a critical role in the activation of macrophages with NETs.

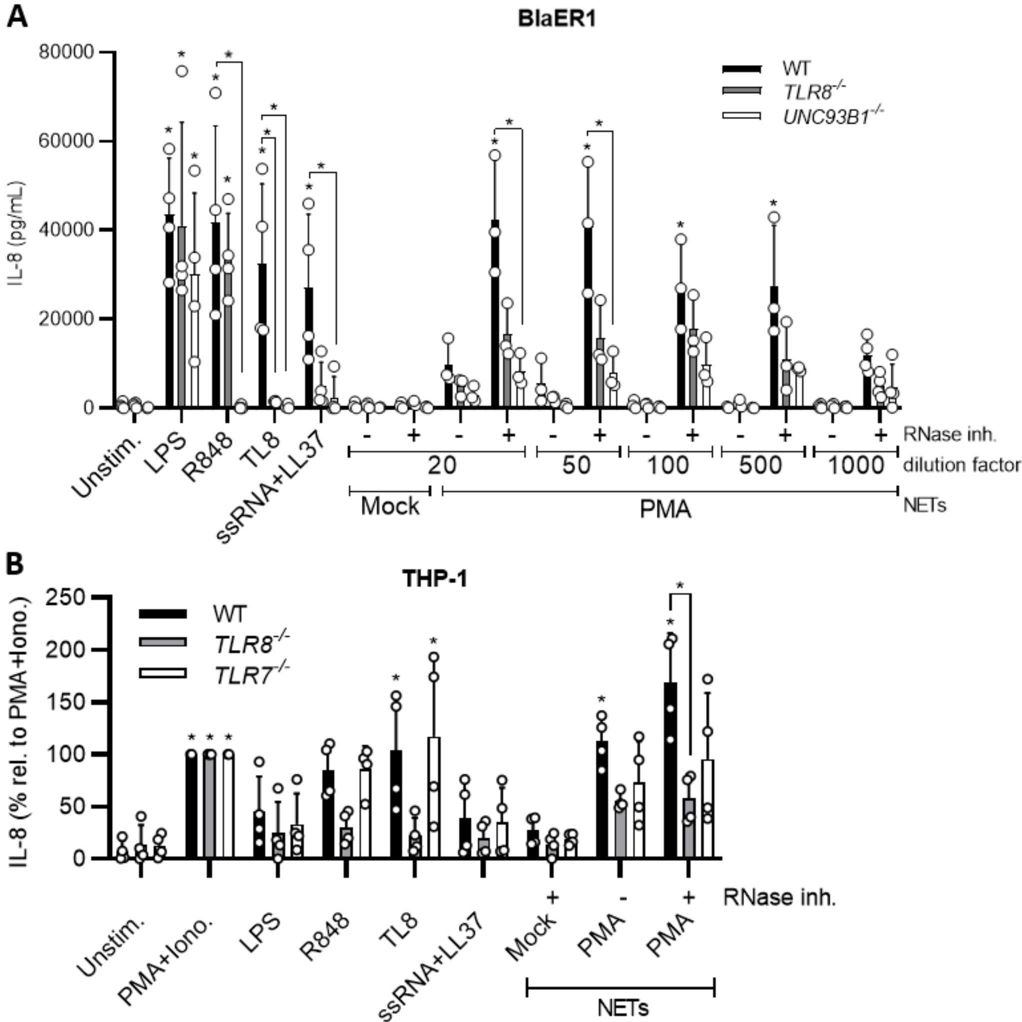


Figure 6: Macrophages respond to naRNA in a TLR8-dependant manner

(**A**) BlaER1 macrophage-like cells (WT, TLR8$^{-/-}$ or UNC93B1$^{-/-}$) were stimulated for 18 h with LPS (0.1 µg/mL), R848 (5 µg/mL), TL8 (100 ng/mL), ssRNA+LL37 or Mock/PMA NETs with or without RNase inhibitor in different dilutions. Combined data of triplicate IL-8 cytokine ELISA (n = 3-4, mean + SD, each dot represents one biological replicate, *p<0.05 according to one-way ANOVA). (**B**) THP-1 cells (WT, TLR8$^{-/-}$ or TLR7$^{-/-}$) were stimulated for 18 h with PMA (25 µg/mL) + Ionomycin (0.375 µg/mL), LPS (0.1 µg/mL), R848 (5 µg/mL), TL8 (40 ng/mL), ssRNA+LL37, Mock NETs with RNase inhibitor (1:50 dilution) or PMA NETs with or without RNase inhibitor (1:50 dilution). Combined data of triplicate IL-8 cytokine ELISA and normalized to PMA + Ionomycin (n = 4, mean + SD, each dot represents one biological replicate, *p<0.05 according to one-way ANOVA).

3.3 Keratinocytes respond to NETs in a naRNA-dependent manner

Neutrophils are highly abundant in the skin especially in the skin of psoriasis patients (Sen et al. 2014) and its known that keratinocytes, the most abundant cells in the skin (Hsu, Li, and Fuchs 2014) are activated by NETs in psoriasis (Shao et al. 2019). We therefore explored if keratinocytes as non-hematopoietic cells with immune functions also get activated by naRNA within the NETs.

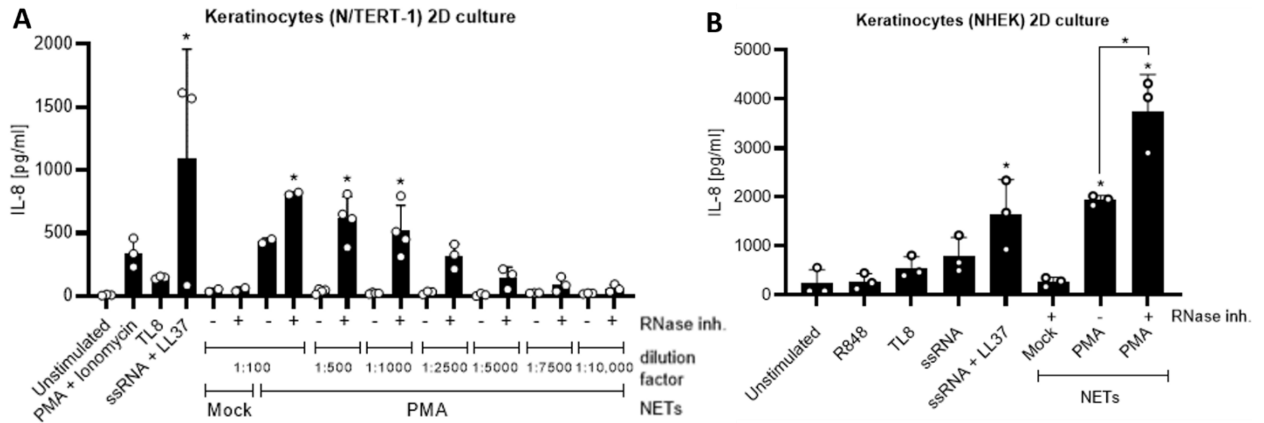
First it was determined if the keratinocytes reacted to naRNA and if this reaction was dose dependent. Therefore, N/TERT-1 keratinocytes which were provided by James Rheinwald, Harvard Medical School, Boston, USA (Dickson et al. 2000; Smits et al. 2017) were analyzed. N/TERT-1 are keratinocytes which were immortalized by expression of hTERT and loss of the pRB/p16^{INK4a} cell cycle control mechanism. The cells were stimulated for 24 h in monolayers with PMA + Ionomycin as positive control, TL8 as TLR8 ligand, ssRNA + LL37 in complex as TLR8 ligand and different NET-contents (Mock or PMA NETs with or without RNase inhibitor) in different dilutions (Figure 7A). The IL-8 response was measured via cytokine ELISA and found to be low for the TLR8 ligand, TL8, whereas ssRNA + LL37 and PMA NETs with stabilized RNA led to a significant release, for PMA NETs even until a 1:1000 dilution PMA NETs without RNase inhibitor only showed an IL-8 release in the lowest dilution of 1:100. Furthermore, the PMA NETs with stabilized naRNA even lead to a measurable IL-8 release in very low doses of 1:7500 in a dose dependent manner, although this was no longer significant.

As N/TER-1 cells are genetically immortalized keratinocytes and therefore differ from primary cells, the effect of NETs as stimuli was also tested on primary normal human epidermal keratinocytes (NHEK). These NHEK cells were stimulated similarly to N/TERT-1 cells for 24 h in monolayer with the same ligands and Mock NETs + RNase inhibitor and PMA NETs with or without RNase inhibitor with a dilution factor of 1:25. The stimulation of RNA stabilized PMA NETs led to a significantly higher release of IL-8 than PMA NETs without RNase inhibitor (Figure 7B).

The skin is a complex organ that consists of several layers of keratinocytes in various differentiation phases (Arda, Göksügür, and Tüzün 2014; Chambers and Vukmanovic-Stejic 2020). In the previous experiments a monolayer of keratinocytes was used to determine the response to NETs. The next step was to validate these results in a so-called 3D human skin equivalent (HSE) (Bitschar et al. 2019), which is more physiological and more comparable to the actual skin. In these 3D HSEs primary keratinocytes isolated from foreskin were seeded on top of a fibroblast layer in the lab of our collaborator, Birgit Schittek. Culturing and an air-lift step led to an 3D model that shows, comparable to the skin, different layers of keratinocytes which mimic natural differentiation phases of human skin. The H&E staining showed that all skin equivalents had a human skin typical pattern with no differences between unstimulated and any stimuli (Figure 7C). For stimulation, the NETs were applied by adding them on top of the HSEs for 24 h. The detection of *CXCL8* mRNA expression levels showed that stimulation with PMA NETs + RNase inhibitor led to a high gene expression of *CXCL8* (Figure 7E). The PMA NETs without RNase inhibitor also induced a significantly lower gene expression compared to PMA NETs with RNase inhibitor. IL-8 was measured in the basal media reservoir. Compared to the unstimulated control, IL-8 concentrations upon stimulation with PMA NETs with or without RNase inhibitor were significantly increased. Furthermore, the PMA NETs with stabilized naRNA even led to a significantly higher IL-8 release than PMA NETs without stabilized naRNA (Figure 7D), indicating RNA is the decisive stimulatory factor. These results demonstrate that in a 3D HSE naRNA activates keratinocytes and induces *CXCL8* expression/IL-8 secretion and thereby validates the results obtained from 2D monolayer keratinocytes that naRNA induces a proinflammatory response in skin cells.

Since NETs consist of many different components, they were digested with RNase A before the stimulation to obtain additional data to validate that naRNA is the actual activator of these processes. Therefore, monolayer NHEKs were stimulated with ssRNA or ssRNA complexed with LL37 as RNA ligands, CpG as DNA control ligand, PMA NETs undigested or digested with RNase A or DNase I and PAM_2CSK_4 as a control TLR2 ligand that is RNA- and DNA-independent. Furthermore, they were stimulated with muramyldipeptide (MDP) as TLR independent ligand. MDP is derived from peptidoglycan motif, which can be found in all bacteria. After 24 h of stimulation the RNA was isolated and the mRNA expression levels of *CXCL8* were analyzed. Therefore, the expression was normalized to an unstimulated control and shown as fold change expression. As results differed in terms of absolute induction values but had the same trend, one representative of three individual experiments is shown (Figure 7F). The PMA NETs induced a strong *CXCL8* expression and digestion with DNase I led to no significant differences. Conversely, when the PMA NETs were digested with RNase A before the stimulation, the expression of *CXCL8* was completely abolished. Furthermore, the NHEKs also responded with higher gene expression to ssRNA alone, which was comparable to ssRNA in complex with LL37. This could indicate that ssRNA does not need to be precomplexed with LL37 to be sensed by keratinocytes.

Taken together these results demonstrate that not DNA but naRNA is the crucial activator in NETs that lead to activation in keratinocytes and that naRNA could be a possible DAMP for these tissue cells.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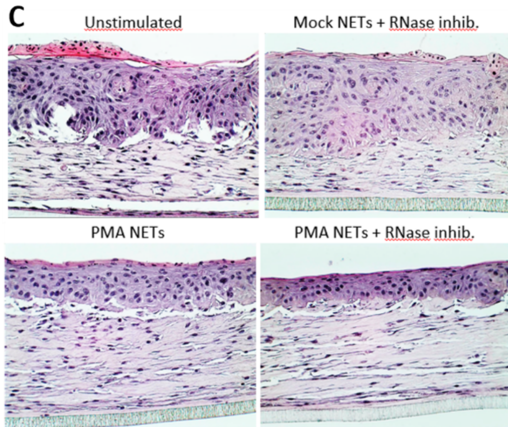
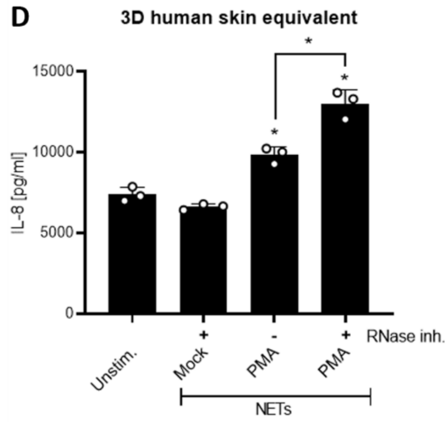
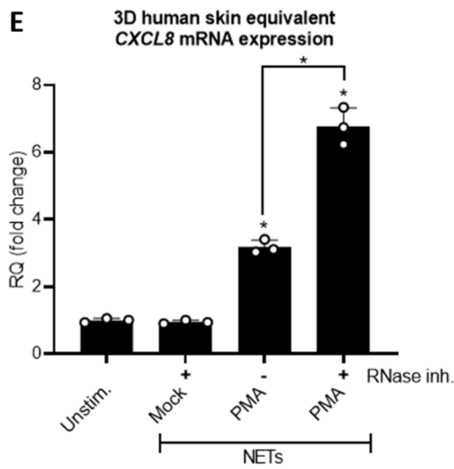
Keratinocytes (N/TERT-1) 2D culture
IL-8 [pg/ml]
Unstimulated
PMA + Ionomycin
TL8
ssRNA + LL37
RNase inh.
dilution factor
1:100
1:500
1:1000
1:2500
1:5000
1:7500
1:10,000
Mock
PMA
NETs
B
Keratinocytes (NHEK) 2D culture
R848
ssRNA
NETs


C
Unstimulated
Mock NETs + RNase inhib.
PMA NETs
PMA NETs + RNase inhib.


D
3D human skin equivalent
IL-8 [pg/ml]
Unstim.
Mock
PMA
RNase inh.
NETs


E
3D human skin equivalent
CXCL8 mRNA expression
RQ (fold change)
Unstim.
Mock
PMA
RNase inh.
NETs

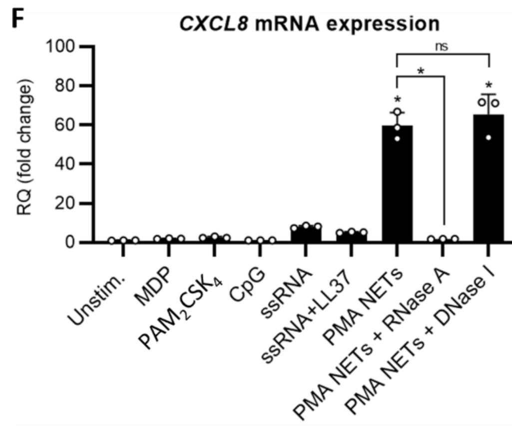

F
CXCL8 mRNA expression
RQ (fold change)
ns
Unstim.
MDP
PAM2CSK4
CpG
ssRNA
ssRNA+LL37
PMA NETs
PMA NETs + RNase A
PMA NETs + DNase I

Figure 7: Keratinocytes react in a dose dependent manner to naRNA

(**A**) N/TERT-1 keratinocytes were stimulated for 24 h with PMA (25 µg/mL) + Ionomycin (0.375 µg/mL), TL8 (200 ng/mL), ssRNA+LL37 or Mock/PMA NETs with or without RNase inhibitor in different dilutions. Combined data of triplicate IL-8 cytokine ELISA (n = 3, mean + SD, each dot represents one biological replicate, *p<0.05 according to one-way ANOVA). (**B**) Primary normal human epidermal keratinocytes (NHEK) were stimulated for 24 h with R848 (20 µg/mL), TL8 (200 ng/mL), ssRNA+LL37, Mock NETs with RNase inhibitor (1:25 dilution) or PMA NETs with or without RNase inhibitor (1:25 dilution). Combined data of triplicate IL-8 cytokine ELISA (n = 3, mean + SD, each dot represents one biological replicate, *p<0.05 according to one-way ANOVA). (**C**) H&E staining of 3D human skin equivalent after stimulation for 24 h with Mock NETs with RNase inhibitor or PMA NETs with or without RNase inhibitor. (**D**) 3D human skin equivalents were stimulated for 24 h with Mock NETs with RNase inhibitor or PMA NETs with or without RNase inhibitor. Representative data of triplicate IL-8 cytokine ELISA (n = 3, mean + SD, each dot represents one technical replicate, *p<0.05 according to one-way ANOVA). (**E**) 3D human skin equivalents were stimulated for 24 h with Mock NETs with RNase inhibitor or PMA NETs with or without RNase inhibitor. Representative data of triplicate CXCL8 mRNA RT-qPCR relative to unstimulated (n = 3, mean + SD, each dot represents one technical replicate, *p<0.05 according to one-way ANOVA). (**F**) NHEKs were stimulated for 24 h with MDP (20 µg/mL), PAM_2CSK_4 (2,54 µg/mL), CpG2006 (5 µM), ssRNA (5.8 µM), ssRNA+LL37 or PMA NETs with or without RNase A or DNase I digestion (1:25 dilution). Representative data of triplicate *CXCL8* mRNA RT-qPCR relative to unstimulated (n = 3, mean + SD, each dot represents one technical replicate, *p<0.05 according to one-way ANOVA). Preparation and stimulation of the 3D human skin equivalent was performed by Jasmin Scheurer, AG Schittek, Division of Dermato-oncology, Department of Dermatology, University Hospital Tübingen.

3.4 naRNA recognition in keratinocytes is MyD88-, TRIF-, NLRP1-, MAVS- and RNase7-independent

Keratinocytes express a broad range of PRRs and TLRs to sense PAMPs and DAMPs (Miller 2008; Rahmani and Rezaei 2016; Kumar 2021). Given that naRNA is a potent activator of proinflammatory responses in keratinocytes and that in neutrophils and macrophages naRNA is sensed TLR8-dependently, the next step was to investigate how naRNA is sensed in keratinocytes. Therefore, sensing of naRNA via TLR8 in keratinocytes was investigated via a so-called 'inhibitory oligonucleotide' (iODN), called IRS661, which is a TLR7 and TLR8 inhibitor (Herster et al. 2020). By adding it to N/TERT-1 cells 1 h before the stimulation, sensing of TLR7 and TLR8 was supposed to be inhibited. For stimulation PAM_2CSK_4 and PMA + Ionomycin as non TLR ligands, ssRNA + LL37 as a RNA ligand, TL8 as a TLR8 ligand and PMA NETs with or without RNase inhibitor in a 1:100 dilution were applied for 24 h to N/TERT-1 cells. The IRS661 inhibitor led to no inhibition of these applied stimuli's except a slight reduction after ssRNA + LL37 stimulation. Contrary, it even increased IL-8 release measured by cytokine ELISA for both PMA NETs (Figure 8A).

Either the IRS661 inhibitor did not work on N/TERT-1 or TLR7 and TLR8 are not involved in the sensing of these ligands. In general the expression in human keratinocytes in a non-inflammatory setting seems low (Li et al. 2013)) and was confirmed by RNAseq (data not shown shared by M. Orgazalli, UMass Med, Worcester, USA). To investigate a dependence on TLR signaling in general, the next step was to target MyD88, an adaptor that is essential for all TLRs except TLR3 (Deguine and Barton 2014). For this a knockdown (KD) of MyD88 was generated using a siRNA and a control siRNA was used to account for off-target effects. Immunoblot confirmed the protein level of MyD88 in N/TERT-1 was strongly downregulated in the specific knockdown condition (Figure 8B), which means that the siRNA created a sufficient knockdown of MyD88. Stimulation of these MyD88 knockdown N/TERT-1 cells with PMA +

Ionomycin, ssRNA + LL37, TL8 or PMA NETs with or without RNase inhibitor nevertheless did not lead to any downregulation of the IL-8 release of any ligand (Figure 8C).

These suggests that MyD88 is not involved in NET sensing in N/TERT-1. But N/TERT-1 are an immortalized cell line and therefore can be different to primary cells. The next step was therefore to repeat the MyD88 knockdown in primary keratinocytes, i.e. NHEK cells. The knockdown in the NHEK cells was created as in the N/TERT-1 with siRNA and the protein levels were downregulated as previously (Figure 8D). The stimulation of these MyD88 knockdown NHEK cells led to similar results compared to MyD88 KD N/TERT-1 results (Figure 8E). Specifically, no significant differences were observed and only the IL-8 concentrations after PAM_2CSK_4 stimulation were slightly reduced as expected, whereas ssRNA+LL37 stimulation led to increased IL-8 secretion. These suggests that keratinocytes sense these RNA ligands through a MyD88-independent and hence TLR7/8-independent pathway.

To exclude the possibility that NET sensing is employed via TLR3, the only TLR not targeted by MyD88 KD, and to find possible alternative pathways, e.g the RIG-I/MDA-5/MAVS (Wicherska-Pawłowska, Wróbel, and Rybka 2021) or NLRP1 (Bauernfried et al. 2021) as ss- or dsRNA sensors, that are responsible, a preliminary screening with different knockdowns created with siRNA were done once. Therefore, TRIF, MAVS and NLRP1 were knocked down in NHEKs and stimulated as before. The TRIF knockdown NHEK cells responded with higher IL-8 release, most of them significantly increased, to all ligands (Figure 8F). This suggests a non-specific effect of the siRNA and does not lead to any conclusion about TRIF in the involvement of naRNA sensing. The stimulation of MAVS knockdown NHEKs did not lead to any differences in the IL-8 release (Figure 8G) except Poly I:C compared to the negative siRNA control. Poly I:C is sensed via RIG-I, which then signals through MAVS, what indicates that a functional KD of MAVS was indeed created. This suggests that MAVS is not involved in sensing NETs/naRNA which activated the cells normally. NLRP1 can sense double-stranded RNA and an activation results in a release of IL-1β (Bauernfried et al. 2021). The NLRP1 knockdown NHEKs were stimulated with Val-boroPro (VbP), a known activator of NLRP1, as a control ligand. The NLRP1 KD cells did not release IL-1β after VbP stimulation, which suggests that this knockdown was successful as well, but it had no impact on naRNA sensing as the PMA NETs did not lead to an IL-1β release (Figure 8H). Additionally, secretion of IL-8 was only significantly reduced in the NLRP1 KD after stimulation with VbP, Poly I:C and Poly I:C in complex with DOTAP (Figure 8I).

The knockdown of MAVS and NLRP1 suggests that NETs do not contain dsRNA and the naRNA within those NETs is not modified into double-stranded RNA for sensing through these pathways. Furthermore, the MyD88 knockdown demonstrates that the sensing of naRNA is independent of TLR signaling including TLR8. Keratinocytes have to sense the naRNA through a different pathway than the neutrophils and macrophages.

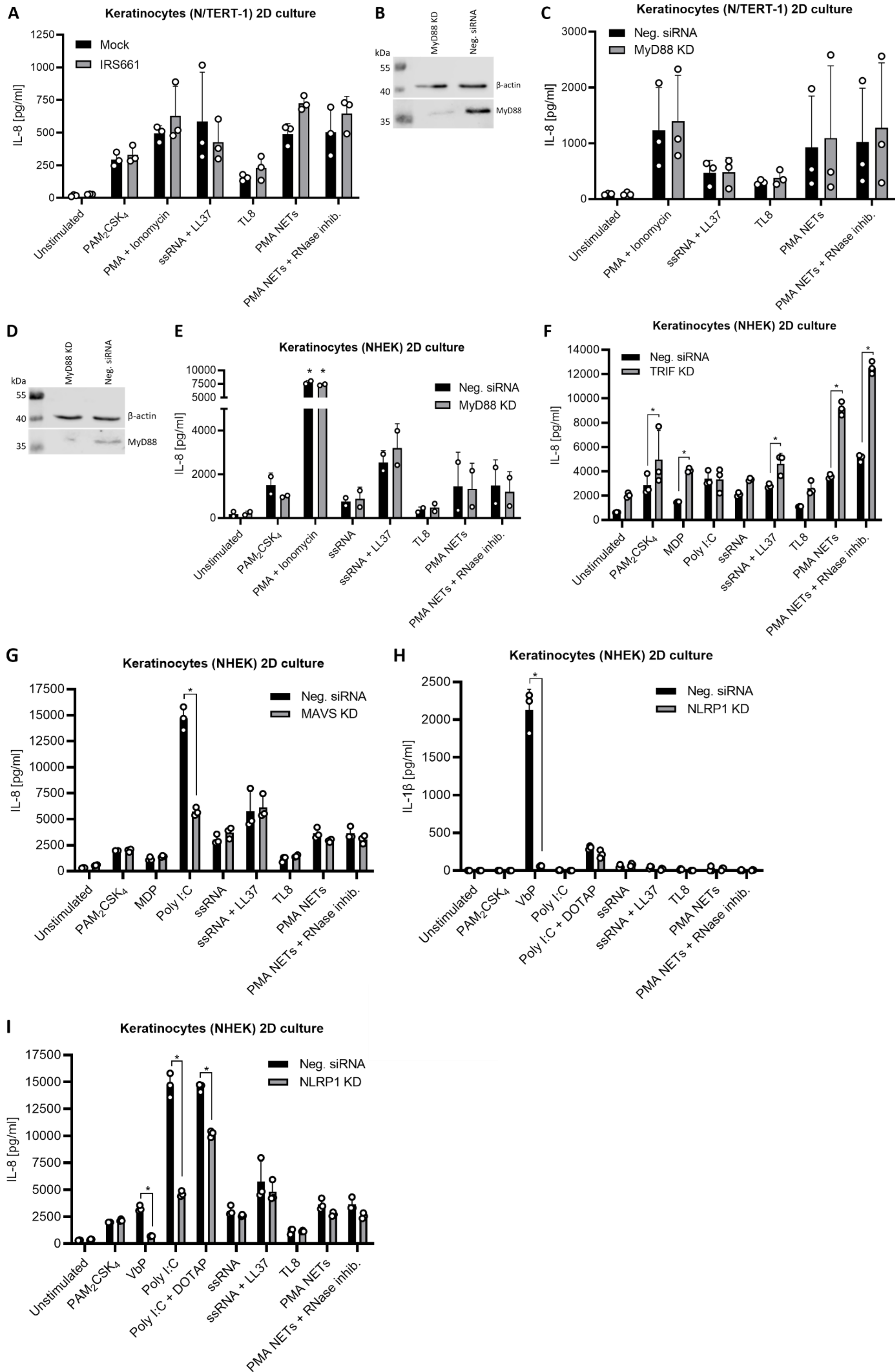

A
Keratinocytes (N/TERT-1) 2D culture
Mock
IRS661
IL-8 [pg/ml]
Unstimulated
PAM2CSK4
PMA + Ionomycin
ssRNA + LL37
TL8
PMA NETs
PMA NETs + RNase inhib.
B
MyD88 KD
Neg. siRNA
kDa
β-actin
MyD88
C
Keratinocytes (N/TERT-1) 2D culture
Neg. siRNA
MyD88 KD
D
E
Keratinocytes (NHEK) 2D culture
ssRNA
F
Keratinocytes (NHEK) 2D culture
TRIF KD
MDP
Poly I:C
G
MAVS KD
H
IL-1β [pg/ml]
NLRP1 KD
VbP
Poly I:C + DOTAP
I

Figure 8: Keratinocytes sense naRNA in a MyD88-, TRIF-, NLRP1-, MAVS- and RNase7 independent manner
(**A**) N/TERT-1 keratinocytes were stimulated for 24 h in the presence with 1 h preincubation or absence of IRS661 (500 nM) with PAM_2CSK_4 (2,54 µg/mL), PMA (25 µg/mL) + Ionomycin (0.375 µg/mL), ssRNA+LL37, TL8 (200 ng/mL), or PMA NETs with or without RNase inhibitor (1:100 dilution). Combined data of triplicate IL-8 cytokine ELISA (n = 3, mean + SD, each dot represents one biological replicate). (**B**) Representative immunoblot of N/TERT-1 lysed cells after transfection of MyD88 siRNA or negative siRNA as control (3,5 nM). Stained for β-actin as loading control and total MyD88 protein. (**C**) Knockdown (KD) of MyD88 created with siRNA (3,5 nM) in N/TERT-1 cells and stimulated for 24 h with PMA (25 µg/mL) + Ionomycin (0.375 µg/mL), ssRNA+LL37, TL8 (200 ng/mL), or PMA NETs with or without RNase inhibitor (1:100 dilution). Combined data of triplicate IL-8 cytokine ELISA (n = 3, mean + SD, each dot represents one biological replicate). (**D**) Representative immunoblot of NHEK lysed cells after transfection of MyD88 siRNA or negative siRNA as control (3,5 nM). Stained for β-actin as loading control and total MyD88 protein. (**E-I**) Knockdown (KD) of MyD88 (3,5 nM), TRIF (3,5 nM), MAVS (10 nM) or NLRP1 (10 nM) created with siRNA in NHEKs and stimulated for 24 h with PAM_2CSK_4 (2,54 µg/mL), MDP (20 µg/mL), VbP (1 µM), Poly I:C (50 µg/mL), PMA (25 µg/mL) + Ionomycin (0.375 µg/mL), ssRNA (5.8 µM), ssRNA+LL37, TL8 (200 ng/mL), or PMA NETs with or without RNase inhibitor (1:25 dilution). Data of triplicate IL-8 or IL-1β cytokine ELISA (n = 1, mean + SD, each dot represents one technical replicate, *p<0.05 according to two-way ANOVA).

3.5 NOD2-RIPK2 signaling mediates sensing of naRNA in primary keratinocytes

Based on these results, we concluded that keratinocytes detect naRNA TLR-independently but through which receptor it is sensed needed to be clarified. The next target to test was nucleotide-binding oligomerization domain 2, short NOD2, which is reported to sense bacterial peptidoglycan structures like MDP (Strober et al. 2006) but, unconventionally, also RNA (Lupfer et al. 2013; Sabbah et al. 2009).

To address this, a knockdown of NOD2 with siRNA in NHEK cells was performed as before and the cells stimulated with PAM_2CSK_4 as NOD2-independent ligand, MDP as a known NOD2 ligand and positive control, and with ssRNA alone, ssRNA in complex with LL37, Mock NETs + RNase inhibitor and PMA NETs pre-treated with or without RNase inhibitor. To determine if the KD was sufficient, the *NOD2* expression was determined. The control KD was normalized to 1 and the NOD2 KD showed an expression rate of 0.4-fold change in all three biological replicates (Figure 9A). It was also tried to determine the change in NOD2 proteins levels but the quality of the antibodies during the immunoblot was not sufficient. After 24 h of stimulation the IL-8 concentrations were measured (Figure 9B). It was observed that ssRNA alone or in complex with LL37 as well as PMA NETs with or without RNase inhibitor led to significant reduced IL-8 release in the NOD2 KD condition compared to the control KD. MDP as control ligand for NOD2 was reduced in the NOD2 KD but not significantly as the response in general was low.

Additionally, after 24 h stimulation of NOD2 KD NHEK cells RNA was isolated to measure mRNA expression levels of *NOD2* and *CXCL8*. These results were not as clear as for the cytokine ELISA. The *NOD2* mRNA expression was significantly reduced for all NOD2 KD samples compared to the negative siRNA control (Figure 9C). Moreover, the results for *CXCL8* mRNA levels differ between the experiments. For one experiment the *CXCL8* mRNA levels were significantly reduced for MDP and PMA NETs with or without stabilized naRNA (Figure 9D). In another experiment, ssRNA alone or in complex with LL37 and PMA NETs without RNase inhibitor had significantly reduced expressions of *CXCL8* mRNA in the NOD2 KD but significantly increased expression levels after stimulation with PMA NETs with stabilized naRNA (Supplementary figure 1B). In a third experiment, the stimulation of NOD2 KD cells with ssRNA and PMA NETs led to significantly reduced *CXCL8* mRNA levels but also significantly increased level after ssRNA+LL37 stimulation. Furthermore, the levels after PMA NETs + RNase inhibitor were reduced but not significantly (Supplementary figure 2B). These three experiments

suggest an involvement of NOD2 in the sensing of ssRNA and naRNA, but these data are not completely clear for the mRNA levels of *CXCL8* after 24 h of stimulation.

To validate these findings, we focused on the NOD2 signaling pathway. The kinase RIPK2 interacts directly with NOD2 downstream in the signaling pathway (Caruso et al. 2014) and can be inhibited with the ATP-competitive inhibitor, GSK583. For the activation of the keratinocytes the IL-8 release was measured via cytokine ELISA of inhibitor-treated vs -untreated cells (Figure 9E). For the stimulation with ssRNA and PMA NETs with and without RNase inhibitor IL-8 release was significantly reduced in GSK583 inhibitor-treated NHEKs. Additionally, MDP stimulation led to reduced IL-8 release during GSK583 inhibition, but this reduction was not statistically significant. The response to the control ligand PAM_2CSK_4 was not affected from the inhibition. These data suggest an involvement of the NOD2-RIPK2 pathway in the sensing of naRNA.

Taken together, the cytokine ELISAs of the NOD2 KD and RIPK2 inhibition together with the qPCR for *CXCL8* mRNA, show an involvement of NOD2 in the sensing of NETs. As this sensing of NETs is naRNA dependent, as previously shown, the data leads to the suggestion that the NOD2-RIPK2 pathway mediates the recognition of naRNA.

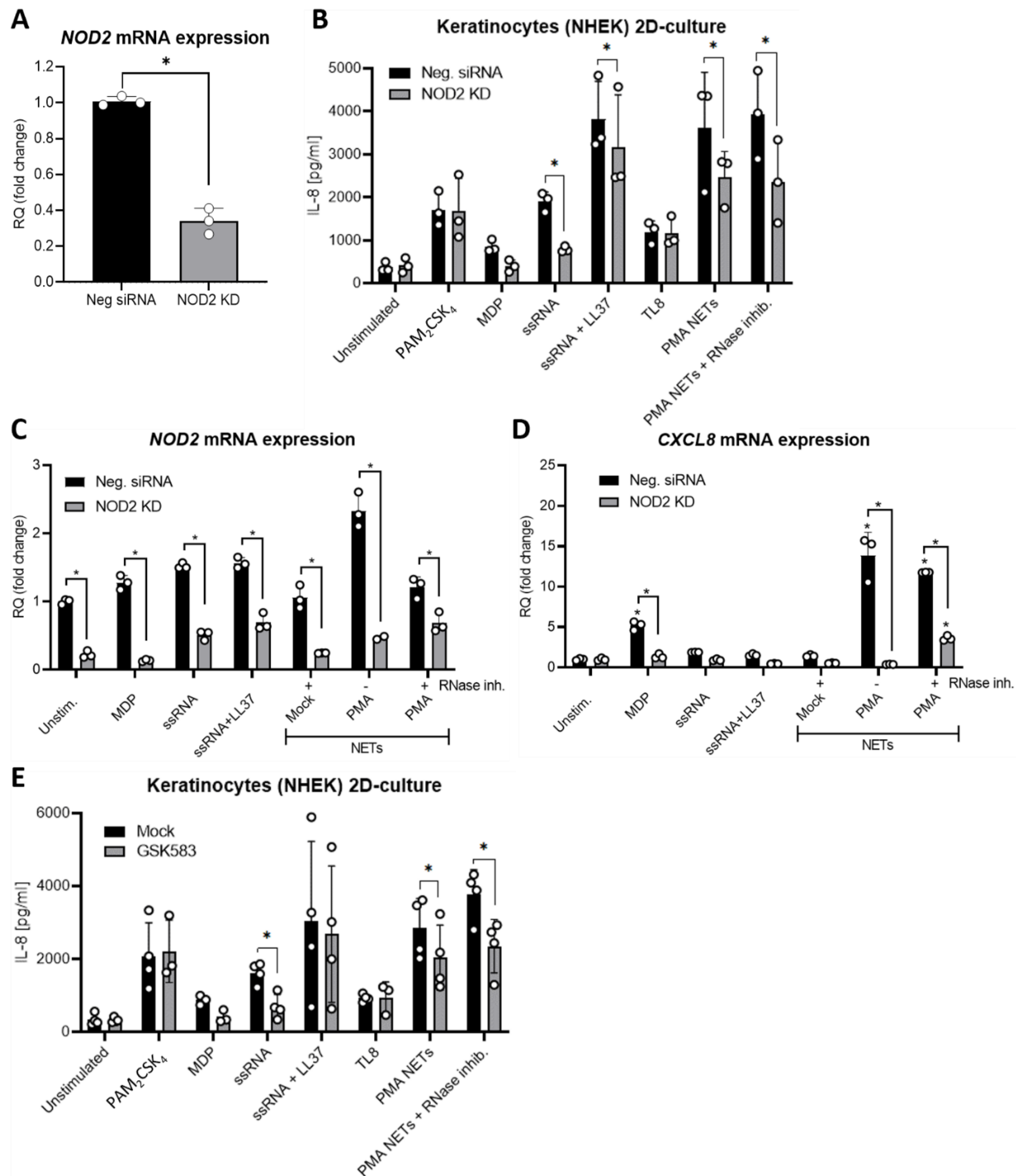


Figure 9: NHEKs sense naRNA via the NOD2-RIPK2 pathway

(**A**) Knockdown (KD) of NOD2 created with siRNA (10 nM) in NHEKs and stimulated for 24 h with PAM_2CSK_4 (2,54 µg/mL), MDP (20 µg/mL), ssRNA (5.8 µM), ssRNA+LL37, TL8 (200 ng/mL), or PMA NETs with or without RNase inhibitor (1:25 dilution). Combined data of triplicate IL-8 cytokine ELISA (n = 3, mean + SD, each dot represents one biological replicate, *p<0.05 according to two-way ANOVA). (**B**) NHEKs were stimulated for 24 h in the presence or absence of RIPK2 inhibitor GSK583 (1 µM) with PAM_2CSK_4 (2,54 µg/mL), MDP (20 µg/mL), ssRNA (5.8 µM), ssRNA+LL37, TL8 (200 ng/mL), or PMA NETs with or without RNase inhibitor (1:25 dilution). Combined data of triplicate IL-8 cytokine ELISA (n = 4, mean + SD, each dot represents one biological replicate, *p<0.05 according to two-way ANOVA). (**C-D**) NHEKs were stimulated for 24 h with MDP (20 µg/mL) + Ionomycin (0.375 µg/mL), ssRNA (5.8 µM), ssRNA+LL37, Mock NETs with RNase inhibitor (1:50) or PMA NETs with or without RNase inhibitor (1:50 dilution). Representative data of triplicate *NOD2* or *CXCL8* mRNA RT-qPCR relative to unstimulated (n = 3, mean + SD, each dot represents one technical replicate, *p<0.05 according to two-way ANOVA).

3.6 naRNA induces a psoriasis-associated gene signature

It is known that NETs are highly abundant in skin lesions of psoriasis and that the frequency of NETs correlates with the severity of the disease (Chiang et al. 2019; Wang and Jin 2020). Furthermore, keratinocytes are a main producer of proinflammatory cytokines in the skin and are involved in the pathogenesis of psoriasis. Given that the naRNA within the NETs can activate keratinocytes in the form of IL-8 release, it was hypothesized that NETs and especially naRNA might lead to upregulated expression of psoriasis-associated genes and thereby promote psoriasis. This hypothesis was tested next.

3.6.1 Induction of psoriasis-associated genes is naRNA-dependent

To address these open questions, NHEKs were stimulated with MDP, ssRNA or ssRNA + LL37 complexed as RNA ligands, CpG as DNA control ligand, PMA NETs undigested or digested with RNase A or DNase I and PAM_2CSK_4 as TLR2 ligand that is RNA- and DNA-independent. After 24 h of stimulation the RNA was isolated and the mRNA expression levels of genes of interest, which can be linked to psoriasis were analyzed. Therefore, the expression for each gene was normalized to an unstimulated control and is shown as fold change expression. Due to working with primary cells, it was not entirely surprising that results differ between biological replicates, but they have the same trend. Therefore, one representative of three individual experiments for each gene is shown (Figure 10A-D). The expression data for the analyzed genes showed for *IL17C, IL36G (*cytokines relevant for psoriasis (Rendon and Schäkel 2019)), *CAMP and RNASE7* (antimicrobial peptides typically elevated in psoriasis (Patra et al. 2018; Rendon and Schäkel 2019)) mRNA levels that PMA NETs induced a strong expression. Additionally, digestion of NETs with RNase A led to a greatly reduced expression of all four genes. This reduction for *IL17C*, *CAMP* and *RNASE7* was significant, whereas for *IL36G* the expression goes down from 43-fold increase to 2.7-fold increase although not statistically significantly. Moreover, digestion with DNase I showed comparable results for *IL17C* and *IL36G* as with undigested NETs but for *CAMP* and *RNASE7* the expression increased significantly. Furthermore, the NHEKs also responded with high gene expression for *IL17C*, *IL36G* and *RNASE7* to ssRNA alone, which is comparable to ssRNA in complex to LL37. This could mean that RNA sensing by keratinocytes induces psoriasis-related gene expression but that, unlike for neutrophils, RNA does not need to be precomplexed with LL37 to be recognized by the NHEKs.

These demonstrates that naRNA, released in the psoriatic skin by NETing neutrophils, could drive the activation of several psoriasis-associated genes in keratinocytes that could fuel the disease.

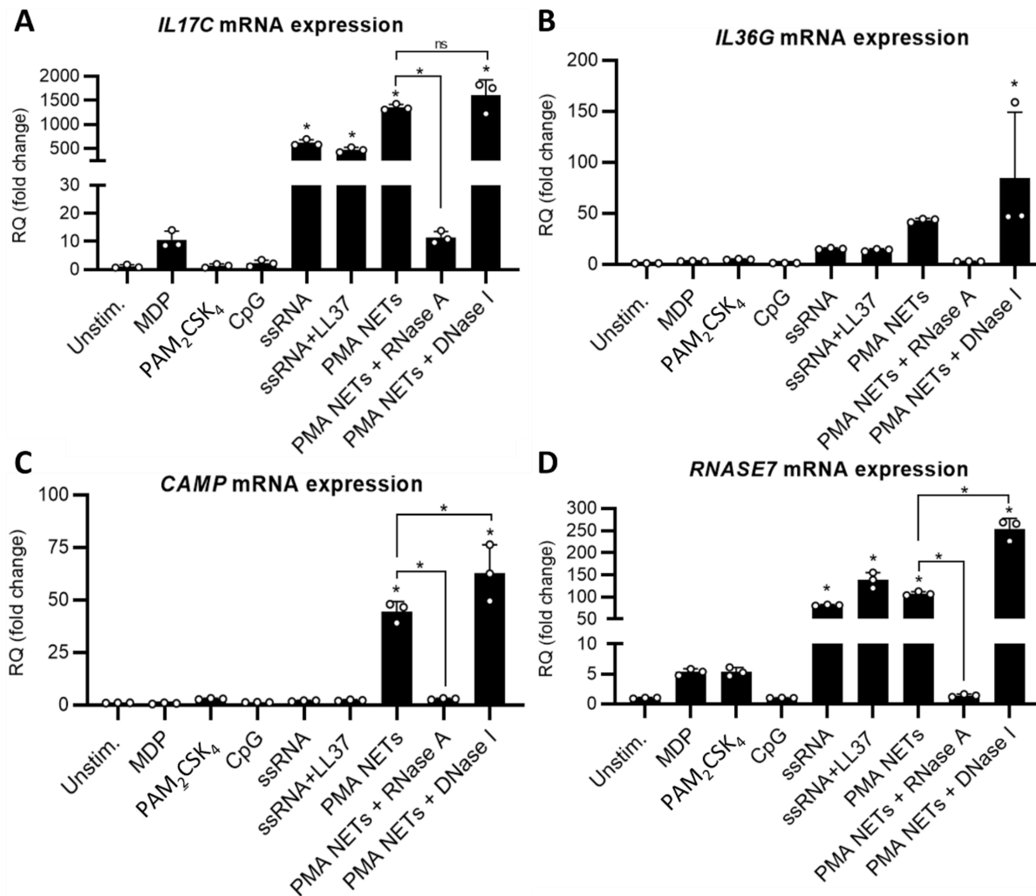


Figure 10: naRNA dependent induction of psoriasis associated genes in NHEKs
(**A-D**) NHEKs were stimulated for 24 h with MDP (20 µg/mL) + Ionomycin (0.375 µg/mL), PAM_2CSK_4 (2,54 µg/mL), CpG2006 + DOTAP (5 µM), ssRNA (5.8 µM), ssRNA+LL37 or PMA NETs with or without RNase A or DNase I digestion (1:25 dilution). Representative data of triplicate *IL17C, IL36G, CAMP* or *RNASE7* mRNA RT-qPCR relative to unstimulated (n = 3, mean + SD, each dot represents one technical replicate, *p<0.05 according to one-way ANOVA).

3.6.2 Upregulation of psoriasis-associated genes in a 3D human skin equivalent

The previous results of psoriasis-associated gene induction were obtained in 2D culture and should next be verified in a physiologically more relevant context. Therefore, the previously tested 3D human skin equivalents were re-tested for *IL17C*, *IL36G*, *CAMP* and RNASE7 expression (Figure 11A-D). As before for monolayer stimulation, the genes of interest were analyzed and values were normalized to unstimulated. The stimulation with PMA NETs + RNase inhibitor led to a significantly increased gene expression of *IL17C*. Conversely, *IL36G* was only slightly elevated after stimulation with PMA NETs + RNase inhibitor but significantly increased after PMA NETs stimulation. Furthermore, both PMA NETs increased *CAMP* gene expression to about 7.2-fold increase, but not significantly with p-values of 0.0545 for PMA NETs and 0.0548 for PMA NETs with RNase inhibitor. In contrast, *RNASE7* gene expression remains at the level of unstimulated and only slightly increased to about 1.2-fold increase with PMA NETs with RNase inhibitor.

Taken together these results demonstrate that in a 3D human skin equivalent that is able to physiologically represent human skin, naRNA activates keratinocytes and induces expression of psoriasis associated genes like *IL17C*, *IL36G* and *CAMP* and thereby strengthen the suggestion of naRNA driven psoriasis-associated gene expression, even if they are not as consistent as obtained from 2D monolayer keratinocytes.

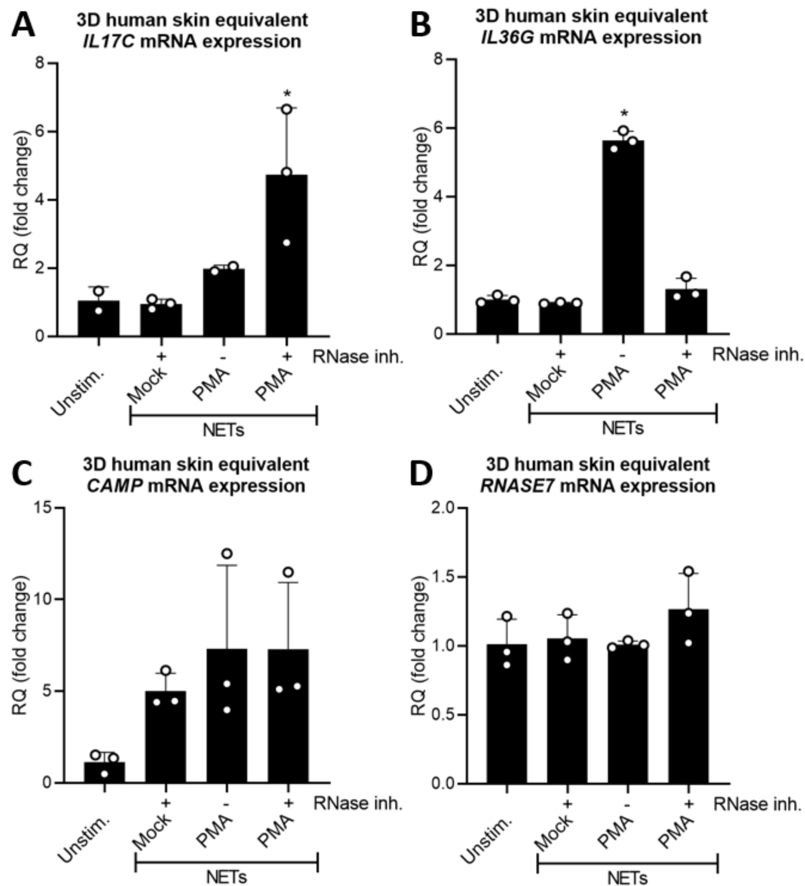


Figure 11: NETs upregulate psoriasis-associated genes in 3D human skin equivalent
(**A-D**) 3D human skin equivalents were stimulated for 24 h with Mock NETs with RNase inhibitor or PMA NETs with or without RNase inhibitor. Representative data of triplicate *IL17C*, *IL36G, CAMP* or *RNASE7* mRNA RT-qPCR relative to unstimulated (n = 3, mean + SD, each dot represents one technical replicate, *p<0.05 according to one-way ANOVA). Preparation and stimulation of the 3D human skin equivalent was performed by Jasmin Scheurer, AG Schittek, Division of Dermato-oncology, Department of Dermatology, University Hospital Tübingen.

3.6.3 NOD2 mediates induction of psoriasis-associated genes by naRNA

It could be demonstrated separately that naRNA is sensed by a NOD2-RIPK2 dependent pathway in keratinocytes and that naRNA can lead to psoriasis-associated gene expression. Activation of NOD2 is reported to lead to induced expression of genes like *IL17C* and *IL36G* (Roth et al. 2014; Shao, Fang, and Wang 2018). Due to this, it was hypothesized that the naRNA sensing via NOD2 directly mediates the expression of the psoriasis associated genes after naRNA recognition.

To test this hypothesis, the previous NOD2 KD experiments were retested for the psoriasis-related genes of interest. As before, the results were not completely consistent. One experiment showed that *IL17C* and *IL36G* were induced after MDP and especially both PMA NETs (Figure 12A + B). The NOD2 KD decreased the induction after MDP and PMA NETs without RNase inhibitor completely. For PMA NETs with stabilized naRNA the expression was significantly decreased from 1204-fold increase to 120-fold increase. This means it was still expressed but 10-fold less in the NOD2 KD. The same could be observed for *IL36G* that was significantly decreased for both PMA NETs, even though PMA NETs with stabilized naRNA was not completely abolished in the NOD2 KD. In addition, the expression of *CAMP* was also greatly increased after both PMA NETs but not after MDP stimulation (Figure 12C). The NOD2 KD decreased this expression significantly for both PMA NETs. The expression pattern of *RNASE7* was

different as both PMA NETs and also the ssRNA with or without LL37 induced *RNASE7* expression (Figure 12D). But in the NOD2 KD the PMA NETs without RNase inhibitor samples were significantly reduced, whereas PMA NETs with RNase inhibitor significantly increased the expression of *RNASE7*. In another experiment, the pattern of *IL17C* induction was comparable (Supplementary figure 1C). But in the third only after MDP stimulation the *IL17C* expression in the NOD2 KD was significantly reduced, whereas after ssRNA+LL37 the expression was significantly increased (Supplementary figure 2C). Furthermore, PMA NETs without RNase inhibitor did not even induce expression and after stimulation with naRNA stabilized PMA NETs a significant increased expression was measured but no differences between the control and NOD2 KD. For *IL17C* expression this means that all three experiments showed an induction of *IL17C* after PMA NETs with RNase inhibitor but only two out of three showed a reduction by the NOD2 KD. The expression of *IL36G* shows a comparable trend, as the second experiment shows a significant reduction of *IL36G* expression after ssRNA with or without LL37 and PMA NETs but not after PMA NETs with RNase inhibitor (Supplementary figure 1D). In contrast, the third experiment showed a significant reduced expression in the NOD2 KD after naRNA stabilized PMA NETs and MDP stimulation but not after the stimulation with the other ligands (Supplementary figure 2D). So that also for *IL36G* only two out of three showed a significant reduction in the NOD2 KD after PMA NETs with RNase inhibitor stimulation. This also applies to *CAMP* expression, as the second experiment showed the same trend as the first one (Supplementary figure 1E) but in the third the expression of *CAMP* in the NOD2 KD was significantly elevated after naRNA stabilized PMA NETs stimulation (Supplementary figure 2E). Additionally, this can also be observed for *RNASE7* expression, as in the second experiment the expression was significantly elevated (Supplementary figure 1F) and in the third significantly downregulated in the NOD2 KD after PMA NETs with RNase inhibitor stimulation (Supplementary figure 2F).

Collectively, for the measured *IL17C*, *IL36G*, *CAMP* and *RNASE7* mRNA levels two out of three experiments showed a significant reduction in case of *IL17C*, *IL36G* and *CAMP* or a significant increase for *RNASE7*. The reasons for these variations are not entirely clear but this nevertheless leads to the suggestion, that *RNASE7* expression is induced by naRNA but not through NOD2. Furthermore, *IL17C*, *IL36G* and *CAMP* seem to be induced by NETs in a NOD2 mediated pattern, but this needs further clarification.

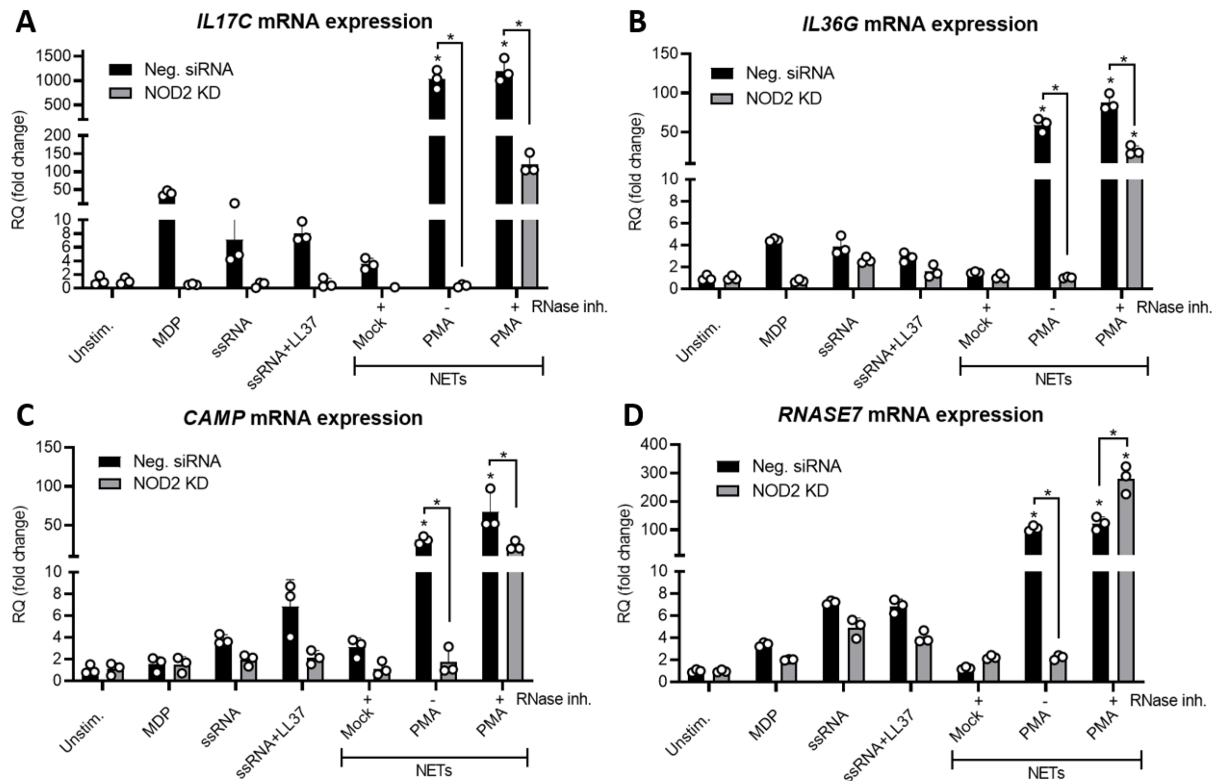


Figure 12: naRNA induced psoriasis-asscociated gene expression is mediated by NOD2
(**A-D**) Knockdown (KD) of NOD2 created with siRNA (10 nM) in NHEKs. The NEHKs were stimulated for 24 h with MDP (20 µg/mL) + Ionomycin (0.375 µg/mL), ssRNA (5.8 µM), ssRNA+LL37, Mock NETs with RNase inhibitor (1:50 dilution) or PMA NETs with or without RNase inhibitor (1:50 dilution). Representative data of triplicate *IL17C, IL36G, CAMP* or *RNASE7* mRNA RT-qPCR relative to unstimulated (n = 3, mean + SD, each dot represents one technical replicate, *p<0.05 according to two-way ANOVA).

3.6.4 IκBζ is involved in naRNA sensing in human keratinocytes

Revealing that NOD2 is sensing the naRNA in keratinocytes needs to be proved further to verify the pathway that is involved. It is reported that IκBζ from keratinocytes drive psoriasis and also lead to an *IL17C* induction (Lorscheid et al. 2019). Previous results showed that NETs lead to a strong induction of *IL17C*. To link those, the activation of IκBζ after stimulation with naRNA was investigated. For this purpose, primary human keratinocytes were stimulated in the lab of our collaborator Daniela Kramer with ssRNA, Mock/PMA NETs with RNase inhibitor in the presence or absence of abemaciclib, a CDK4/6 inhibitor that inhibits IκBζ. The mRNA gene expression levels were measured after 1 h and 24 h. After 1 h the activation of IκBζ, shown by *3hgZeta* (binds to exon 6 of IκBζ) mRNA induction, was upregulated for ssRNA and PMA NETs with RNase inhibitor but not for the Mock NETs with RNase inhibitor (Figure 13A). CCNA2 is a marker for CDK4/6 inhibition that is reduced after abemaciclib treatment (Jin et al. 2018; Cornwell et al. 2023). A reduced CCNA2 induction was obtained for all samples with the inhibitor although the Mock and PMA NET samples alone already have a reduced value, which was reduced even more with the inhibitor (Figure 13B). Furthermore, the mRNA expression levels for *CXCL8* and *IL17C* were highly upregulated after naRNA stabilized PMA NET stimulation but not when the CDK4/6 inhibitor was present (Figure 13C and D). The expression of *IL36G* was not induced after 1 h, as all samples, including the unstimulated, had comparable expression rates, but these were significantly reduced in all samples with abemaciclib (Figure 13E). After 24 h the mRNA levels of *IκBζ* were significantly downregulated for the unstimulated and Mock/PMA NETs with RNase inhibitor samples

and ssRNA sample was completely abolished but this was not significant (Figure 13F). For *CXCL8* expression after 24 h ssRNA and PMA NETs with RNase inhibitor led to an upregulation and these were reduced with the CDK4/6 inhibitor for PMA NETs and completely gone for ssRNA (Figure 13H). The expression level after 24 h for *IL36G* was significantly increased for the PMA NETs with RNase inhibitor sample and this expression was significantly reduced in presence of the CDK4/6 inhibitor (Figure 13J). Only for *IL17C* after 24 h the CDK4/6 inhibitor could not reduce the high mRNA expression levels after the naRNA stabilized PMA NETs stimulation, as one of the three technical replicates was elevated (Figure 13I).

Taken together, these data indicate that naRNA leads to an activation of IκBζ, which then leads to an activation of the psoriasis associated genes *IL17C*, *IL36G* and *CXCL8*. These activation by naRNA is a fast process that already after 1 h lead to an upregulated expression of IκBζ target genes.

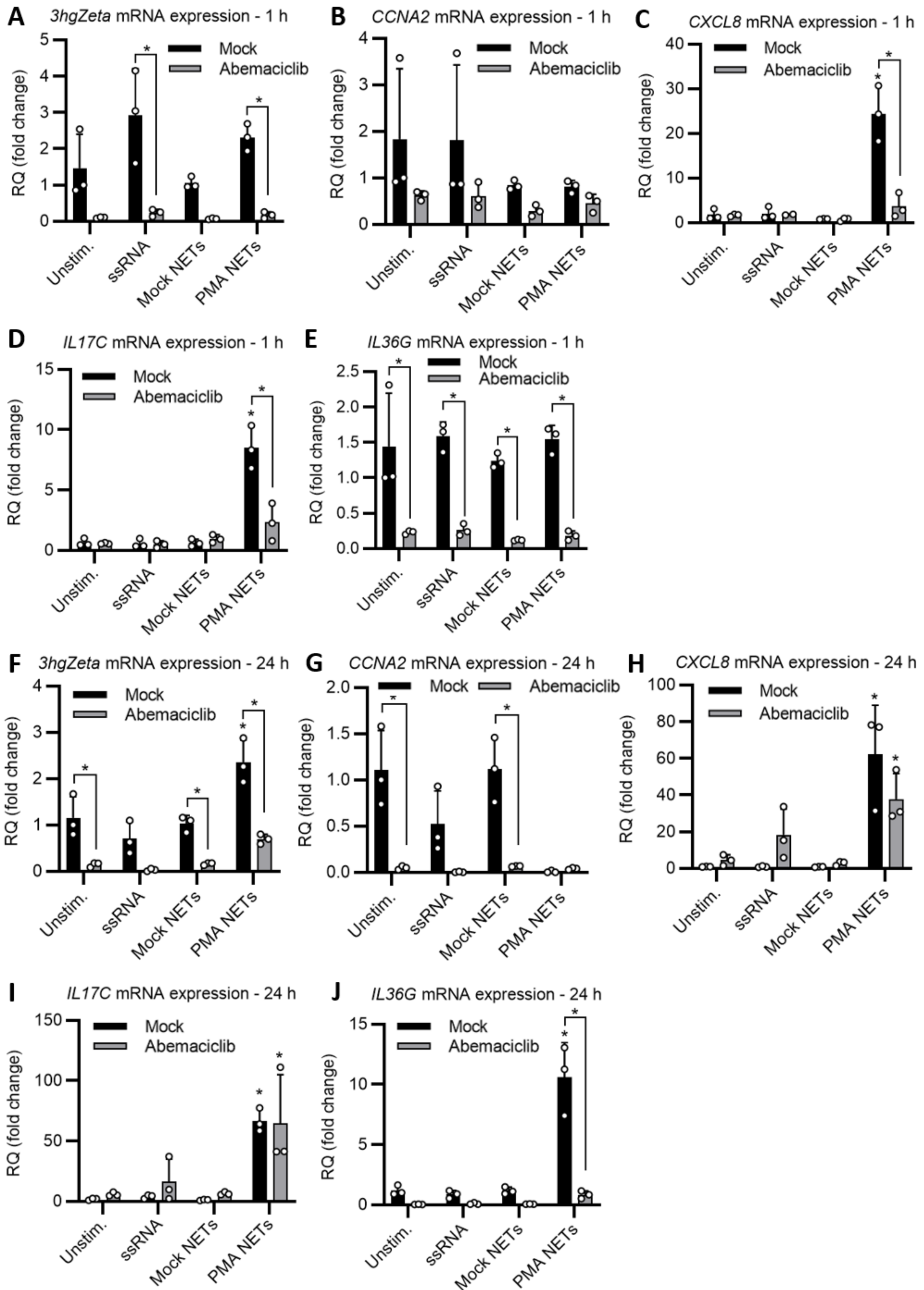

A
3hgZeta mRNA expression - 1 h
RQ (fold change)
Mock
Abemaciclib
Unstim.
ssRNA
Mock NETs
PMA NETs
B
CCNA2 mRNA expression - 1 h
C
CXCL8 mRNA expression - 1 h
D
IL17C mRNA expression - 1 h
E
IL36G mRNA expression - 1 h
F
3hgZeta mRNA expression - 24 h
G
CCNA2 mRNA expression - 24 h
H
CXCL8 mRNA expression - 24 h
I
IL17C mRNA expression - 24 h
J
IL36G mRNA expression - 24 h

Figure 13: naRNA sensing leads to IκBζ activation in human keratinocytes

(**A-E**) Primary keratinocytes were stimulated in the presence with 3 h preincubation or absence of abemaciclib for 1 h with ssRNA (5.8 µM) or Mock/PMA NETs with RNase inhibitor (1:50 dilution). Data of triplicate *3hgZeta*, *CCNA2*, *CXCL8*, *IL17C or IL36G* mRNA RT-qPCR relative to unstimulated (n = 1, mean + SD, each dot represents one technical replicate, *p<0.05 according to two-way ANOVA). (**F-J**) Primary keratinocytes were stimulated in the presence with 3 h preincubation or absence of abemaciclib for 24 h with ssRNA (5.8 µM) or Mock/PMA NETs with RNase inhibitor (1:50 dilution). Data of triplicate *3hgZeta*, *CCNA2*, *CXCL8*, *IL17C or IL36G* mRNA RT-qPCR relative to unstimulated (n = 1, mean + SD, each dot represents one technical replicate, *p<0.05 according to two-way ANOVA). Experiments with abemaciclib were performed by Berenice Fischer, PhD student of AG Kramer, Department of Dermatology, University Medical Centre of Johannes Gutenberg University Mainz.

3.6.5 Induction of psoriasis-associated genes in murine keratinocytes

So far, all experiments were done with human keratinocytes. The next step was to investigate if murine keratinocytes reacted similarly to a naRNA stimulus, also with a view to a later genetic validation. To address this, primary murine C57BL/6 WT keratinocytes isolated from the mouse tail in the lab of our collaborator Daniela Kramer in Mainz, were stimulated with ssRNA and Mock/PMA NETs with stabilized naRNA. As in the human keratinocytes, *IκBζ* mRNA levels were upregulated significantly after PMA NET stimulation in the murine keratinocytes (Figure 14A). Stimulation with ssRNA led to a significant upregulation of *Il17c, Cxcl5* and *Defb4* (Figure 14B, F and H), whereas PMA NETs with RNase inhibitor led to a significant increase of mRNA expression levels of *Il17c*, *Cxcl1*, *Cxcl2*, *Cxcl10* and *Il23a* (Figure 14B, D, E, G and I). The expression of *Il36g* was only slightly elevated after ssRNA and PMA NET stimulation. This shows that not only human but murine keratinocytes as well react to naRNA and also in murine keratinocytes *IκBζ* gets upregulated after sensing NETs, which then leads to a fast and strong upregulation of IκBζ proinflammatory target genes.

To address the question, if these naRNA mediated expression of proinflammatory genes is NOD2 dependent, primary murine C57BL/6 WT or NOD2$^{-/-}$ keratinocytes, isolated from the mouse tail in the lab of our collaborator Daniela Kramer were stimulated with ssRNA. After 1 h of stimulation the expression was determined. It was observed that the expression of *Il17c*, *Cxcl2* and *Cxcl10* was significantly decreased in the NOD2 KO keratinocytes compared to the WT keratinocytes after the ssRNA stimulation (Figure 15A, D and F). Furthermore, the expression of *Il36g* and *Defb4* was significantly increased for the WT keratinocytes after ssRNA stimulation compared to unstimulated WT cells and it was also increased for the NOD2 KO cells but not significantly (Figure 15B and G). Although the expression of *Il36g* and *Defb4* is lower in the NOD2 KO cells compared to the WT keratinocytes it was not statistically significant. In contrast, the expression of *Cxcl1*, *Cxcl5* and *Il23a* was increased after ssRNA stimulation but no differences between WT and NOD2 KO keratinocytes were observed (Figure 15C, E and H).

In summary, these data indicate that the ssRNA-mediated induction of proinflammatory genes in murine keratinocytes is NOD2-dependent, suggesting that murine and human keratinocytes react similar. But this needs to be further verified as the murine keratinocytes were only tested once.

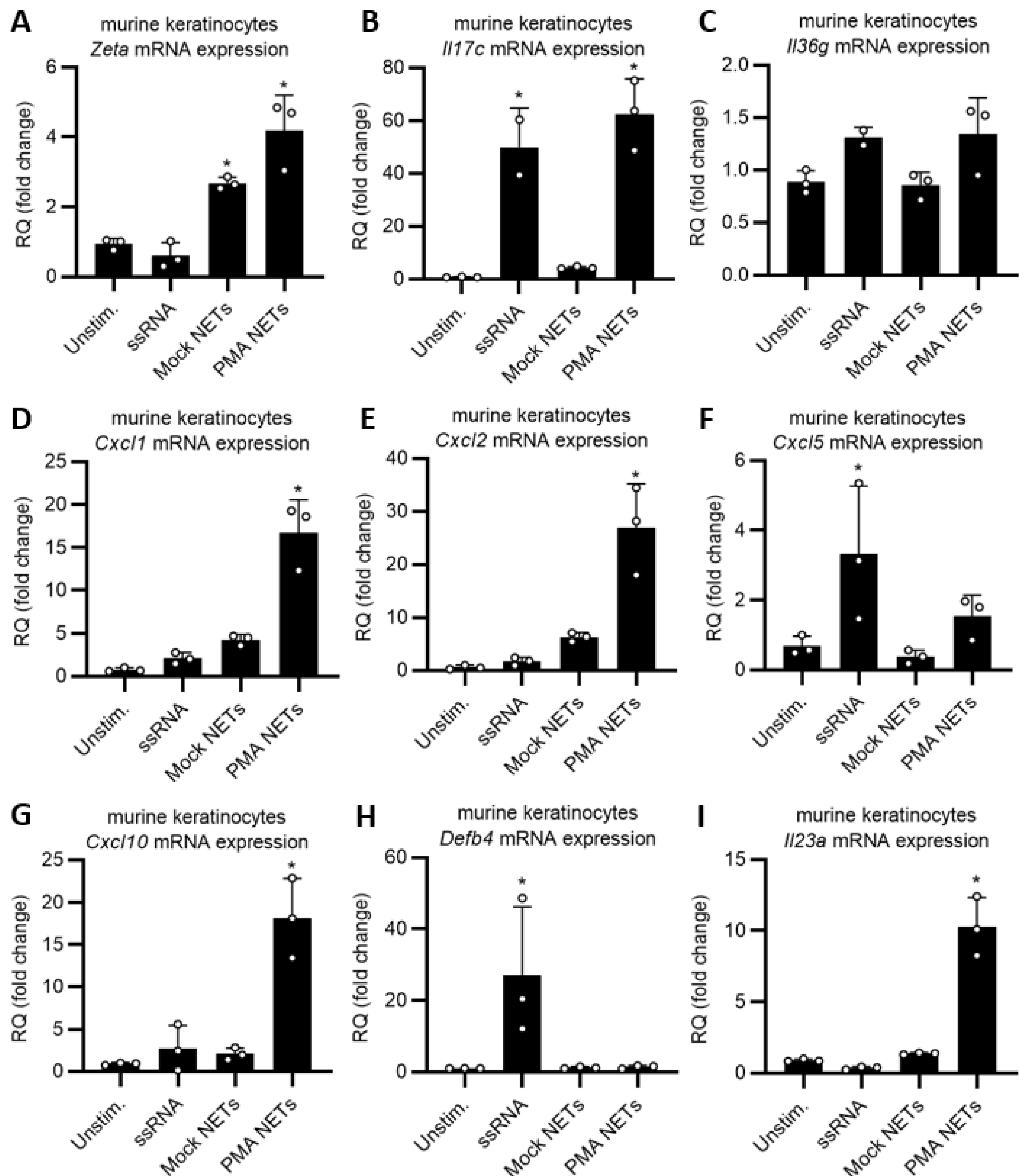


Figure 14: naRNA induces proinflammatory gene expression in murine keratinocytes

(**A-I**) Primary murine C57BL/6 WT keratinocytes were stimulated for 1 h with ssRNA (5.8 µM) or Mock/PMA NETs with RNase inhibitor (1:100 dilution). Data of triplicate *Zeta*, *Il17c*, *Il36g, Cxcl1, Cxcl2, Cxcl5, Cxcl10, Defb4* or *Il23a* mRNA RT-qPCR relative to unstimulated (n = 1, mean + SD, each dot represents one technical replicate, *p<0.05 according to one-way ANOVA). Experiments with murine keratinocytes were performed by Berenice Fischer, PhD student of AG Kramer, Department of Dermatology, University Medical Centre of Johannes Gutenberg University Mainz.

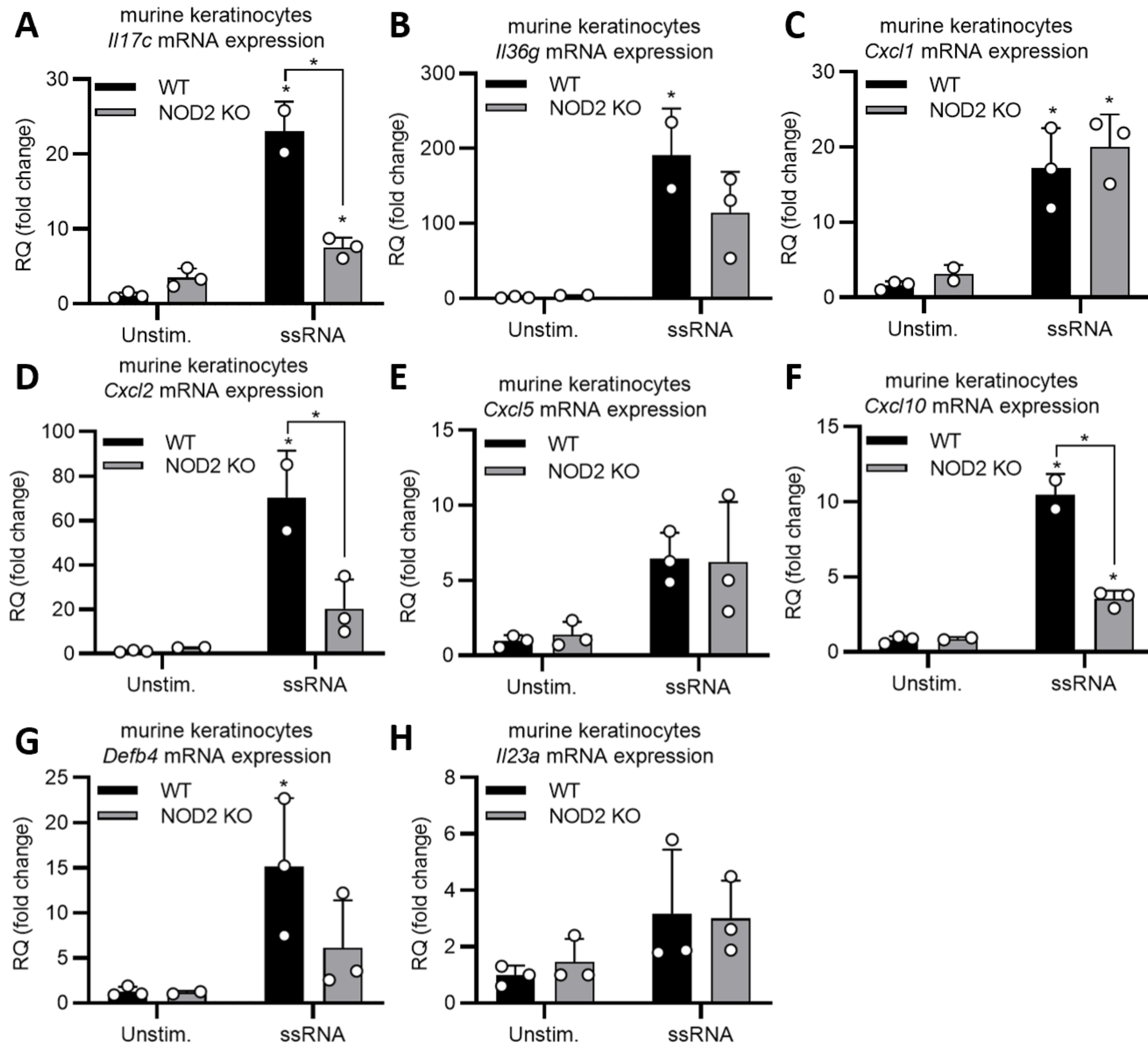


Figure 15: Murine NOD2 KO keratinocytes reaction to naRNA stimulation

(**A-H**) Primary murine C57BL/6 WT keratinocytes or primary murine NOD2$^{-/-}$ (NOD2 KO) keratinocytes were stimulated for 1 h with ssRNA (5.8 µM). Data of triplicate *Il17c*, *Il36g, Cxcl1, Cxcl2, Cxcl5, Cxcl10, Defb4* or *Il23a* mRNA RT-qPCR relative to WT unstimulated (n = 1, mean + SD, each dot represents one technical replicate, *p<0.05 according to one-way ANOVA). Experiments with murine keratinocytes were performed by Berenice Fischer, PhD student of AG Kramer, Department of Dermatology, University Medical Centre of Johannes Gutenberg University Mainz.

3.7 Outlook: Dermal fibroblasts sense NETs in a RIPK2-independent manner

The skin consists of several cell types. A strongly represented type besides the keratinocytes are fibroblasts. They not only synthesize the extracellular matrix but also collagen and play an important role in wound healing (Talbott et al. 2022). Last but not least it should be investigated if naRNA also leads to an activation of fibroblast via the NOD2-RIPK2 pathway, like in the keratinocytes.

To clarify if fibroblasts can sense NETs, if naRNA is the crucial activator in this process and if the naRNA is sensed via the NOD2-RIPK2 pathway, a fibroblast cell line called R2F/TERT-1 was used. This cell line was provided by James Rheinwald, Harvard Medical School, Boston, USA (Dickson et al. 2000). The cells were stimulated for 24 h in the presence or absence of the RIPK2 inhibitor GS583 with several control ligands, ssRNA with or without LL37, TL8, R848, MDP as NOD2 control ligand, Tri-DAP as NOD1 control

ligand and 1:50 and 1:100 dilutions of Mock and PMA NETs with or without RNase inhibitor. For cellular responsiveness the IL-8 release was measured. The obtained IL-8 values showed that the R2F/TERT-1 cells responded to PMA NET content and when the naRNA was stabilized with even higher IL-8 release (Figure 16). But the GSK583 inhibitor had no effect on this IL-8 release. In addition, the cells did not respond at all to a MDP stimulation, what could suggest that these cell line do not express the NOD2 receptor. Only the NOD1 ligand Tri-DAP led to an IL-8 release that could be significantly decreased to the background level with the GSK583 inhibitor. Moreover, none of the RNA ligands led to an activation of the R2F/TERT-1 cells.

Taken together these data demonstrate that the R2F/TERT-1 cell line can sense naRNA but in a RIPK2 independent way. It remains to be tested whether TLRs might instead play a role like in neutrophils and macrophages.

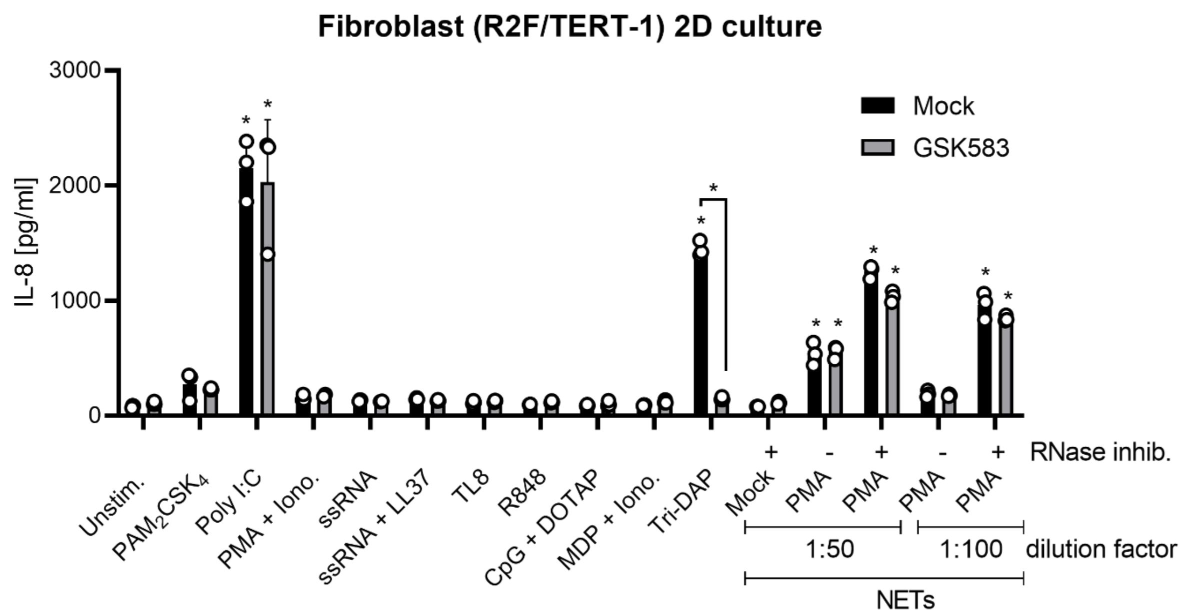


Figure 16: Fibroblast recognize NETs in a RIPK2 independent manner

R2F/TERT-1 fibroblasts were stimulated in the presence with 1 h preincubation or absence of GSK583 for 24 h with PAM_2CSK_4 (2,54 µg/mL), Poly I:C (50 µg/mL), PMA (25 µg/mL) + Ionomycin (0.375 µg/mL), ssRNA (5.8 µM), ssRNA+LL37, TL8 (200 ng/mL), R848 (10 µg/mL), CpG + DOTAP (5 µM), MDP (20 µg/mL) + Ionomycin (0.375 µg/mL), Tri-DAP (10 µg/mL), Mock NETs with RNase inhibitor (1:50) or PMA NETs with or without RNase inhibitor in different dilutions. Representative data of triplicate IL-8 cytokine ELISA (n = 3, mean + SD, each dot represents one technical replicate, *p<0.05 according to two-way ANOVA).

4. Discussion

In this study, I demonstrated that the RNA within NETs is an activator of different cell types – namely macrophages, keratinocytes and fibroblasts – associated with immune responses. In particular, proinflammatory cytokine release of macrophages partly depends on the recognition of endosomal TLR signaling, especially TLR7 and TLR8 signaling. Additionally, keratinocytes as most abundant cell type in the skin and a type of immune-like cell respond to naRNA with the initiation of psoriasis associated gene expression. It could be demonstrated that trough naRNA the expression of *IL17C*, *IL36G*, *CXCL8*, *CAMP* and *RNASE7* is increased. My findings suggest that sensing of naRNA, which leads to psoriasis-associated gene expression in these keratinocytes is mediated by the NOD2-RIPK2 pathway. A preliminary experiment also showed that IκBζ, which is reported to lead to psoriasis-associated gene expression, is activated upon sensing of naRNA in keratinocytes. Furthermore, not only human but also murine keratinocytes respond to naRNA by increased expression of various genes, like *Il17c*, *Cxcl1*, *Cxcl2*, *Cxcl10* and *Il23a*. In contrast, fibroblasts as another important cell type of the skin react to NETs, albeit not via RIPK2. These findings are summarized in figure 17, although the fibroblasts are not shown as so far it could only be shown that they react to NETs but not if this is due to naRNA and via which receptor. Additionally, the results are discussed in the following together with remaining open questions, as well as the open questions that arose from this work.

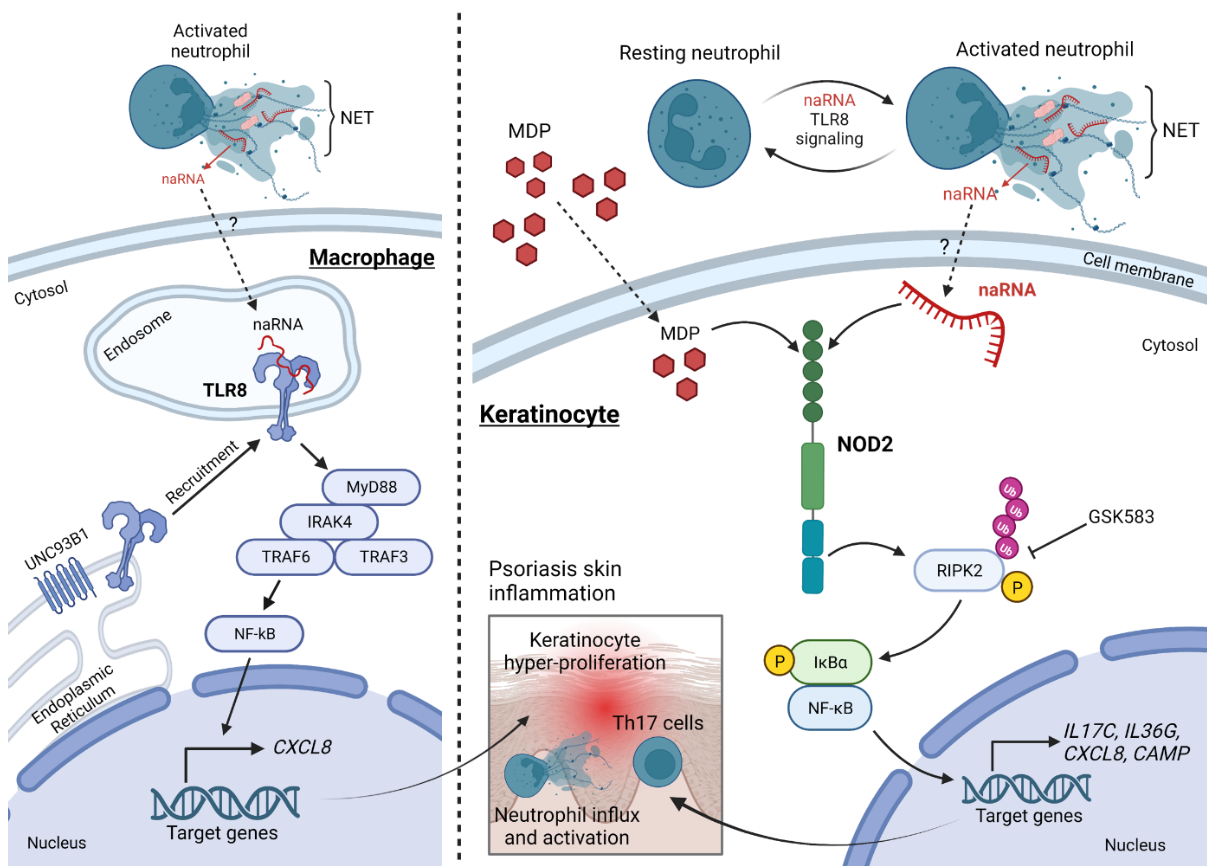


Figure 17: naRNA triggered reactions via TLR8 in macrophages and NOD2 in keratinocytes
naRNA from NETs gets transported into the cytoplasm of macrophages (left side) and keratinocytes (right side). Thereby, macrophages recognize naRNA via TLR8, which must first be recruited via UNC93B1. NaRNA mediated activation leads to signal transduction, resulting in induction of *CXCL8* expression and release of IL-8. This IL-8 secretion contributes to psoriatic skin inflammation. In keratinocytes (right side) naRNA is recognized by NOD2. This leads to a signal transduction via RIPK2, that can be inhibited by GSK583. The activation of the NOD2-RIPK2 pathway leads to induction of target genes that are associated with psoriasis, like *IL17C*, *IL36G*, *CXCL8* and *CAMP*. These cytokines and AMPs have the potential to further fuel the skin inflammation in psoriatic skin lesions by attracting and activating further immune cells, like PMNs and Th17 cells. Created with BioRender.com.

4.1 Macrophages fuel naRNA induced proinflammatory reactions

The macrophage-like cells BlaER1 and THP1 showed that macrophages respond to naRNA in a TLR7- and TLR8-dependent way by releasing high amounts of the proinflammatory cytokine IL-8 (Figure 6). Thereby, one if these RNA sensing TLRs seems to be enough to sense the naRNA and induce the proinflammatory reaction that leads to release of IL-8. This is not entirely surprising as RNAs that can activate both receptors have been described, e.g. RNA40 (Heil et al. 2004; Obermann et al. 2021). On the other hand, unlike in monocyte cell lines, primary human immune cells express either TLR7 or TLR8, but with similar specificity for the same RNA stimuli (Cervantes et al. 2012; Georg and Sander 2019). In further experiments a double knockout of $TLR7^{-/-}$ and $TLR8^{-/-}$ macrophages should be stimulated with naRNA to determine that the naRNA is only sensed by these two receptors or if other receptors are also involved in the recognition, e.g. NOD2. This was attempted but the knockout cells still responded to control ligands for TLR7 and TLR8, like TL8-506 and R848. Consequently, no statement could be made about this hypothesis so far. Additionally, IL-8 is only one of many cytokines that can be produced by macrophages. Which cytokines get also triggered to be released after sensing of naRNA needs to be further evaluated to determine which role the sensing of naRNA by macrophages could play in disease progression. Therefore, the expression of target genes of the TLR7 and TLR8 pathway could be analyzed by RT-qPCR and the amount of the released cytokine could be determined by ELISA or a cytokine plex. Cytokines that are regulated by these two receptors that could be of interest in the context of macrophages and psoriasis are IL-1β, IL-12, IL-23 and TNF-α (McCoy and O'neill 2008; de Marcken et al. 2019; Georg and Sander 2019; Kamata and Tada 2022). These cytokines attract further immune cells like, Th1 and Th17 cells which could then lead to a further cytokine release and increased inflammation and disease progression.

The remaining question is how the naRNA gets to the endosomal TLR receptors in the macrophages. A possible explanation could be resent data of our group, which show that RNA and LL37 complex within NETs (Bork et al. 2022). Together with the previous findings of our group, that LL37 leads to internalization of RNA and activation of TLR8 in naïve PMNs (Herster et al. 2020), this could suggest that the naRNA is transferred by LL37 into macrophages and maybe even into naïve PMNs and activate the endosomal TLR8. To test this hypothesis the uptake via a live cell imaging of fluorescent labeled naRNA and LL37 in macrophages or other cell types, like PMNs, could be investigated.

Furthermore, it remains unclear whether other cells of the immune system are able to recognize naRNA, via which receptor and how they respond to it. In this context, it would be particularly interesting to investigate how plasmacytoid dendritic cells (pDCs), which also accumulate in psoriasis lesions (Tian and Lai 2022), respond to naRNA. Since pDCs are considered to be a link between the innate and adaptive immune systems, they are also thought to play a central role in psoriasis. Therefore, should pDCs react with a proinflammatory response to naRNA, this could lead to a further amplification of inflammation. To investigate this, pDCs should be stimulated with NETs and naRNA, similarly to the macrophages in this work. To check for receptors that could be involved, a knockout of the most promising targets NOD2 or TLR7 or TLR8 would be an option. Thereby, TLR7 would be even more interesting in pDCs than TLR8, as they express predominantly TLR7 (Hornung et al. 2002; Mitchell, Chintala, and Dey 2018). However, other innate immune cells, such as natural killer cells, should also be stimulated with naRNA to determine if and how they respond.

4.2 TLR independent and NOD2-RIPK2 dependent sensing of naRNA in keratinocytes

Stimulation of keratinocytes, N/TERT-1 and NHEK cells, revealed that they respond highly sensitively to NET-content with preserved naRNA (Figure 7A and B). This could be shown in 2D and 3D culture experiments by the induced expression of *CXCL8* and release of IL-8 (Figure 7D-F). This demonstrates that low concentrations of naRNA are enough to trigger a reaction in keratinocytes. In contrast to PMNs and macrophages, the recognition of naRNA is not mediated through a TLR sensing as the TLR7 and TLR8 inhibitor IRS661 did not reduce the IL-8 release after NET-content stimulation and MyD88 knockdown as well had no effect on the naRNA sensing. Furthermore, the screening of several knockdowns of proteins that are described to be involved in different RNA sensing pathways did not result in a decreased IL-8 release for MAVS and NLRP1 or had side effects like in the case of TRIF, so that it could not be considered (Figure 8). Although the RNA sensing TLRs 7 and 8 are not constitutively expressed, they can be induced through Ca^{2+}-induced differentiation in case of TLR7 (Li et al. 2013; Klymenko et al. 2017) or by LL37, which induces TLR7 and TLR8 expression in keratinocytes (Miura et al. 2022), although I did not formally show this. The fact that the keratinocytes did not respond to TL8-506, strengthens our assumption that TLR8 is not involved in the sensing.

LL37 is also part of the NETs (Radic and Marion 2013). Furthermore, the stimulation of keratinocytes with PMA NETs induced *CAMP* expression (Figure 10C), what could suggest that the produced LL37 after naRNA recognition is able to induce the TLR7 and TLR8 expression. Although these two receptors seem not to be involved in the naRNA sensing, their expression could lead to enhanced sensing of RNA from other sources like bacteria. This could further enhance proinflammatory reactions. Ways to test this would be to stimulate keratinocytes with PMA NETs or LL37 and either together with the stimulation or afterwards infect the cells with bacteria. Furthermore, the same could be applied for immune cells like macrophages that could be treated with LL37 or the supernatant of keratinocytes. If the sensing is enhanced the expression and release of cytokines should be amplified, and in the case of macrophages, this could also result in lower numbers of bacteria than without additional LL37, e.g. by phagocytosis.

Additionally, naRNA leads to increased *CAMP* expression in keratinocytes. The resulting increased amount of LL37 could further enhance the effect of naRNA in the skin on NET-formation of naïve PMNs or release of cytokines by macrophages by making more naRNA available for TLR8 sensing. This could further escalate the self-amplification of the NET-formation in the skin and fuel the psoriasis disease progression.

Further experiments showed that naRNA sensing is mediated through the NOD2-RIPK2 pathway, as inhibition of RIPK2 significantly reduced the IL-8 release and knockdown of NOD2 with a siRNA the IL-8 release and *CXCL8* expression (Figure 9). Two previous studies could already show that NOD2 and RIPK2 are associated with the sensing of single stranded RNA from viruses (Sabbah et al. 2009; Lupfer et al. 2013) but it was never shown for keratinocytes or other sources like the RNA from NETs. This suggests that the main RNA sensor in keratinocytes could be NOD2. But this needs to be validated further as some remaining open question need to be clarified. For this validation an activation of RIPK2 should be shown as RIPK2 in the activated form is phosphorylated and ubiquitinated. This was addresses via immunoblot and co-immunoprecipitation but the quality of the antibodies for phosphorylated RIPK2 was not sufficiently.

Moreover, it should be investigated if NOD2 and RIPK2 directly interact after the sensing of naRNA. As it could be shown that NOD2 directly interacts with RIPK2 after the activation by ssRNA (Lupfer et al. 2013). This could be tested with a co-immunoprecipitation or pulldown assay were either RIPK2 or NOD2 are isolated and if both proteins can be detected in an immunoblot this confirms the hypothesis. If not, this would suggest that either they do not interact with each other or another protein is acting between NOD2 and RIPK2. Whereby it is very unlikely, as also RIPK2 inhibition leads to reduced IL-8 release and it is shown in various studies that NOD2 interacts directly with RIPK2 (Trindade and Chen 2020). Further, these findings should be validated with knockout cells of NOD2 or RIPK2. The signaling after a stimulation with ssRNA, PMA NETs or isolated naRNA should then be entirely gone if it is based solely on NOD2 or RIPK2. If it is only reduced but not completely gone, this indicates that the sensing is not exclusively due to NOD2 and RIPK2. Especially after a stimulation with isolated naRNA as this does not contain any other NET components. Moreover, a stimulation of a 3D human skin equivalent would be necessary to confirm the results from the 2D-culture in a physiologically more relevant context. In an optimal setting these 3D human skin equivalents would also be tested with keratinocytes with a knockout of NOD2 or RIPK2. A knockdown in these models would be difficult to realize as the knockdown is only stable for a few days and the model needs 23 days to grow before they can be stimulated and how efficient a knockdown is in such a model after growing, or whether it is possible at all, was never described. It was also addressed to inhibit HSEs with the RIPK2 inhibitor GSK583, but this was unsuccessful as the MDP control still resulted in expression of the target genes.

Additionally, it is reported that MDP sensing via NOD2 relies on a previous phosphorylation of MDP by NAGK (Stafford et al. 2022). It remains unclear if the RNA from the NETs binds directly to NOD2 and if the RNA needs to be modified like the MDP. To investigate the direct binding of RNA and NOD2 the RNA could be labeled with a fluorophore and NOD2 stained with a labeled antibody, followed by a life cell imaging to determine a colocalization. Furthermore, a co-immunoprecipitation could also be done with NOD2 and RNA to check for the direct interaction. If sufficient RNA, known to interact with NOD2, could be isolated in this way, sequencing of the RNA might be of interest to look for a specific sequence or other requirements that the RNA must have in order to be bound by NOD2. In this way it could be determined if the RNA maybe needs to be rich in uridine, like the RNA ligands that can be bound by the TLR8 (Ostendorf et al. 2020). To use a knockout of NAGK could be interesting to check if NAGK also modifies RNA, although this is unlikely as it would need a binding site for RNA that is not described.

Another question that arose from the data is, that the NOD2 is a cytoplasmic receptor. So, the RNA from the NETs needs to get inside the cell to be recognized by NOD2. How this is mediated and by which proteins or through which mechanism remains unclear. MDP as ligand of NOD2 has several ways to get to the cytosolic receptor. Usually the peptidoglycan encounter phagocytosis and lysosomal digestion before it may get transported to the cytosol (Trindade and Chen 2020). However, a first step to investigate the transport of naRNA into the cell could be a life cell imaging of RNA labeled with a fluorophore and markers for different cell compartments to check were the RNA goes, which mechanism would be possible and what is nearby. The finding that naRNA in keratinocytes is sensed by the NOD2-RIPK2 pathway helps to understand how RNA can be sensed in the skin but open questions about the complete pathway, the properties and requirements of the RNA to be sensed and how the RNA comes to the NOD2 receptor remains unclear and needs to be investigated.

An additional question that arises is, whether other cells can also sense naRNA via NOD2 or if this mechanism is keratinocyte-specific. There are other proteins like tristetraprolin (TTP), a RNA binding protein, which is regulating the mRNA levels of several cytokines and that acts different in other cell

types. Andrianne et al. showed 2017 by deletion of TTP in keratinocytes, that this leads to exacerbated inflammation in psoriasis models. This is different compared to ablation of TTP in dendritic cells or myeloid cells. By this they demonstrated that TTP has a keratinocyte-intrinsic role (Andrianne et al. 2017). Therefore, it cannot be excluded that this also applies for a NOD2-intrinsic role in keratinocytes, since PMNs and macrophages appear to recognize naRNA by TLR8. In this context, fibroblasts from the cell line R2F/TERT-1 as another major cell type of the skin were stimulated in the presence of the RIPK2 inhibitor GSK583 with naRNA but this did not reduce the IL-8 release (Figure 16). Moreover, they do not respond to MDP as NOD2 ligand or TL8-506 as TLR8 ligand, leading to the suggestion that they do not express NOD2 and TLR8 or they are not functional. This could be due to the fact that it is a cell line, because it is described that fibroblasts express NOD2 and the impact of this sensing in fibroblasts on diseases like Crohn's disease (Nayar et al. 2021). This suggests that this cell line may not suitable for this purpose and the experiments should be repeated with primary fibroblasts. More cell types need to be tested before it can be clearly stated that NOD2 has an intrinsic role only in keratinocytes.

If the RNA sensing in keratinocytes is NOD2-mediated, this could have major implications as it would help understand how keratinocytes respond to RNA, especially host-derived RNA. New therapies could be developed for diseases associated with abnormal keratinocyte behavior that target the NOD2 signaling pathway. This could have an impact not only on diseases associated with NOD2 and the skin, such as the Blau syndrome, but also on others not previously associated with NOD2, such as psoriasis.

4.3 Psoriasis disease progression fueled by naRNA sensing keratinocytes?

The sensing of naRNA was further analyzed to reveal more genes that can be associated with the activation of the NOD2-RIPK2 pathway by naRNA. In this context, the psoriasis associated genes *IL17C*, *IL36G*, *CAMP* and *RNASE7* were found to have increased expression levels in addition to *CXCL8.* This expressions completely relies on the RNA within the NETs as a RNase A digest before the stimulation to remove the naRNA leads to background expression levels of these genes (Figure 7F, Figure 10). The expression of these genes was also analyzed in the 3D human skin equivalents. They showed an increased expression of *IL17C* and *CAMP* (Figure 11A and C). Although the expression of *CAMP* was not significantly elevated but close to that with a p value of 0.0548 for PMA NETs with RNase inhibitor. Furthermore, it showed for *IL36G* not quite so clear results as in the 2D culture experiments, as it was only significantly elevated for PMA NETs but not for PMA NETs with RNase inhibitor (Figure 11B). Why it was not elevated for PMA NETs with RNase inhibitor as in the 2D culture remains unclear. The same applies to the *RNASE7* results (Figure 11D) as they were not elevated at all. It could be possible that the reaction of the keratinocytes in the more physiologically relevant 3D model is different to the 2D culture. One possible explanation could be that the NETs were applied on the top of the 3D human skin equivalents, where fully differentiated keratinocytes are located, the corneocytes. Usually, these cells are dead and form a barrier and so it could be possible that these cells do not sense the naRNA or only low amounts of the NETs and naRNA penetrate into the 3D model, where they could be sensed by active keratinocytes. This could explain why *IL36G* and *RNASE7* expression was different between 3D and 2D experiments. But this would suggest that maybe lower amounts are enough to still trigger *IL17C* and *CAMP*. If it was the case that only low amounts of the PMA NET stimulus got to the right place for sensing within the 3D models, it could maybe explain why especially for *IL17C* the 2D experiments lead to over 1000-fold increase after PMA NETs stimulation (Figure 10A) but for the 3D human skin equivalents only to 4.7-fold increase (Figure 11A). Therefore, it should be retested by adding the stimulants to the media in which the 3D human skin equivalents are placed. This may allow

a better infiltration of the stimulants into the 3D model. Ideal would of course also be a 3D model that incorporates neutrophils, in order to check the effect of NETs produced *in situ*.

As it was shown that the naRNA was sensed by NOD2, it was further analyzed if the expression of these psoriasis associated genes can also be connected to the activation of NOD2, as it was described that NOD2 activation leads to *CXCL8* production (Warner et al. 2014) and that *S. aureus* leads to expression of *IL17C* in keratinocytes via the NOD2 receptor (Roth et al. 2014). Further 2D culture experiments with the NOD2 knockdown were controversial as the expression patterns were not identical between the repeats (Figure 12, Supplementary figure 1, Supplementary figure 2). Therefore, further repeats are necessary to clarify the involvement of NOD2 in the expression of *IL17C*, *IL36G*, *CAMP* and *RNASE7*. But these experiments suggest together with the work of Roth et al. from 2014 that *IL17C* is expressed after naRNA sensing by NOD2. Further, also *CAMP* and *IL36G* seem to be regulated by the NOD2 pathway, whereas *RNASE7* was in two of the three repeats upregulated in the NOD2 KD compared to the control siRNA. This could suggest that even though it could be shown with the RNase A digest that the increased expression of *RNASE7* is dependent on the naRNA (Figure 10D), this is not mediated through NOD2. Furthermore, this could lead to the suggestion that the knockdown of NOD2 seems to even increase the sensing of naRNA through another receptor, which is regulating the *RNASE7* expression. This needs to be clarified by further experiments with keratinocytes from a NOD2 knockout. Not only would this show whether the other three genes are regulated by NOD2, but it could also verify that the expression of *RNASE7* is not regulated by NOD2, if it is still expressed after stimulation of NOD2 KO keratinocytes. Which other receptor could be involved in the sensing of naRNA in keratinocytes is unclear and needs to be investigated. Further RNA sensing receptors that are not tested yet are MDA5, RIG-I and PKR, but these usually only bind dsRNA, which is not the case for naRNA. It could also be possible that NOD1 is involved in the sensing of naRNA as it is comparable in structure with NOD2 and it was already described that also NOD1 can bind RNA although only dsRNA (Vegna et al. 2016; Wu, Zhang, et al. 2020), but maybe it has an unknown function in the sensing of naRNA.

So far only a few genes were tested for an induction of their expression after naRNA stimulation. Keratinocytes can express a broad range of cytokines and NOD2 is linked to multiple pathways that regulate inflammatory responses through the activation of type I IFNs and NF-κB (Trindade and Chen 2020). Therefore, many other cytokines, like IL-1, IL-6, CCL2, CCL5 and IFN-β among many other target genes of the NF-κB and type I IFN pathways (Hoesel and Schmid 2013; McNab et al. 2015) could be interesting to be investigated for their involvement in naRNA sensing and to check which of these are regulated by NOD2. Additionally, beyond the analysis of the expression of the genes via RT-qPCR, it should also be tested how much of the respective cytokine is released by the keratinocytes, this includes IL-17C, IL-36γ, LL37 und RNase7 as so far only the amount of released IL-8 is determined. Knowing that these cytokines and possibly others get produced after naRNA sensing, it still has to be found out in which extend these cytokines fuel the psoriasis progression in the skin. It is known that keratinocytes play a major role in psoriasis (Ortiz-Lopez, Choudhary, and Bollag 2022) but how big the impact on the disease progression is remains unknown and is hard to address as psoriasis is not fully understood. But it could be tested how potent this cytokine cocktail is in attracting other immune cells, like PMNs, macrophages and T-cells. This could be possibly tested by swarming assays in which different concentrations of supernatant from stimulated keratinocytes are used to see if and how many and how fast these cells migrate towards this supernatant that contains the cytokines. Further, it could also be tested if these cytokines are able to not only attract immune cells but also activate

them. This could be tested by either co-culturing these cells together with previously stimulated keratinocytes or by stimulating these cells with the supernatant from stimulated keratinocytes and look by PMNs for NET-formation or via FACS for activation markers like CD62L or in case of macrophages at activation markers like CD69 or CD83.

In addition, this described role of NOD2 in the context of naRNA sensing and psoriasis-associated gene expressions could lead to new approaches for a psoriasis therapy. A possible *in vivo* method to test this would be to use NOD2 KO mice in a psoriasis model. Typically, this model is induced by applying imiquimod on the skin. If the NOD2 KO mice reacts less or even with no psoriasis phenotype, this would indicate a major role of NOD2 in psoriasis and show how effective possible treatments could be.

Furthermore, the novel RNA polymerase I inhibitor CX-5461 is described to suppress IL17 and other cytokine expressions, resulting in no psoriasis phenotype in mice after imiquimod treatment (Wu et al. 2023). This shows the effect and impact of IL-17 on the disease progression of psoriasis. Additionally, an inhibitor for NOD2, RIPK2 or any other involved protein of this pathway, that is yet to be fully determined, could show how big the impact of these sensing in the psoriasis development and progression is and thereby also potentially act as a possible treatment for patients that suffer from psoriasis.

4.4 Could IκBζ be a downstream target of the NOD2-RIPK2 pathway?

This work could show that naRNA is sensed via NOD2 and RIPK2, but the complete pathway has still to be determined. It is known that NOD2 is interacting directly with RIPK2, which is then signaling either via TRAF3 and IRF7 to induce IFN-β signaling or via LUBAC, TAK1 and then either through the MAPK pathway or via the complex of NEMO, IKKα and IKKβ, which activate the NF-κB signaling (Figure 3B) (Caruso et al. 2014; Trindade and Chen 2020). Furthermore, IκBζ from keratinocytes is reported to be a driver of psoriasis and it leads to *IL17C* induction (Lorscheid et al. 2019). Additionally, it is described that upregulated IκBζ expression from biopsies of patients with psoriasis is coincided with high *IL36G* expression levels (Müller et al. 2018). Because both *IL17C* and *IL36G* expression are upregulated by naRNA, we hypothesized that NETs lead to an activation of IκBζ and that the NOD2-RIPK2 pathway is connected with IκBζ. We used the CDK4/6 inhibitor abemaciclib that inhibits *IκBζ* expression and observed a downregulated expression of *CXCL8*, *IL17C* and *IL36G* already 1 h after the stimulation (Figure 13). This showed not only that these genes are already induced 1 h after the stimulation, but it also further demonstrates that IκBζ was inhibited and these cytokines as well. This suggests that IκBζ is a downstream target in the pathway after sensing of naRNA through NOD2 and RIPK2. But further experiments are needed as this experiment was only performed once and it does not clearly show that these proteins are signaling through the same pathway. It could be possible that the CDK4/6 inhibitor has an off-target effect on other proteins that are involved. To show a connection of NOD2, RIPK2 and IκBζ a knockout of NOD2 would be suitable. This knockout should not sense naRNA and then also should not lead to expression of IκBζ if it is downstream of NOD2. In this way off-target effects of inhibitors or siRNAs are prevented as only signaling of NOD2 is abolished.

If it can be confirmed that CDK4/6 inhibitors reduce the cytokine release from naRNA sensing by NOD2, such inhibitor could be used as a therapeutic agent against psoriasis. This could be helpful in two ways, since on the one hand the cytokine production by keratinocytes could be decreased, which would weaken the inflammation. On the other hand, it is reported for PMNs that the transport of NE to the nucleus is blocked by CDK4/6 inhibitors, which prevents NET-formation (Amulic et al. 2017; Tan, Aziz, and Wang 2021). This would decrease NET-formation and thereby the release of naRNA, which could

then not activate keratinocytes and the production of cytokines such as IL-17C, IL-36γ and others. And even if a few NETs are still formed and naRNA is shown to be a very potent activator in low doses, these would then not lead to a cytokine production as the sensing pathway on keratinocytes would also be inhibited. Therefore, CDK4/6 inhibitors could be a potent treatment for psoriasis patients.

4.5 NOD2 in the context of psoriasis

Mutations, polymorphisms or substitutions within the NOD2 gene are associated with several diseases, like Blau syndrome, early-onset sarcoidosis, Crohn's disease or NOD2-associated autoinflammatory disease (NAID), also called Yao syndrome (Caso et al. 2015; Yao, Li, and Shen 2019). Moreover, also dysregulated signaling of NOD2 is associated with diseases like type 2 diabetes and asthma (Trindade and Chen 2020). In addition, a correlation between Crohn's disease and psoriasis was observed, as these two diseases occur more frequently together in the same person than would be predicted by chance (Lee, Bellary, and Francis 1990). But so far only one study could observe a susceptibility for the NOD2 gene in correlation to psoriatic arthritis (Rahman et al. 2003), on the other hand several excluded NOD2 as susceptibility gene for psoriasis as no relevant correlations of polymorphisms could be associated to psoriasis (Borgiani et al. 2002; Young et al. 2003; Plant et al. 2004; Zhu et al. 2012). This does not exclude a dysregulation of this pathway as no studies address proteins that are downstream of NOD2 in the context of psoriasis or investigate a possible dysregulation of this pathway.

There are studies as well that address a connection between NOD2 and TLR sensing. *In vitro* and *in vivo* it could be observed that treatment of TLR receptors with ligands from a viral infection or LPS lead to a NOD2 enhanced signaling in a type-I IFN dependent manner (Caruso et al. 2014). But it seems also to work the other way round as it is described that NOD2 modulate and potentiate signals that are transmitted via TLR2, TLR3 and TLR4 (Netea et al. 2005). This was investigated with human peripheral blood mononuclear cells (PBMCs) but not with keratinocytes. In addition, NOD2 stimulation in mice leads to a mostly Th2-cell dependent response but a priming of Th1, Th2, and Th17 cells was observed if a co-stimulation with TLR ligands was performed (Fritz et al. 2007; Magalhaes et al. 2008). It would be interesting to determine, if NOD2 signaling is able to do the same in keratinocytes as this could be potentiate the inflammation in psoriatic skin lesions and either escalate the disease or even be a possible trigger for psoriasis.

The stimulation of NOD2 additionally promotes, by recruitment of ATG16L1, the formation of autophagosomes that lead to increased bacterial killing and antigen presentation (Cooney et al. 2010; Homer et al. 2010). As psoriatic lesions are reported to be infiltrated by many different bacteria strains (Rendon and Schäkel 2019) this NOD2 contribution could be a mechanism that is enhanced and is amplifying the inflammation, due to possibly higher bacteria killing rate and enhanced antigen presentation, which leads to further activation of immune cells that are abundant in psoriatic skin lesions.

Another activating mechanism for NOD2 could be the Ca^{2+} flux within a cell. In colon epithelial cells it was observed that cellular perturbations that are related to the flux of Ca^{2+} and lead to increased intracellular Ca^{2+} concentrations, resulted in NOD2 activation. Although the mechanism behind this activation is not clear yet, it is proposed that changes in the intracellular Ca^{2+} flux trigger an internalization of peptidoglycan (Molinaro et al. 2019). As the keratinocytes in psoriasis are hyperproliferating but not fully differentiate it could be that also their Ca^{2+} flux within the cell is disturbed, so that this could enhance the NOD2 activation in keratinocytes, e.g. by increased uptake of naRNA.

Taken together, this demonstrates many possible ways in which NOD2 could contribute to psoriasis and the disease progression. But it remains unclear which of these mechanisms applies to psoriasis and in which extend this could fuel or even trigger the disease. Therefore, more research is necessary to investigate this. A possible way to investigate this could be *in vivo* experiments with NOD2 knockout mice to see what role NOD2 overall plays. If NOD2 contributes to the disease, a first test with curcumin might be useful as a potential treatment, since it directly targets NOD2. It was observed to reduce the expression of NOD2 in fibroblasts, resulting in decreased production of proinflammatory cytokines (Akbari-Papkiadehi et al. 2023). But curcumin may not be the best treatment because it only reduces NOD2 expression. Whether this reduction would be sufficient to reduce inflammation or if there are more promising agents for treatment remains to be determined.

4.6 Mouse vs human keratinocytes: Same recognition of naRNA?

The results for human keratinocytes showed that naRNA is sensed via NOD2. But to test this in a more complex system, which consists of the cooperation of many different cell types, *in vivo* experiments are necessary. Furthermore, possible treatments strategies and reagents need to be tested first in mice. Therefore, it was tested if the naRNA sensing pathway via NOD2 is exclusive for human keratinocytes or if it is conserved. To address this, mouse keratinocytes were stimulated and the expression of various proinflammatory cytokines were tested (Figure 14). However, it was not possible to test *CXCL8*, one of the major cytokines of this work that was tested in the human keratinocytes, as mice lack the IL-8 gene. It could be shown that not only the expression of the IκBζ gene was induced by NETs but also *Il17c*, *Cxcl1*, *Cxcl2*, *Cxcl10* and *Il23a*. Furthermore, in a second experiment keratinocytes of WT and NOD2 KO mice were stimulated and the expression of *Il17c*, *Cxcl2* and *Cxcl10* was significantly reduced in the NOD2 KO cells stimulation with ssRNA (Figure 15). In addition, also the expression of *Il36g* and *Defb4* was reduced in the NOD2 KO cells but not significantly. This suggests that also murine keratinocytes recognize ssRNA via the NOD2 pathway and that this sensing leads to production of psoriasis associated genes, just as in human keratinocytes. Therefore, it seems that this NOD2 sensing mechanism is conserved between humans and murine keratinocytes. But this has to be verified further as it was only a single experiment and with ssRNA and not with NET-content.

4.7 Role of RNases in naRNA sensing

This work presents some significant influences of naRNA on cells of the immune system and keratinocytes. The major signaling pathways for the sensing of naRNA are NOD2 in keratinocytes and TLR8 in PMNs and macrophages. For the TLR8 to recognize RNA ligands special RNases are necessary. So it is described that the lysosomal endoribonuclease RNase T2 *in cellulo* is crucial to provide RNA ligands from an exogenous source that can be detected by TLR8 (Greulich et al. 2019) and further that RNase T2 and RNase 2 cooperate to digest RNA from various sources, resulting in ligands that can be detected by TLR8 (Ostendorf et al. 2020). Thereby the RNA ligands have to be rich in uridine and guanosine to activate the TLR8 (Tanji et al. 2015). The sequence of the naRNA and to which products it could be digested by RNases would be important to determine, as it would allow to make more specific predictions about the potency of naRNA to activate the TLR8 receptor and the resulting inflammation. Additionally, treatment with RNase A was described to prevent NET-formation and disease progression (Lasch et al. 2020; Früh et al. 2021) but one has to consider that this could also lead to enhanced signaling, when the RNA products from the digestion are then sensed by the RNA receptors TLR8 or NOD2. The same applies to a treatment with DNase to digest the backbone of the NETs. This DNase treatment is used by patients with cystic fibrosis but together with other treatments.

This makes it impossible to define the effect of the DNase on NETs (Liew and Kubes 2019). Here, one has to consider, if the backbone of the NETs, to which the naRNA adheres, is digested, this could lead to a higher availability of the naRNA and thus to an enhanced proinflammatory reaction. In summary, this means it has to be considered if RNases in principle can be used as treatment or if they promote the inflammation and also which RNase could be used as various RNases exist that each have different cleavage sizes and activities and also the human body expresses many different ones that play crucial roles in various pathologies (Wu, Xu, et al. 2020). In addition, RNases could be active in the whole body and would not be limited to the disease area, resulting in non-specific effects like altered gene expression, translation or regulation (Castro et al. 2021; Siraj 2022). Clearly, there will be a dose dependency at work here and so far there is no evidence that NOD2 or other cytoplasmic RNA sensors require RNA degradation (Chan and Jin 2022). Therefore, RNases as treatment for diseases associated with naRNA are maybe not the best opportunity as other therapeutical agents could be more promising.

But RNases do not only play a role in the context of macrophages or as a treatment. Keratinocytes do not express RNase 2 or RNase T2, instead they express RNase 7 and RNase 5, which are both part of the RNase A superfamily (Simanski et al. 2012; Rademacher et al. 2019). RNase 5 has a lower ribonucleolytic activity compared to RNase 7 (Gao and Xu 2008). Additionally, RNase7 is also an antimicrobial peptide with functions against a broad range of microorganisms and is part of the growth control mechanism in the skin (Rademacher et al. 2019). The ribonucleolytic activity could suggest that RNase 7 and RNase5 secreted by keratinocytes replace the RNases from macrophages and in the skin maybe promote the naRNA sensing by digesting it into ligands that are available for RNA receptors. This would not be limited to keratinocytes, as maybe also ligands for the TLR8 arise from this digestion. Especially as keratinocytes are the major cell type in the skin and even if only low amounts are secreted, the enormous number of cells could lead to a significant amount of RNases. To test this, it would be interesting to digest naRNA with either RNase7, RNase 5 or both and stimulate cells that do not express RNases but the RNA sensing receptors TLR8 or possibly NOD2. Suitable for this experiment would be human embryonic kidney (HEK) cells transfected with the respective receptor. This would not only show if ligands for the RNA receptors are produced, but also if NOD2 has special requirements to sense naRNA that are maybe fulfilled through one of these RNases. Furthermore, RNase 7 is not only constantly released and has the ability to digest RNA, it is able to bind DNA and RNA to form a complex (Rademacher et al. 2019; Kopfnagel et al. 2020), just like LL37 does. Thereby, naRNA is inducing the expression of RNase 7 in keratinocytes (Figure 10D) but this inductions seems not NOD2 dependent (Figure 12D), although this is not completely clear and has to be verified. It would be interesting to determine if RNase 7 binds to naRNA and if this complex mediates or promotes the sensing in keratinocytes or any other cell type like macrophages or PMNs. If it does this, it could be possible that RNase 7 could achieve the same tasks and inflammatory potential as LL37. Furthermore, it would be interesting to investigate as RNase 7 is a ribonuclease, if the RNA is more resistant to nuclease degradation, when it is in complex, as it is the case for LL37 (Ganguly et al. 2009). In this context it would be necessary to investigate the stability and the overall life span of naRNA further. To test this, NET-formation could be induced and supernatant with RNases from stimulated keratinocytes or macrophages or recombinant proteins of RNase7, RNase 2 or RNase T2 could be added. Afterwards over time the fluorescent signal from the naRNA detected via microscopy should be reduced and disappear completely if these RNases digest the naRNA. This would allow to determine how robust the naRNA within the NETs is. Of course, naRNA is not only digested by RNases to be removed. Other cells such as macrophages can take up NETs to remove them. This could also be tested to determine how

fast NETs and naRNA can be removed by adding macrophages to NETs under a microscope. As macrophages produce RNases this would show both mechanisms for removal of NETs, via RNases and phagocytosis. Information about the stability and the clearance rate of naRNA would also influence possible treatment options with RNases against naRNA associated inflammations.

4.8 naRNA as possible Vita-DAMP

RNA has a short life span and is removed fast, which lead to the hypothesis of our group that naRNA could be a DAMP, initiating an early response to clear pathogens and induce a proinflammatory milieu (Bork et al. 2022). But maybe naRNA is not only a DAMP, it could be a possible vita-DAMP. For the body it is important to discriminate between a danger that is alive or dead and it adjusts the immune response to the level of threat it encounters. So far, two vita-MAMPs are described, bacterial mRNA and c-di-adenosine mono-phosphate. These two vita-MAMPs are associated with microorganisms that are alive and lead to unique viability-associated innate immune responses that are not triggered if stimulated with killed bacteria. But the mechanism behind it is not fully understood (Blander and Barbet 2018; Ugolini and Sander 2019). It could be hypothesized that naRNA also act as a vita-DAMP that reports the infiltration of pathogens, caught in their web-like structure, created during the NET-formation. But the mechanism of vita-MAMPs needs to be further investigated to make a statement about naRNA as a vita-DAMP.

4.9 Conclusion

In this study it was shown that the NET component naRNA, which is not much investigated or described, has a major impact in activating proinflammatory reactions in various skin associated cell types. This naRNA is able to activate PMNs via the TLR8 to induce further NET-formation, starting a self-amplifying mechanism. Furthermore, also macrophages get activated by naRNA through the TLR8. Interestingly, keratinocytes, the major cell type of the skin, recognize naRNA through the NOD2-RIPK2 pathway with an involvement of IκBζ. This activation of keratinocytes results in a naRNA dependent induction of psoriasis associated genes like *IL17C*, *IL36G* and *CAMP*, that have the potential to fuel the disease progression of psoriasis. Furthermore, naRNA could be a crucial component for the psoriasis progression. But not all open questions could be completely clarified and many more questions arose from this work, so that more research is necessary to confirm these findings. However, further insights into the proinflammatory potential of naRNA could eventually lead to new treatments of naRNA or NET-associated diseases.

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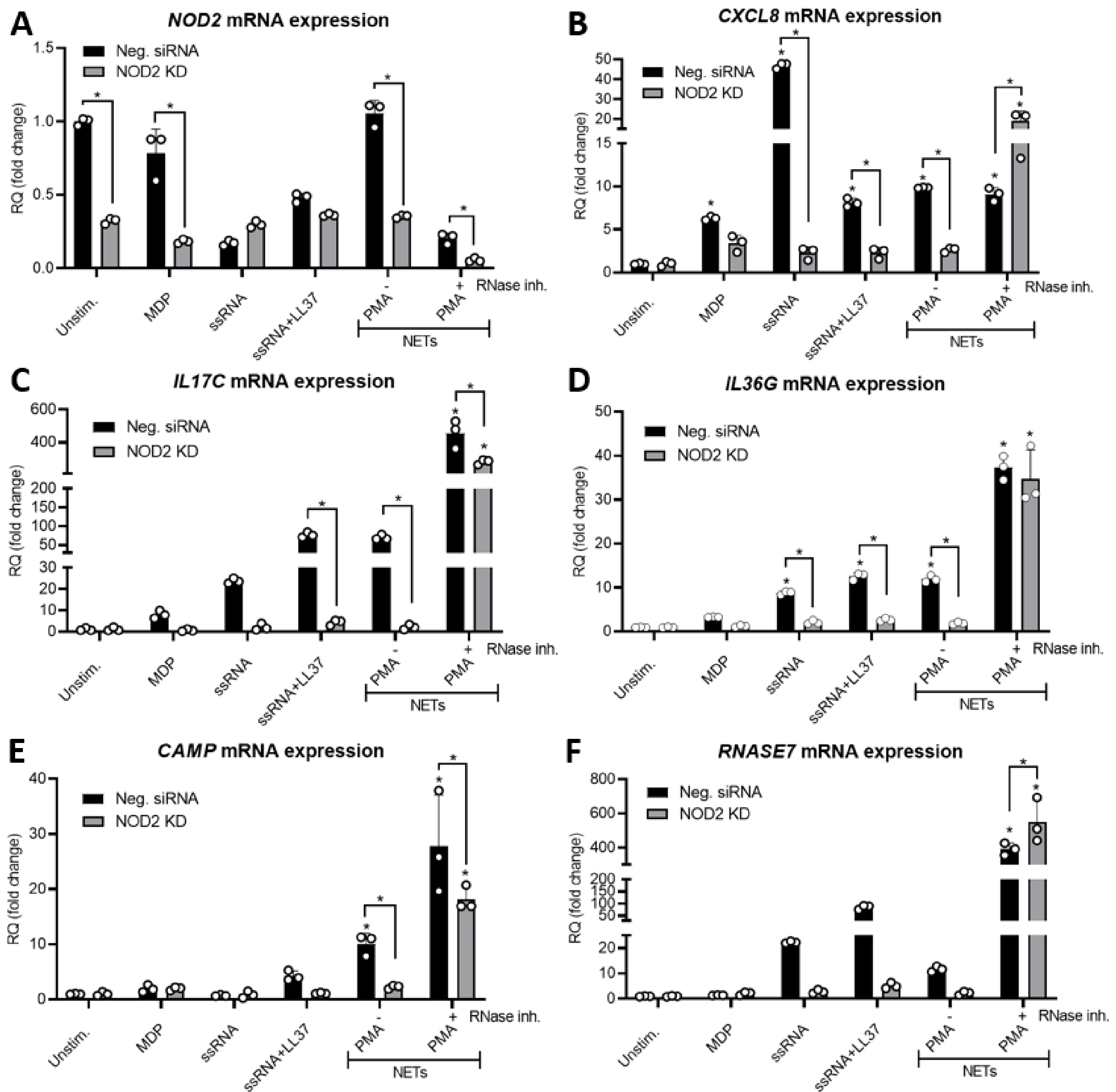
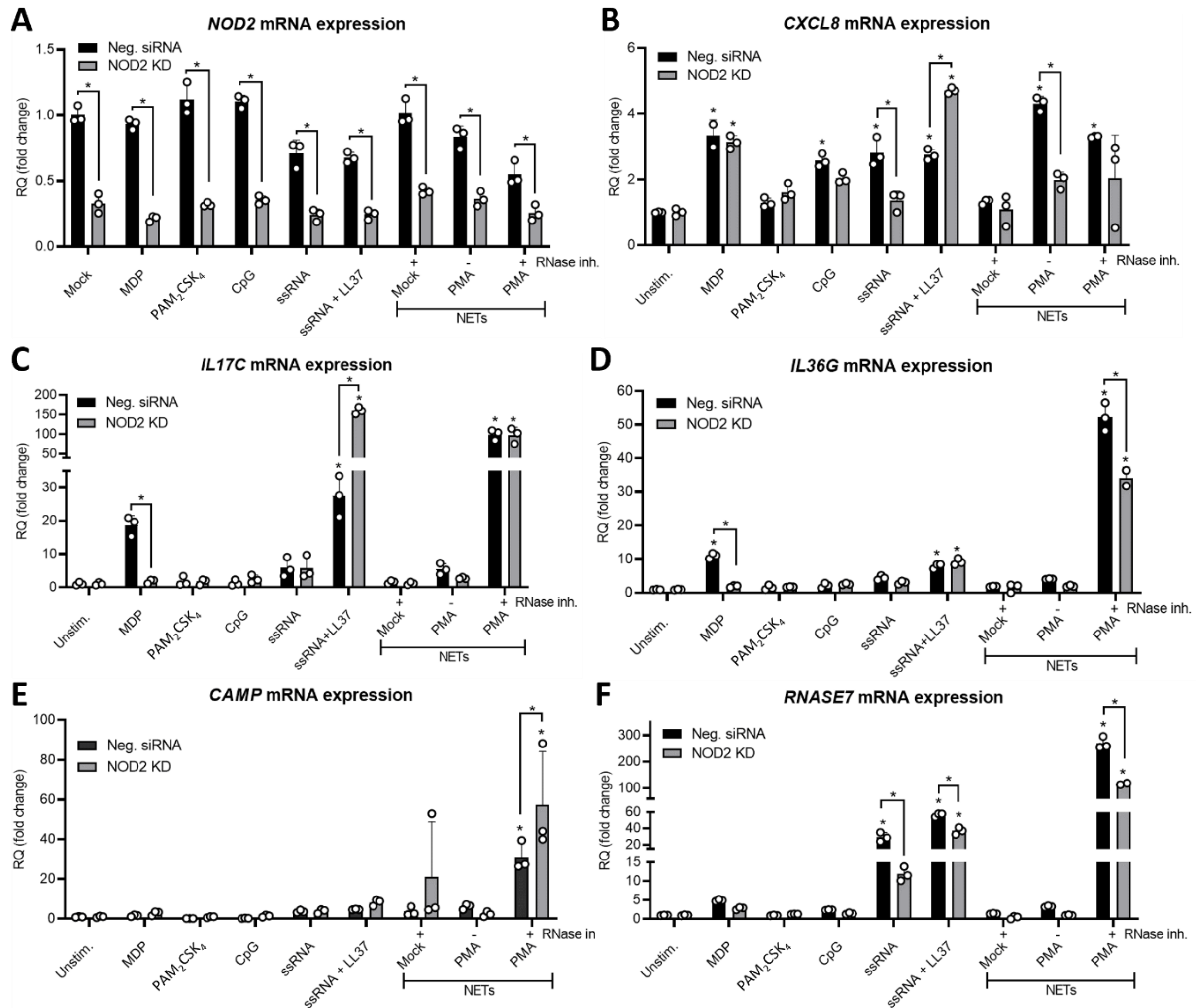
6. Appendix

6.1 NOD2 mediated induction of proinflammatory genes by naRNA (further experiments)



Supplementary figure 1: NOD2 mediated induction of proinflammatory genes by naRNA (second experiment)
A-F) NHEKs were stimulated for 24 h with MDP (20 µg/mL) + Ionomycin (0.375 µg/mL), ssRNA (5.8 µM), ssRNA+LL37 or PMA NETs with or without RNase inhibitor (1:50 dilution). Representative data of triplicate *NOD2*, *CXCL8*, *IL17C, IL36G, CAMP* or *RNASE7* mRNA qPCR relative to unstimulated (n = 3, mean + SD, each dot represents one technical replicate, *p<0.05 according to two-way ANOVA).



Supplementary figure 2: NOD2 mediated induction of proinflammatory genes by naRNA (third experiment)
A-F) NHEKs were stimulated for 24 h with MDP (20 µg/mL) + Ionomycin (0.375 µg/mL), PAM_2CSK_4 (2,54 µg/mL), CpG2006 + DOTAP (5 µM), ssRNA (5.8 µM), ssRNA+LL37, Mock NETs with RNase inhibitor (1:50 dilution) or PMA NETs with or without RNase inhibitor (1:50 dilution). Representative data of triplicate *NOD2*, *CXCL8*, *IL17C, IL36G, CAMP* or *RNASE7* mRNA qPCR relative to unstimulated (n = 3, mean + SD, each dot represents one technical replicate, *p<0.05 according to two-way ANOVA).

Abbreviations

Symbol	Name
ADA	Assay Diluent A
ADAR1	Adenosine deaminase RNA-specific binding protein
AIM2	Absent in Melanoma 2
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosintriphosphat
BAFF	B-cell activating factor
BCR	B-cell receptor
BM	Bone marrow
BSA	Bovine serum albumin
°C	degree celsius
CARD	Caspase activation and recruitment domain
CCL	CC-chemokine-ligand
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
cGAS	Cyclic GMP-AMP Synthase
CGD	Chronic granulomatous disease
CIITA	class II histocompatibility complex transactivator
CLR	C-type lectin-like receptor
CpG	Cytosine-phosphate-guanine
CXCL	C-X-C motif chemokine
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FCS	Fetal bovine serum
g	Gram
GM-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSDMD	Gasdermin D
h	Hours
HD	Healthy donors
H&E	Hematoxylin and eosin
hEGF	Human epidermal growth factor
HEK	Human embryonic kidney
HLA	Histocompatibility leukocyte antigens

HRP	Horseradish peroxidase
HSE	Human skin equivalent
ICAM	Intercellular adhesion molecule
IF	Immunofluorescence
IFI16	Interferon-inducible 16
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1
IFN	Interferon
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IL-1R	IL-1 receptor
IL-1RA	IL-1 receptor antagonist
IL-36R	IL-36 receptor
IKK	Inhibitory-κB Kinase
iODN	Inhibitory oligodeoxynucleotide
IRAK	IL-1R-associated kinases
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
kb	Kilobases
KC	keratinocyte-derived chemokine
KD	Knock-down
kg	Kilogram
KO	Knock-out
L	Liter
LGP2	Laboratory of genetics and physiology-2
LRR	Leucine rich repeats
LPS	Lipopolysaccharide
LUBAC	Linear ubiquitin chain assembly complex
M	Molar
MAL	MYD88-adaptor-like protein
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signaling
M-CSF	Macrophage-colony stimulating factor
MDA5	Melanoma differentiation-associated antigen 5
mDC	Myeloid dendritic cells
MDP	Muramyl dipeptide
MEK	MAPK/ERK kinase
mg	Milligram
MHC	Major histocompatibility complex
min	Minutes
MINCLE	Macrophage inducible Ca^{2+}-dependent lectin receptor
MIP-2	Macrophage-inflammatory protein 2
miRNA	Micro RNA

ml	Milliliter
mM	Millimolar
MMP-9	Matrix metalloproteinase-9
MPO	Myeloperoxidase
MYD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NAGK	N-acetylglucosamine kinase
NAID	NOD2-associated autoinflammatory disease
NAIP	NLR family apoptosis inhibitory protein
NAP1	Nucleosome Assembly Protein 1
naRNA	NET-associated RNA
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NF-κB	Nuclear Factor-κB
ng	Nanogram
NHEK	Normal human epidermal keratinocytes
NK	Natural killer
NLR	NOD-like receptor
nM	Nanomolar
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NRF2	Nuclear factor erythroid 2–related factor 2
NSP	Neutrophil serine protease
OAS1	2'-5'-oligoadenylate synthetase 1
ODN	Oligodeoxyribonucleotides
ORN	Oligoribonucleotides
PAD4	Peptidyl arginine deiminase 4
PAMP	Pathogen-associated molecular pattern
PASI	Psoriasis area and severity index
PBS	Phosphate-buffered saline
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
PDK1	Phosphoinositide-dependent kinase-1
PI3K	Phospho¬inositide 3 kinase
PKC	Protein kinase C
PKR	Protein kinase R
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphonuclear neutrophil
Poly I:C	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
R848	Resiquimod
RA	Rheumatoid arthritis
RIG-I	Retinoic acid-inducible gene I
RIPK	Receptor-interacting serine/threonine-protein kinase
RLR	RIG-I like receptor

RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
s	Seconds
S. aureus	*Staphylococcus aureus*
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
ssRNA	Single-stranded RNA
STAT3	Signal transducer and activator of transcription 3
STING	Stimulator of interferon genes
TAB	TAK1-binding proteins
TAK1	TGFβ-activated kinase 1
TAM	Tyro3, Axl, and Mer receptors
TANK	TRAF family memberassociated NF-kB activator
TBK	TANK-binding kinase
TCR	T-cell receptor
TGF	Transforming growth factor
Th	T helper
TIR	Toll-Interleukin 1 receptor
TIRAP	TIR-associated protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factors
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T cell
TREM-1	Triggering receptor expressed on myeloid cells
TRIF	TIR domain-containing adaptor protein inducing IFN-β
TSLP	Thymic stromal lymphopoietin
TTP	Tristetraprolin
U	Units
µg	Microgram
µl	Microliter
µM	Micromolar
UNC93B1	Unc-93 homolog B1
VbP	Val-boroPro
VCAM-1	vascular cell adhesion molecule 1
WT	Wild-type

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Statutory Declaration

Ich erkläre hiermit, dass ich die zur Promotion eingereichte Arbeit mit dem Titel:

'NOD2-RIPK2 signaling mediates psoriasis-associated gene expression by naRNA from neutrophil extracellular traps in primary human keratinocytes'

selbständig verfasst, nur die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen (alternativ: Zitate) als solche gekennzeichnet habe. Beiträge anderer wurden im Text als solche gekennzeichnet und sind zusätzlich in nachstehender Tabelle der mitwirkenden Wissenschaftlern gelistet. Ich erkläre, dass die Richtlinien zur Sicherung guter wissenschaftlicher Praxis der Universität Tübingen (Beschluss des Senats vom 25.5.2000) beachtet wurden. Ich versichere an Eides statt, dass diese Angaben wahr sind und dass ich nichts verschwiegen habe. Mir ist bekannt, dass die falsche Abgabe einer Versicherung an Eides statt mit Freiheitsstrafe bis zu drei Jahren oder mit Geldstrafe bestraft wird.

Mitwirkende Wissenschaftler

Scientist	Affiliation	Data created	Data shown in this thesis in Figure
Francesca Bork	Group of Prof. Weber, University of Tübingen	PMN stimulation and imaging	5B and C
Jasmin Scheurer	Group of Prof. Schittek, University Hospital Tübingen	3D human skin equivalent	Preparation of 3D skin equivalents (HSEs) used for 7C and D, 11A-D
Birgit Sauer			
Birgit Schittek			
Berenice Fischer	Group of Prof. Kramer, University Medical Centre of Johannes Gutenberg University Mainz	CDK4/6 inhibition Murine WT and $NOD2^{-/-}$ keratinocytes stimulation	13A-J 14A-I, 15A-H
Title of paper:	Release of the pre-assembled naRNA-LL37 composite DAMP re-defines neutrophil extracellular traps (NETs) as intentional DAMP webs		
Status in publication process:	Biorxiv; DOI: https://doi.org/10.1101/2022.07.26.499571		

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