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Expression and function of the P2X7 receptor in human immune cells

Dissertation

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Table of content

1 Aims of the study

The P2X7 receptor is a trigger of pro-inflammatory responses and a key player in the development of inflammatory diseases. In mice, P2X7-specific nanobodies have proven effective as tools for P2X7 imaging and receptor modulation as well as to cure disease in a variety of P2X7-mediated disease models. The aim of this project is to assess the potential of nanobodies directed against human P2X7 for receptor staining and modulation in the human immune system.

Specific aims are:

- To assess the expression of P2X7 in the different human immune cell subsets and in cell lines of interest
- To assess the expression of P2X7 in human T cells and monocytes after cell stimulation
- To assess the potential of P2X7 nanobodies for the inhibition of P2X7 function in human cells

This project was carried out in close cooperation with Dr. Welbeck Danquah and Prof. Dr. Friedrich Koch-Nolte, who manufactured and kindly provided the P2X7-specific nanobodies used in this study.

2 Introduction

2.1 The human immune response

The human organism constantly faces a variety of threats like pathogenic microbes or the formation of tumor or autoreactive cells. The immune system encompasses a network of immune cells and molecules that protect the human body by eliminating pathogens while tolerating the self-antigens of the host.

The innate branch of the immune system constitutes the first barrier against pathogens. Cells of the innate immune response recognize pathogen-associated molecular patterns (PAMPS) like lipopolysaccharides (LPS), peptidoglycans and lipoteichoic acids by a limited repertoire of germline-encoded pattern-recognition receptors (PRRs) that includes the family of Toll-like-receptors (TLR) (Schenten and Medzhitov 2011). This allows a prompt but relatively unspecific immune reaction. Moreover, the innate antigen presenting cells (APCs) initiate the adaptive immune response via antigen presentation. Full activation of the adaptive immunity requires several days but provides a highly specific immune response to a particular antigen. Cells of the adaptive immunity express a highly diverse antigen receptor repertoire and undergo clonal expansion upon specific antigen recognition in the presence of additional activation signals. The adaptive immunity creates an immunological memory that allows an enhanced immune response to subsequent encounters with the same pathogen. The innate-like immunity has properties of both the innate and adaptive immunity. Innate-like cells have a limited TCR repertoire, although originating from the same lymphoid precursors like the adaptive cells, and their responses show innate-like as well as typically adaptive properties.

Invading pathogens like bacteria quickly encounter monocytes/macrophages, granulocytes, DCs and NK cells. Neutrophil granulocytes are of myeloid origin and make 50-75 % of the total leucocytes. They kill pathogens rather unspecific using well-known techniques likes phagocytosis and the production of reactive oxygen species (ROS) as well as the unique technique of Neutrophil extracellular traps (NETs) (Mayadas, Cullere et al. 2014). NETing neutrophils trap pathogens in a net of decondensed chromatin fibers, in which antimicrobial acting molecules like myeloperoxidase, LL-37, S100A, lactoferrin-chelating proteins and neutrophil elastase are integrated (Papayannopoulos and Zychlinsky 2009). At the same time, NETosis is a form of cell death since the involved components are released upon dissolution of granule and nuclear membranes. Neutrophils characteristically express IgG-receptor CD16

that mediates antibody-dependent cell-mediated cytotoxicity, phagocytosis and cell proliferation (Mayadas, Cullere et al. 2014).

Monocytes originate from myeloid precursor cells in the bone marrow and fetal liver under the influence of cytokines like macrophage colony-stimulating factor (M-CSF) and migrate into the peripheral blood where they make an approximate 10 % of the blood leukocytes. Monocytes migrate into the peripheral tissue along chemoattractant gradients and are able to differentiate into macrophages or monocyte-derived subtypes of dendritic cells (moDCs) (Leon and Ardavin 2008). They recognize PAMPs of bacterial cell walls via a multitude of TLRs and promote an inflammatory environment by releasing pro-inflammatory cytokines including IL-1β, IL-6 and Tumor necrosis factor α (TNF-α), prostaglandins and complement. Alike neutrophil granulocytes monocytes are highly phagocytic for bacteria. Moreover, they are professional antigen-presenting cells (APS) and key activators of the adaptive immune response. After internalization of a pathogen monocytes present antigen fragments to naïve T cells on MHC (Major histocompatibility complex) class I and II molecules in the secondary lymphatic organs (lymph nodes, spleen, tonsils, Peyer Plaques). At the same time, they provide further activation signals through interaction of costimulatory molecules CD80 and CD86 with structures on the T cell surface (Ginhoux and Jung 2014).

Microglia are the resident mononuclear phagocytes of the central nervous system (CNS) and make an approximate 10 % of the cells of the adult human brain. They differentiate from myeloid progenitor cells that have migrated to the CNS during the embryonic period. Their main task is the maintenance of homeostasis and elimination of the body's own waste material like cell debris in the brain and spinal cord. They are also key players in the immune defense of the CNS and upon activation through DAMPs or pro-inflammatory cytokines including IFN-γ, microglia cells can release a variety of inflammatory mediators including TNF-α and IL-1β, chemoattractants and cytotoxic ROS (Ransohoff and Cardona 2010).

DCs are the most efficient APCs and play a key role in the initiation of the adaptive immune response. While patrolling in the periphery of the body (e.g. respiratory or gastrointestinal mucosa, dermis etc.) the DC is optimally capable of tissue adherence as well as detection and capturing of pathogens using its dendrites and a large number of adhesion molecules. After internalization of a pathogen, DCs migrate to the T cell and B cell zones of lymphoid organs for antigen-presentation and activation of the naïve lymphocytes (Ito, Amakawa et al. 2002, Geissmann, Manz et al. 2010). DCs can be subdivided into the conventional myeloid DCs (mDCs), which characteristically express CD11c, and the small subset of plasmacytoid DCs (pDCs), which express instead the IL-3 receptor (CD123). Recognition of viral antigens and

self nucleic acids by TLR7 and TLR9 on pDCs triggers the secretion of large quantities of type I IFNs.

Natural killer cells belong to the group of innate lymphoid cells and characteristically express CD56. They play a minor role in the defense against bacteria but are eminent in the protection against viral infections and tumor formation. NK cells recognize downregulation in MHC expression in virus-infected cells, a common mechanism of viruses to escape detection by the adaptive immune system. They also recognize opsonization and respond to interleukins like IL-12 and IL-15 released by virus-infected cells and distinct surface patterns of tumor cells. NK cells secret granzymes and perforins for target cell destruction or bind to Fas ligand, therefore triggering apoptosis. Release of cytokines like IFN-γ leads to destruction of tumor cells and stimulation of other immune cells including macrophages (Vivier, Tomasello et al. 2008).

Eosinophil and basophil granulocytes are further representatives of the innate immunity. These cell types play a minor role in the defense against bacteria; however, they are key players in the defense against parasites and in the pathomechanism of allergic reactions (Min, Brown et al. 2012, Wen and Rothenberg 2016).

T cells and B cells mediate the adaptive immunity and constitute 25-40% of the blood leukocytes. T cells originate from hematopoietic stem cells in the bone marrow and colonize the thymus. Here they progress through a number of differentiation stages to acquire the ability of recognizing foreign antigen while tolerating self-antigen. The fully functional T cells leave the thymus and spread into the periphery. In case a CD4+ T cell (also T helper cell, Th) encounters its specific antigen during antigen presentation, the T cell receptor (TCR)/CD3 complex and co-receptor CD4 bind to the MHC class II/antigen complex strongly. In addition, costimulatory molecules CD80 and CD86 expressed on the monocytes and DCs bind to CD28 on the T cell. The combination of these two signals activates the T cell, which will start releasing IL-2. IL-2 acts in an autocrine and paracrine way by binding to the IL-2 receptor expressed on the same cell or on T cells in the proximity and maintaining proliferation. Activated Th cells can differentiate into effector T cells Th1, Th2, Th17 or periphery-derived regulatory T cells, according to the cytokines released in their immediate environment. Th1 cells develop under the influence of IL-12 and produce IFN-γ, which activates macrophages and CD8+ T cells. Interleukin-4 (IL-4) contributes to the development of Th2 cells, which are crucial for the defense of extracellular parasites and play an important role in autoimmunity. Whereas Th2 cells were initially considered to activate B cell cells, a

further subset of Th cells, the T follicular helper cells (Tfh cells), which are located within the B cell follicles in the secondary lymphatic organs, were recently discovered as important B cell activators as they provide signals crucial for isotype switching and somatic hypermutation (Sahoo, Wali et al. 2016). Th17 cells are triggered by IL-6 and TGF-β. They produce Interleukin 17A and F (IL-17A and F), which particularly attract and activate neutrophil granulocytes (Vignali, Collison et al. 2008).

In the absence of IL-6, TGF-β also triggers the formation of periphery-derived regulatory T cells by inducing the de novo expression of transcription factor forkhead box P3 (FoxP3) (Chen, Jin et al. 2003). FoxP3 is the most important regulatory gene for the differentiation of Tregs not just in the periphery, but also in the thymus during the development of thymus derived Tregs (Hori, Nomura et al. 2003). Controlling the immune response is essential for preventing damage to the body and the major task of Tregs. Regulatory T cells are characterized by the constitutive expression of the IL-2R and the lack of IL-7R, therefore harboring the $CD4^+CD25^+CD127^-$ phenotype. Besides the secretion of immunosuppressive cytokines including IL-10, IL-35 and TGF-β, Tregs are able to degrade pro-inflammatory ATP into anti-inflammatory adenosine using the ectoenzymes CD39 and CD73, thereby limiting inflammatory reactions (Rissiek, Baumann et al. 2015). They can also dampen T cell activation and proliferation through deprivation of IL-2 by binding this important T cell growth factor to the high affinity CD25 receptor, which they express constitutively. Tregs are further able to induce apoptosis in APCs using granzymes and perforins and they are involved in inhibition of maturation and function of DCs (Vignali, Collison et al. 2008)

$CD8^+$ T cells (also cytotoxic T cells, Tc) play a major role in the elimination of virus-infected cells and tumor cells. This cell type recognizes its specific antigen via presentation on MHC I and expresses CD8 as co-receptor. The CD8 receptor is mainly expressed as α/β heterodimer but occurs as unconventional α/α homodimer as well. While the conventional $CD8\alpha\beta^+$ T cells are found throughout the human body, the unconventional $CD8\alpha\alpha^+$ T cells are predominantly found within the population of intestinal intraepithelial lymphocytes and still not much is known about their development pathway and distinct tasks. Recent data suggests a thymic development along a maturation pathway called “agonist selection”, at which autoreactive $TCR\alpha\beta^+$ $CD4^-CD8^-$ thymocytes are specifically preserved (Pobezinsky, Angelov et al. 2012, McDonald, Bunker et al. 2015). These cells quickly migrate into the intestinal mucosa, where they differentiate into $CD8\alpha\alpha^+$ T cells. This suggests an activated transcriptional program for tissue residency, and indeed the universal homing receptor Hobit, also found on liver-resident NK cells and Natural Killer T cells, is expressed by $CD8\alpha\alpha^+$ T

cells (Verstichel, Vermijlen et al. 2017). Unconventional CD8 T cells rather recognize self than foreign antigen and multiple studies showed, that they do not just recognize classical MHC I but a broad spectrum of MHC haplotypes as well (McDonald, Bunker et al. 2015).
B cells develop in the bone marrow and fetal liver and circulate in the blood and lymphatic organs, where they are responsible for the humoral immune response. This cell type expresses the B cell receptor (BCR), a membrane-standing immunoglobulin that recognizes its specific antigen in a native form, along with the B cell co-receptor complex consisting of CD19, CD21 and CD81, and has the unique ability to produce antibodies (abs). Unlike the TCR, that can only recognize its specific antigen as peptide presented by APCs on MHC class I or II molecules, the BCR can bind proteins, glycoproteins, polysaccharides and even whole viruses or bacterial cells that are presented by APCs or float freely. B cells can be activated in a T cell-dependent and T cell-independent manner. T cell-dependent activation takes place in the follicular zone of secondary lymphatic organs while T cell-independent activation is restricted to the marginal zones. After encounter of its specific T cell-dependent antigen, the B cell can take up, process and present this antigen to Tfh cells as a peptide fragment on MHC II molecules. The Tfh cell binds to this MHC II/antigen complex and releases stimulatory cytokines including IL-4 and IL-21. A further B cell activation signal is provided through interaction of the B cell receptor CD40 with CD40 ligand expressed on the Tfh cell (Sahoo, Wali et al. 2016). T cell-independent antigens such as bacterial lipopolysachharides activate B cells without Th cell signaling via TLRs or extensive cross-linking of the BCR. Upon activation, B cells undergo clonal expansion and can differentiate into ab-producing plasma cells or memory B cells. Mature naïve B cells express immunoglobulin (Ig) M and IgD as part of the B cell receptor. IgM is furthermore the first ab class produced in response to a pathogen and an early indicator for acute infection. Structural modification of the constant domain of the heavy chain of the membrane-standing abs by deletional DNA recombination (class switch recombination) allows isotype switching to IgA, IgE or IgG, which occurs quickly after B cell activation. This process facilitates an improved effector function that is adapted to the situational requirements: IgA is predominantly found in the mucosa of the gastrointestinal, respiratory and urogenital tract, IgE plays a crucial role in the defense against parasites and the pathomechanism of allergic reactions and IgG is the main ab produced for the defense of bacterial and viral infections (Stavnezer and Schrader 2014).

The innate-like lymphocytes represent a link between the innate and adaptive immunity. They include Mucosa-associated invariant T cells (MAIT cells), TCRγδ cells and invariant natural

killer T cells (iNKT cells). MAIT cells represent 5-10 % of the peripheral T cell compartment, and mostly reside in mucosal tissue and in the liver. MAIT cell activation displays adaptive as well as innate characteristics, as it is mediated via the semi-invariant TCR Vα7.2-Jα33/12/20 and a variety of TLRs. The semi-invariant TCR Vα7.2-Jα33/12/20 is restricted to a non-classical MHC molecule, the MHC-I related molecule 1, which does not present peptide fragments, but rather vitamin B-based molecules. Interestingly, initiation of full MAIT cell activity requires not just TCR activation but additional antigen binding to TLRs (Chen, Wang et al. 2017). Moreover, cytokines including IL-17 released by T cells and IL-18 released by monocytes can activate MAIT cells directly (van Wilgenburg, Scherwitzl et al. 2016). Upon activation, MAIT cells produce pro-inflammatory cytokines like IFN-γ, IL-17 and TNF-α as well as cytotoxic granzymes and they are eminent in the protection against bacterial and viral infections (Le Bourhis, Martin et al. 2010, Meierovics, Yankelevich et al. 2013, Booth, Salerno-Goncalves et al. 2015, van Wilgenburg, Scherwitzl et al. 2016). In contrast to the majority of T cells, that expresses a TCR composed of one α- and one β-glycoprotein chain, TCRγδ cells express a TCR consisting of a γ- and a δ- chain. They represent a small subset of T cells in the periphery (1–6%) and are predominantly found in the intestine, dermis, lung and uterus. Similar to MAIT cells, TCRγδ cells express a mono- or oligoclonal TCR. Unlike TCR αβ cells, the majority of TCRγδ cells are not restricted to classical MHC molecules and recognize unconventional antigens including alkyl diphosphonates without presentation by professional APCs (Vantourout and Hayday 2013). Vγ9Vδ2^{+} cells, which are the main subset within the peripheral blood TCR γδ compartment, recognize hydroxymethyl-but-2-enyl-pyrophosphate, an intermediate in the alternative deoxyxylulose (non-mevalonate) pathway of cholesterol synthesis, which is produced by a multitude of different bacteria and also eukaryotes including Plasmodium species. At the same time, Vγ9Vδ2^{+} T cells recognize isopentenyl pyrophosphate 24, an intermediate in the mevalonate pathway of eu- and prokaryotic cells and a self-phosphoantigen (Morita, Jin et al. 2007). Vδ1^{+} T cells typically reside in peripheral tissue and little is known about their ligands so far. Of note, these cells recognize stress-induced self-antigen MHC class I polypeptide-related sequence A/B and glycolipids presented by CD1c (Siegers and Lamb 2014). Vδ1^{+} T cells are critical in tumor surveillance and show cytotoxic activity against hematopoietic and solid tumors including leukemia (Correia, Fogli et al. 2011), neuroblastoma (Schilbach, Frommer et al. 2008) and colon carcinoma (Wu, Wu et al. 2015). Vδ1^{+} T cells are moreover involved in the defense and control of viral infections including infection with the Cytomegalo virus (Knight, Madrigal et al. 2010), Hepatitis C virus (Agrati, D'Offizi et al. 2001) and HI virus (Fenoglio, Poggi et al.

2009, Hudspeth, Fogli et al. 2012). iNKT cells are hardly represented in human peripheral blood, and express an invariant TCR Vα24-Jα18 along with NK receptors. In contrast to "classical" T cells, they recognize glycolipid antigens that are presented on CD1d molecules. Upon activation, iNKT cells are able to stimulate NK cells, DCs and other immune cells by rapidly secreting large amounts of cytokines including IFN-γ and IL-4 and providing further signals through expression of co-stimulatory molecules like CD40L. Due to these unique features, iNKT cells play an important role in the defense of bacteria, viruses, fungi and parasites (Kinjo, Kitano et al. 2013).

2.1.1 Danger associated molecular patterns (DAMPs)

Inflammatory conditions are concomitant to tissue damage and cell death due to e.g. hypoxia, hyperthermia and release of cytotoxic substances. Damaged or dying cells alert their vicinity by releasing danger associated molecular patterns (DAMPs) into the extracellular space during cell lysis. This happens in an uncontrolled way, based on diffusion. Intact immune cells presumably also release DAMPs in a controlled fashion, though the exact mechanisms remain to be elucidated. Similar to PAMPs DAMPs bind to PRRs and other receptors and transmit pro-inflammatory signals. DAMPS can be a very heterogeneous group of molecules, including proteins like high mobility group box 1, heat shock protein and the S100 protein family, nucleic acids, mono- and polysaccharides like hyaluronan and purine metabolites like ATP (Sharma and Naidu 2016). ATP fulfills a diversity of functions in the intra- and extracellular milieu: it is used as the main energy currency throughout the body and represents an integral part of a variety of signal transduction ways at the same time. In addition to passive release, intact immune cells can actively release ATP by pannexins and connexin hemichannels upon activation. Extracellular ATP mediates its pro-inflammatory effects through binding to a variety of nucleotide receptors including P2Y1, P2Y2 and P2Y11 and to all members of the P2X receptor family (Idzko, Ferrari et al. 2014).

2.2 The purine receptor family

The purine receptor family comprises three different subclasses: P1, P2Y and P2X. Purine receptors are widely distributed among all vertebrate animals and particularly expressed in immune cells but also further cells throughout the body including endothelial, smooth muscle, gut and colon cells and cells of the nervous system (Surprenant and North 2009, Kaczmarek-Hajek, Lorinczi et al. 2012). P1 receptors are G-protein-coupled and comprise four different

types (A1, A2A, A2B and A3), activated by Adenosine (Fredholm, AP et al. 2011). P2Y receptors are as well G-protein-coupled but bind several nucleotides including ATP, Adenosine diphosphate (ADP), Nicotinamide adenine dinucleotide (NAD) and Uridine triphosphate (UTP). Eight mammalian P2Y receptors have been cloned so far (P2Y1, 2, 4, 6, 11, 12, 13 and 14). P2X receptors comprise seven different types (P2X1-7) and clearly differ from P1 and P2Y receptors regarding form and structure. P2X receptors are ionotropic and share ATP as the main endogenous agonist (Idzko, Ferrari et al. 2014). They consist of two hydrophobic membrane-spanning domains, a large highly glycosylated extracellular domain containing ten conserved cysteine residues, and an intracellular amino (N)- and carboxyl (C)-terminus. Eleven to 13 exons encode the respective full-length P2X proteins, with P2X4 as the shortest (384 amino acids) and P2X7 as the longest (595 amino acids) proteins (Burnstock 2007). P2X receptors are trimers and each of the three subunits can bind one ATP molecule. Binding of ATP triggers subunit rearrangement and opening of the ion channel allowing influx and efflux of cations including $Ca2^{+}$, Na^{+} and K^{+}. While most P2X receptors are homomeric, P2X1 can form heterotrimers with P2X2, P2X4 and P2X5 subunits and P2X2 can even form heterooligomers with subunits of P2X3. The seven receptor subtypes all display unique abilities as well as differences in ligand-sensitivity and ion- and nucleotide selectivity (Idzko, Ferrari et al. 2014).

2.2.1 The P2X7 receptor

The gene coding for P2X7 in humans is located on chromosome position 12q24 (Sluyter and Stokes 2011). P2X7 is a homotrimeric receptor with each of the three subunits displaying a molecular weight of 72kDa and consisting of 595 amino acids (Kaczmarek-Hajek, Lorinczi et al. 2012). The shape of a subunit resembles that of a dolphin with two fins embracing the two neighboring subunits (Karasawa and Kawate 2016). The three intracellular carboxyl tails of the trimeric receptor are 120-200 amino acids longer in comparison to that of the other P2X receptors and represent a structural idiosyncrasy. The tail extension was determined to function as a regulatory gating module, potentially increasing the sensitivity of P2X7 channel gating to intracellular regulation (Becker, Woltersdorf et al. 2008). ATP is the main endogenous agonist of P2X7 and binds in a pocket formed by two interacting subunits (Idzko, Ferrari et al. 2014). NAD+ is an alternative agonist for murine P2X7. In this case Mono-ADP-ribosyltransferase 2.2 (ART2.2) expressed on murine T cells transfers the ADP-ribose moiety

of NAD+ to Arg125 of the P2X7 protein thus positioning the ADP moiety into the ligand binding pocket, which also activates the receptor (Scheuplein, Schwarz et al. 2009).

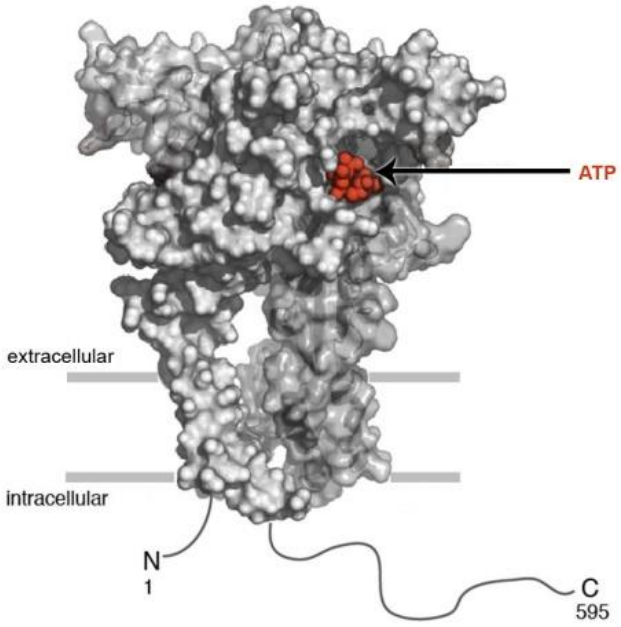


Fig. 1: 3D model of the P2X7 receptor. The P2X7 receptor is a homotrimer, each of the three subunits comprising 595 amino acids and resembling the shape of a dolphin embracing the two neighboring subunits with their fins. As a simplification, the intracellular N- and C-termini for only one of the three subunits are shown schematically as two black lines. ATP (shown in red) binds its binding pocked formed by two interacting subunits (adapted from Danquah 2012).

2.2.1.1. Immune responses triggered by P2X7 activation

ATP-mediated P2X7 activation triggers a variety of immune responses:

1. Release of pro-inflammatory cytokines

P2X7 activation promotes the rapid release of pro-inflammatory cytokine IL-1β from monocytes/macrophages (Pelegrin and Surprenant 2006), DCs (Englezou, Rothwell et al. 2015) and microglial cells (Sanz, Chiozzi et al. 2009). Upon binding to PRRs, PAMPs like LPS promote the transcription of the IL-1β gene and the production of the inactive 31 kDa pro-IL-1β. P2X7-mediated efflux of K+ in response to ATP binding triggers the assembly and activation of the inflammasome, which consists of the NACHT, LRR and PYD-containing protein 3 (NALP3) and the apoptosis-associated speck-like protein containing a CARD (ASC), resulting in the cleavage of pro-caspase 1 into active caspase 1. Caspase 1 in turn cleaves pro-IL1-β into the active 17 kDa form IL1-β, which is finally released from the cell (Fig. 2). The mechanisms behind IL1-β release remain poorly understood: one explanation

might be exocytosis via a specialized lysosomal compartment (Andrei, Margiocco et al. 2004); another model proposes the release through shedding of plasma membrane derived microvesicles (MacKenzie, Wilson et al. 2001). More recent data suggests an involvement of Gasdermin D (GSDMD), which is cleaved between its N- and C-terminus by inflammatory caspases including caspase 1, 4, and 5, and assembles in large oligomeric complexes within the cell membrane after insertion of its N-terminus, therefore building a pore that allows the secretion of IL-1β and probably IL-18 (Ding, Wang et al. 2016). Moreover, Ca^{2+} influx resulting from P2X7 activation mediates the formation of Ca^{2+}/Calmodulin (Calm) complexes, which activate the phosphatase calcineurin. Calcineurin dephosphorylates the transcription factor nuclear factor of activated T-cells (NFAT), which then translocates into the nucleus and activates the transcription of IL-2 (Fig. 2) (Yip, Woehrle et al. 2009).

2. Pore formation

Activation of P2X7 is associated with pore formation allowing the influx of $Ca2^{+}$ and Na^{+} ions and efflux of K^{+} ions as well as the uptake of larger molecules up to a molecular weight of 900 Da (Surprenant and North 2009, Idzko, Ferrari et al. 2014). Different mechanisms are in discussion: One the one hand dilatation of the P2X7 ion channel itself might mediate the in- and efflux. On the other hand, P2X7 activation might lead to direct interaction with plasma membrane spanning glycoprotein Pannexin-1, which builds non-selective hexameric channels (Gulbransen, Bashashati et al. 2012). As mentioned before, the latest findings revealed GSDMD as an important element in pore formation following P2X7 mediated inflammasome activation, which is in turn tightly connected to P2X7 binding (Ding, Wang et al. 2016).

3. Ectodomain shedding of cell surface proteins

P2X7 activation catalyzes the rapid loss of diverse ectodomains. Upon P2X7 activation, proteases of the A Disintegrin And Metalloproteinase (ADAM) family, such as ADAM 10 and 17, proteolytically cleave a variety of cell surface molecules after P2X7 activation including CD62L, CD27, CD23, IL-6 receptor (IL-6R) and TNF-α (Fig 2) (Blobel 2005, Moon, Na et al. 2006, Le Gall, Bobe et al. 2009, Garbers, Janner et al. 2011). Interestingly, the membrane proteins may display new functional activities and extended pro-inflammatory potentials after shedding: The membrane bound IL-6R is inter alia expressed by monocytes and transmits pro-inflammatory signals upon binding of cytokine IL-6. Cleaved by ADAM 17 the soluble IL-6R can extend its pro-inflammatory activity through a mechanism called “trans-signaling” (Rose-John 2012). Endothelial cells not expressing the IL-6R and normally

unresponsive to IL-6 can be stimulated by the soluble IL-6R/IL-6 complex and secrete mononuclear cell attracting cytokine 1 (DeLeo 2007). Trans signaling is presumably also involved in the induction of Th17 cells, as studies demonstrated an increased IL-6-mediated induction of Th17 cells in the presence of IL-6R (Dominitzki, Fantini et al. 2007). CD27 is a TNF-α receptor expressed on T, B and NK cells that binds CD70, which is expressed on activated T and B cells. Soluble CD27 enhances activation and proliferation in T cells (Huang, Jochems et al. 2013), induces IgG production in B cells (Dang, Nilsson et al. 2012) and increased serum concentrations are found in systemic lupus erythematosus (Font, Pallares et al. 1996), B cell malignancies (Ciccarelli, Yang et al. 2009) and HIV-1 infection (De Milito, Aleman et al. 2002). The IgE receptor CD23 is expressed on B cells and cleaved by ADAM 10. Soluble CD23 positively regulates IgE synthesis and presumably enhances allergic immune responses (Cooper, Hobson et al. 2012). Adhesion molecule CD62L (L-selectin) is commonly found on lymphocytes and mediates attachment of T and B cells to high endothelial venules in the secondary lymphatic organs as well as to endothelium at sites of inflammation. After being cleaved by ADAM 17, soluble CD62L (sCD62L) promotes the maturation of DCs via upregulation of TLR4 (Ye, Liu et al. 2017) and several diseases correlate with high serum concentrations of sCD62L including active systemic lupus erythematosus (Font, Pallares et al. 1996), bronchial asthma (Nadi, Hajilooi et al. 2015) and acute and chronic forms of leukemia (Olejnik 1999, Sopper, Mustjoki et al. 2017).

4. Externalization of phosphatidylserine and induction of cell death

The redistribution of phosphatidylserine (PS) on the cell membrane is well known to promote phagocytic clearance of apoptotic cells (Fadok, Bratton et al. 2000). Prolonged P2X7 activation triggers irreversible PS externalization and apoptotic cell death in T cells. Conversely, short P2X7 activation (<30 min.) induces reversible PS externalization (Fig 2) and membrane blebbing, but no cell death (Mackenzie, Young et al. 2005). Bleb formation is a phenomenon at which parts of the cell membrane reversibly protrude and retract. The regulation and physiological relevance of P2X7-associated membrane blebbing are not clarified yet (Qu and Dubyak 2009).

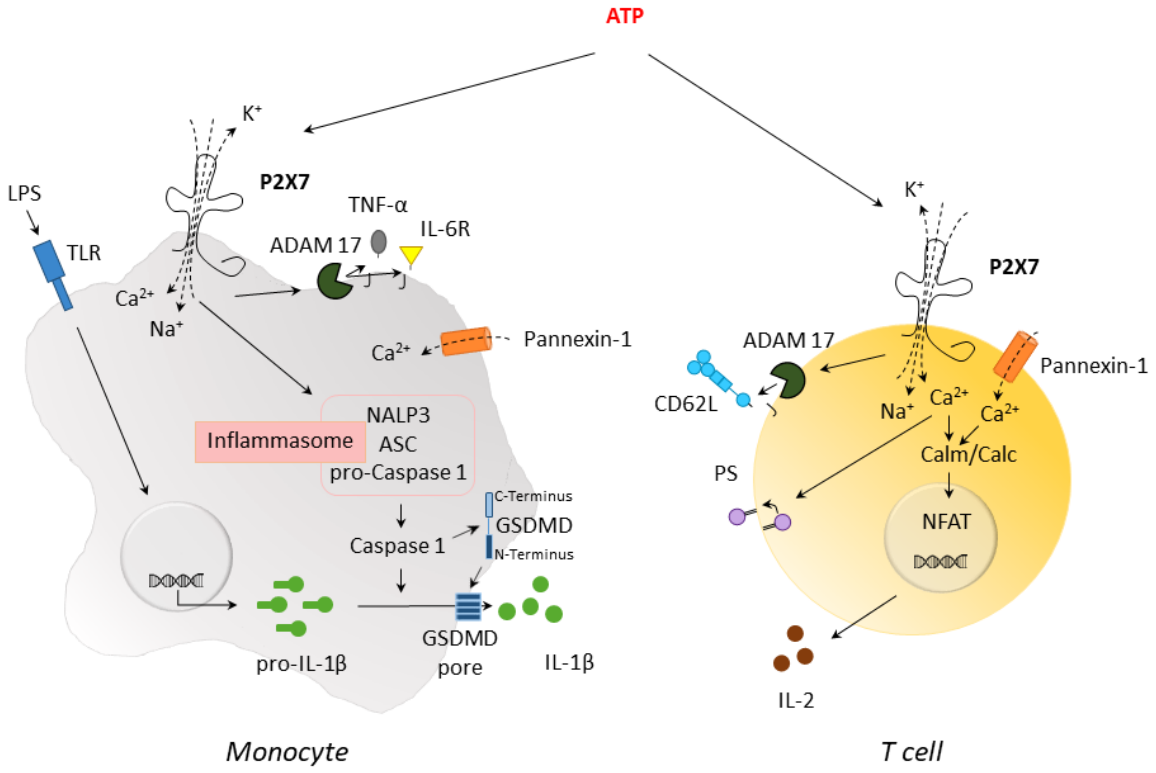


Fig. 2: Immune responses triggered by P2X7 activation. Gating of P2X7 effects the efflux of K^+ ions and the influx of Na^+ and Ca^{2+} ions. Pannexin-1 pore formation also allows the influx of molecules. In the monocyte the subsequent activation of the inflammasome, consisting of the NACHT, LRR and PYD-containing protein 3 (NALP3) and the apoptosis-associated speck-like protein containing a CARD (ASC), leads to cleavage of pro-Caspase 1 into Caspase 1. Caspase 1 cleaves Gasdermin D (GSDMD) between its N- and C-Terminus, which leads to GSDMD pore formation after insertion of the N-Terminus into the cell membrane. Interaction of the Toll-like receptor (TLR) with lipopolysaccharides (LPS) triggers the production of pro-IL-1β. Caspase 1 cleaves pro-IL-1β into active IL-1β, which is released from the cell via the GSDMD pore. A Disintegrin And Metalloproteinase 17 (ADAM 17) cleaves Tumor necrosis factor α (TNF-α) and Interleukin 6 receptor (IL-6R) from the cell surface. In the T cell P2X7 activation effects the shedding of ectodomain CD62L via A Disintegrin And Metalloproteinase 17 (ADAM 17) and the influx of Ca^{2+} ions triggers the externalization of Phosphatidylserine (PS). The increased intracellular concentration of Ca^{2+} also effects the formation of Ca^{2+}/Calmodulin (Calm) complexes, which activate phosphatase Calcineurin (Calc). Calcineurin dephosphorylates the transcription factor nuclear factor of activated T-cells (NFAT), which translocates into the nucleus and activates the transcription of IL-2. IL-2 is finally released from the cell (adapted from Danquah 2012).

2.3 P2X7 in disease

The P2X7 receptor is a key mediator in a multitude of immunological reactions. Thus, it is not surprising, that P2X7 is involved in the pathogenesis of a variety of diseases, including autoimmune and infectious diseases as well as tumor growth (see Table 1). Induction of genetic P2X7-deficiency and pharmacological receptor manipulation are common methods to assess the role of P2X7 in disease development in vitro and in vivo. The following table gives an overview over a number of diseases, where an impact of P2X7 on disease development

was determined. The experiments were performed in vivo using mouse/rat models of disease and injury. Three studies marked with "in vitro" were performed using cell lines.

Disease model	Approach	Reagent/Type of KO	Result	Source
Autoimmune diseases				
Autoimmune encephalomyelitis	P2X7 blockade	Brilliant Blue G	Reduced astrogliosis, improved neurological symptoms	(Grygorowicz, Welniak-Kaminska et al. 2016)
Autoimmune exocrinopathy	P2X7 blockade	A438079	Reduced salivary gland inflammation	(Khalafalla, Woods et al. 2017)
Autoimmune glomerulonephritis	P2X7 blockade	nanobody 13A7-HLE	Reduced severity of nephritis	(Danquah, Meyer-Schwesinger et al. 2016)
Autoimmune myocarditis	P2X7 blockade	A740003	Resistance to myocarditis development	(Zempo, Sugita et al. 2015)
Type I Diabetes	P2X7 deficiency	P2X7 KO mice	Resistance to Diabetes I induction	(Vieira, Nanini et al. 2016)
Systemic Lupus erythematosus	P2X7 blockade	Brilliant Blue G	Reduced severity of Lupus nephritis	(Zhao, Wang et al. 2013)
Neoplastic diseases				
Bronchial carcinoma	P2X7 blockade (in vitro)	A438079 or AZ10606120	Decreased migration of human H292 and PC-9 lung cancer cells	(Takai, Tsukimoto et al. 2014)
Colon carcinoma	P2X7 blockade	Periodate-oxidized ATP (oATP)	Decrease in tumor growth	(Adinolfi, Raffaghello et al. 2012)
Glioblastoma multiforme (GM)	P2X7 activation (in vitro)	ATP or 2'(3')-O-(4-benzoylbenzoyl)-ATP (Bz-ATP)	Potentiation of cytostatic effect of Temozolomide on human GM stem-like cells	(D'Alimonte, Nargi et al. 2015)
Breast cancer	P2X7 deficiency (in vitro)	P2X7 knockdown in T47D cancer cells	Decreased invasion and migration of human T47D cancer cells	(Xia, Yu et al. 2015)
Melanoma	P2X7 blockade	AZ10606120	Decrease in tumor growth	(Adinolfi, Raffaghello et al. 2012)

Neuroblastoma	P2X7 blockade	AZ10606120 or A740003	Decrease in tumor growth	(Amoroso, Capece et al. 2015)
Pancreatic ductal adenocarcinoma	P2X7 blockade	AZ10606120	Decrease in tumor growth	(Giannuzzo, Saccomano et al. 2016)
Infectious diseases				
Chlamydia muridarum	P2X7 deficiency	P2X7 KO mice	Enhanced bacterial growth in cervical and vaginal epithelial cells, enhanced inflammation in endocervix, oviduct and mesosalpingeal tissues	(Darville, Welter-Stahl et al. 2007)
Chlamydia psittaci	P2X7 activation	ATP	Inhibition of bacterial growth in macrophages	(Coutinho-Silva, Stahl et al. 2003)
Leishmania amazonensis	P2X7 deficiency	P2X7 KO mice	Increased parasitic burden in cutaneous lesions, increased lesion size	(Figliuolo, Chaves et al. 2017)
Mycobacterium tuberculosis	P2X7 deficiency	P2X7 KO mice	Increased bacterial burden in lung tissue	(Santos, Rodrigues-Junior et al. 2013)
Toxoplasma gondii	P2X7 deficiency	P2X7 KO mice	Increased parasitic burden in macrophages	(Lees, Fuller et al. 2010)
CNS/PNS				
Alzheimer's disease	P2X7 blockade	Brilliant Blue G	Reduced amyloid plaque formation in brain hippocampal structures	(Miras-Portugal, Diaz-Hernandez et al. 2015)
Amyotrophic lateral sclerosis	P2X7 blockade	Brilliant Blue G	Enhanced motor neuron survival, reduced microgliosis, improved motor performance	(Apolloni, Amadio et al. 2014)
Cerebral ischemia	P2X7 blockade	Brilliant Blue G	Reduced infarct volume, attenuated motor symptoms	(Cisneros-Mejorado, Gottlieb et al. 2015)
Huntington's disease	P2X7 blockade	Brilliant Blue G	Inhibition of neuronal apoptosis,	(Diaz-Hernandez,

			reduced motor-coordination deficits	Diez-Zaera et al. 2009)
Neuropathic pain	P2X7 deficiency	P2X7 KO mice	Inhibition of mechanical and thermal hypersensitivity	(Chessell, Hatcher et al. 2005)
Parkinson's disease	P2X7 blockade	Brilliant Blue G	Decreased loss of dopaminergic neurons	(Wang, Xie et al. 2017)
Temporal lobe epilepsy	P2X7 blockade	JNJ-47965567	Reduced spontaneous seizures, reduced gliosis	(Jimenez-Pacheco, Diaz-Hernandez et al. 2016)
Heart/Vessels				
Atherosclerosis	P2X7 deficiency	P2X7 KO mice	Reduced plaque formation	(Stachon, Heidenreich et al. 2017)
Heart transplantation	P2X7 blockade	oATP	Long-term heart transplant survival	(Vergani, Tezza et al. 2013)
Myocardial ischemia-reperfusion injury	P2X7 blockade	Brilliant Blue G	Reduced cell death, increased myocardial function	(Granado, Amor et al. 2015)
Lung				
Allergic airway inflammation	P2X7 blockade	KN62	Resistance to bronchial asthma	(Muller, Vieira et al. 2011)
Cigarette-smoke-induced lung inflammation and emphysema	P2X7 deficiency	P2X7 KO mice	Reduced lung inflammation, prevention of emphysema	(Lucattelli, Cicko et al. 2011)
Lung fibrosis	P2X7 deficiency	P2X7 KO mice	Reduced lung inflammation and fibrosos	(Riteau, Gasse et al. 2010)
Neurogenic pulmonary edema (NPE)	P2X7 blockade	Brilliant Blue G	Prevention of NPE after subarachnoid hemorrhage	(Chen, Zhu et al. 2014)
Kidney				
Ischemic acute kidney injury	P2X7 blockade	A438079	Reduced renal dysfunction and tubular damage	(Yan, Bai et al. 2015)
Salt-sensitive hypertension	P2X7 deficiency	P2X7 KO mice	Reduced renal hypertension, reduced kidney inflammation and fibrosis	(Ji, Naito et al. 2012)
Gastrointestinal tract				

Chemically-induced colitis	P2X7 blockade	A438079	Reduced inflammation in colon tissue	(Wan, Liu et al. 2016)
Liver				
Non-alcoholic steatohepatitis	P2X7 deficiency	P2X7 KO mice	Reduced liver inflammation and fibrosis	(Das, Seth et al. 2013)
Skin				
Allergic contact dermatitis	P2X7 blockade	nanobody 13A7	Reduced skin inflammation	(Danquah, Meyer-Schwesinger et al. 2016)
Muscle				
Duchenne muscular dystrophy	P2X7 blockade	A438079	Improved muscle and bone structure, reduced inflammation, fibrosis and cognitive impairment	(Sinadinos, Young et al. 2015)
Bone				
Osteoarthritis	P2X7 blockade	AZD9056	Reduced inflammation and pain	(Hu, Yang et al. 2016)

Table 1: P2X7-mediated diseases and conditions. Studies were performed in vivo using mouse/rat models of disease and injury. Three studies marked with "in vitro" were performed using cell lines. "P2X7 blockade" = pharmacological inhibition of P2X7. "P2X7 activation" = pharmacological activation of P2X7. "P2X7 deficiency" = induced genetic P2X7 deficiency. "Reagents/Type of KO" indicates the specific P2X7 antagonist/agonist used for P2X7 blockade/activation and the respective type of KO used for induction of P2X7 deficiency.

P2X7 signaling is a friend and foe in disease development: On the one hand, P2X7 activation triggers exuberant immune responses, leading to immune-mediated pathology like post-ischemic inflammation, chronic inflammatory processes including autoimmune and allergic disorders, and to fibrotic remodeling. P2X7 signaling also promotes tumor growth, though the underlying mechanisms remain to be clarified. P2X7-deficiency and pharmacologic P2X7 blockade effectively prevented disease development in various conditions as summarized in Table 1. On the other hand, sufficient P2X7 activation is essential in the defense of infectious diseases caused by intracellular bacteria and protozoa. In these cases, P2X7 stimulation, prevented disease progression. In addition, P2X7 activation enhances the cytostatic effect of chemotherapy in disease models of brain cancer, despite the effect of tumor growth promotion mentioned previously (see Tab. 1 for reference). Taken together, these findings emphasize the complex effects of P2X7 signaling in the human body and reveal P2X7 as a highly attractive

therapeutic target. P2X7 antagonists are promising therapeutic drugs in acute and chronic inflammation as well as neoplastic diseases while P2X7 agonists might represent a novel class of therapeutics in infectious diseases.

2.4 Nanobodies as tools for P2X7 research and potential therapeutic drugs

In the last years, a range of substances targeting P2X7 for therapeutic purposes has been generated. Small molecule P2X7-antagonists had first shown promising results in animal models but clinical trials revealed the need for improvement e.g. regarding toxicity and selectivity (Keystone, Wang et al. 2012, Stock, Bloom et al. 2012). Antibodies (abs) got into focus as anti-P2X7 therapeutics as they combine favorable characteristics like high specificity and simple pharmacodynamics (Bumbaca, Boswell et al. 2012). Nanobodies (nbs) are a novel class of proteins derived from Llama-produced IgG2 and IgG3 abs. Besides the classical IgG1 abs (cAbs), consisting of light and heavy chains, Camelids are able to produce IgG2 and IgG3 subclasses, lacking the light chains and CH1 domains, referred to as heavy chain only abs (hcAbs). Whereas conventional abs recognize their specific antigen with a paratope formed by the variable domains of the light and heavy chains, hcAbs use a single variable domain (variable domain of the heavy chain only antibody: VHH) for antigen recognition. The VHHs are referred to as “nanobodies” due to their small size. Distinct structural properties including their size enable nbs to reach epitopes located in crevices of protein surfaces that are inaccessible for conventional antibodies. Further advantageous characteristics of nbs are the high stability regarding changes in temperature and pH level, a high solubility, good tissue penetration and a quick systemic clearance as well as low immunogenicity and easy production in eu- and prokaryotic cells at relatively low costs ((Wesolowski, Alzogaray et al. 2009, Siontorou 2013, Unciti-Broceta, Del Castillo et al. 2013, Bannas, Well et al. 2014).

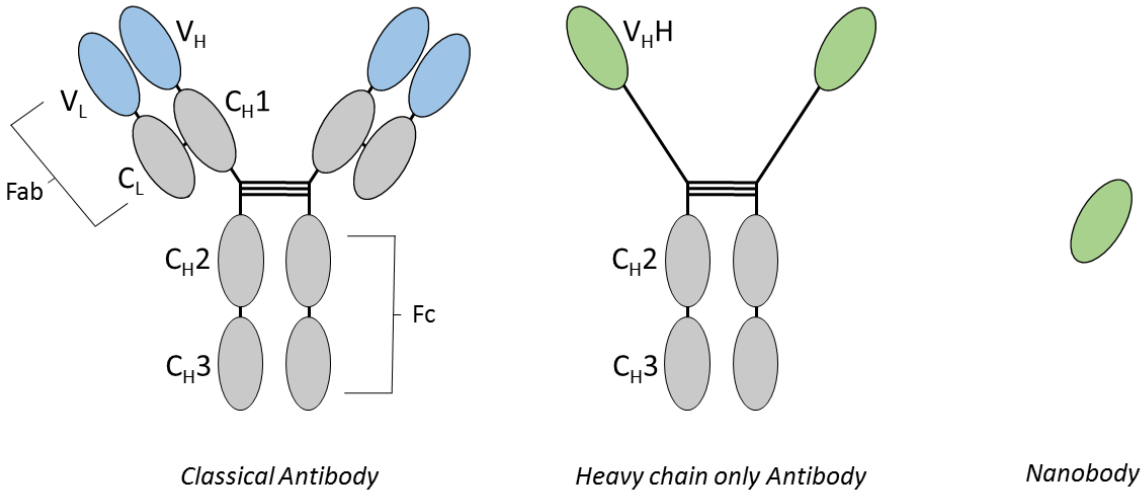


Fig. 3: Classical antibody (cAb), heavy chain only antibody (hcAb) and nanobody (nb). (a) cAbs consist of two light and two heavy polypeptide chains that are connected by disulfide bonds. Each light chain is composed of one variable and one constant domain (V_L and $C_{L)}$, each heavy chain is composed of one variable domain (V_H) and three constant domains (C_H1 - 3). The fragment of antigen binding (Fab) consists of the combined V_L- C_L and V_H-C_H1, the fragment of crystallization (Fc) is formed by C_H2 – 3. Combination of one V_L and one V_H forms the antigen binding paratope whereas the Fc fragment mediates the effector function. **(b)** hcAbs only consist of two heavy chains lacking the C_H1 domain. The variable domain of the heavy chain only antibody (V_HH) mediates the antigen recognition. **(c)** The V_HHs are referred to as "Nanobodies" (adapted from Danquah 2012)

Five different nbs by Ablynx are currently being tested in human clinical trials. The most advanced trial, Anti-von Willebrand Factor-Nanobody Caplacizumab, has successfully completed Phase III study on acquired Thrombotic Thrombocytopenic Purpura. Caplacizumab blocks incorrectly processed von-Willebrand-Factor multimers by binding to its A1 domains therefore preventing the adhesion of platelets and formation of blood clots (Ablynx website). Nbs targeting P2X7 have not yet been tested in human clinical trials and there is a remaining need for research on these. Nevertheless, in-vivo experiments with mice indicated the therapeutic utility of nbs in P2X7-mediated diseases: in mouse models of experimental glomerulonephritis and experimental allergic contact dermatitis the protective effect of anti-P2X7 nbs on disease development and progression could already be demonstrated. Moreover, the anti-P2X7 nb Dano1 showed a 1000fold higher potency in preventing pro-inflammatory cytokine release in endotoxin-treated human blood, than small-molecule P2X7 antagonists currently tested in human clinical trials (Danquah, Meyer-Schwesinger et al. 2016).

2.4.1 Nanobody formats

One great advantage of the nb technology is the possibility of flexible reformatting. In that manner, the properties of the nbs can be modified purposefully e.g. regarding potency and half-life. The nbs used in our project were manufactured and kindly provided by Dr. Welbeck Danquah and Prof. Friedrich Koch-Nolte. The group of Prof. Koch-Nolte has generated a series of highly specific mouse and human anti-P2X7 nbs that allow visualization of P2X7 on cell surfaces and promote inhibiting or enhancing effects on P2X7 function. They have designed nbs in different formats: monomers, dimers and nb-fragment of crystallization (nb-Fc) fusion proteins. Nb-dimers were constructed by genetic linkage of two nb-monomers with a 35-amino acid linker. This nb construct has an increased receptor affinity and higher functional potency compared to the single monomers. Moreover, the in vivo half-life of certain nb-dimers could be extended by genetic linkage to an albumin-binding nb. The fusion proteins were constructed by the fusion of two nb monomers with the Fc domain of a mouse or rabbit IgG (Scheuplein, Rissiek et al. 2010). Analog to the nb-dimers this nb construct displayed an increased receptor affinity and a higher functional potency compared to the original nb-monomer. In addition they facilitated P2X7 detection using Fluorescence activated cell sorting (FACS) by labeling the Fc domain with a fluorochrome (Danquah, Meyer-Schwesinger et al. 2016).

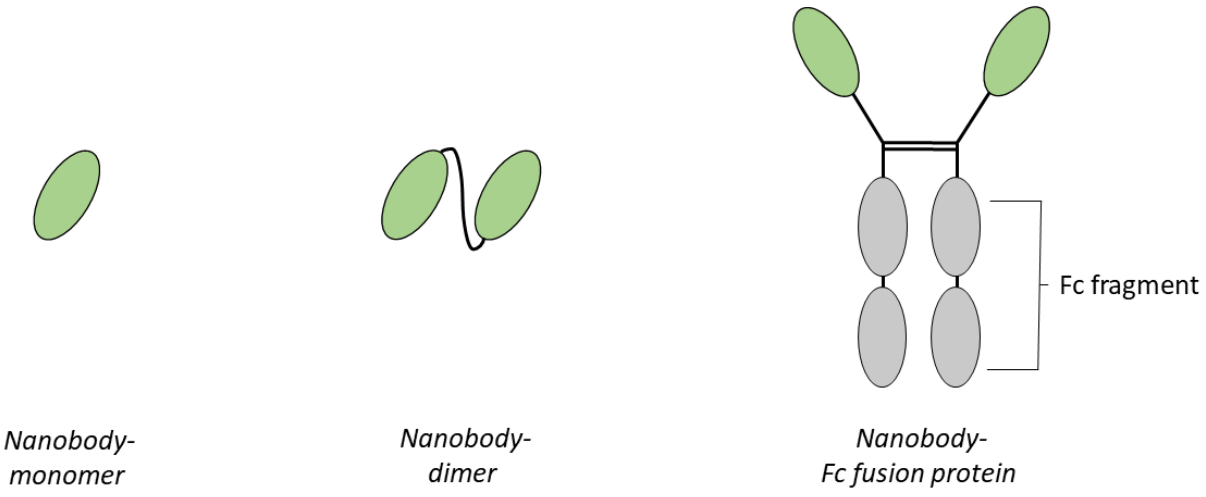


Fig. 4: Nanobodies in monomeric, dimeric and Fc fusion protein formats. (adapted from Danquah 2012**)**

3 Materials and Methods

3.1 Materials

3.1.1 Media and supplements

Bovine serum albumin (BSA)	PAA
Dimethyl sulfoxide cell culture grade (DMSO)	Applichem
DMEM + GlutaMAX	Gibco
Dulbecco's Phosphate Buffered Saline (PBS)	Gibco
Dulbecco's Modified Eagle Medium (DMEM)	Gibco
Ethylenediaminetetraacetic acid-Trypsin	Gibco
Fetal calf serum (FCS)	Biochrome AG
Hanks' balanced salt solution (+ Ca^{2+}/+ Mg^{3+})	Gibco
L-Glutamine, 200 mM	Gibco
Lymphocyte Separation medium	Biochrome AG
Penicillin/Streptomycin	Gibco
RPMI 1640	Gibco
Tween-20	ICI-Americas

3.1.2 Complete media, solutions and buffers

Immune cell culture medium:	10% FCS 1% Penicillin-Streptomycin L-Glutamine 2 mmM in RPMI
Microglia culture medium:	10% FCS 1% Penicillin-Streptomycin in DMEM + GlutaMAX
Neuron culture medium:	10 % FCS 1% Penicillin-Streptomycin 2 mM Glutamine 1 mM Pyruvate in DMEM
Freezing medium:	65% RPMI 25% FCS

	10% DMSO
FACS buffer:	0,1% BSA 0,02% sodium azide in 1x PBS
Washing buffer:	0,05 % Tween-20 in 1x PBS
Stop solution:	0,5 M H_2SO_4 in deionized H_2O

3.1.3 Cell lines

Human microglia cells: CHME-5 (Janabi, Peudenier et al. 1995)

Kindly provided by Division of Neuroscience, Institute of Experimental Neurology, San Raffaele Scientific Institute

Human neuroblastoma cells: SH-SY5Y (Biedler, Roffler-Tarlov et al. 1978)

Kindly provided by Division of Neuroscience, Institute of Experimental Neurology, San Raffaele Scientific Institute

3.1.4 Reagents for cell stimulation

Adenosinetriphosphate (ATP)	Sigma-Aldrich
Anti-CD3 (clone OKT3)	Bioxcell
Ionomycin	Sigma-Aldrich
Lipopolysaccharides (LPS)	Sigma-Aldrich
Probenecid	Sigma-Aldrich
Retinoic Acid (RA)	Sigma-Aldrich

3.1.5 Cytokines and growth factors

IFN-γ	R & D
rh GM-CSF	PeproTech
rh IL-2	Hoffmann-Roche
rh IL-4	Biolegend

3.1.6 Reagents for cell culture

Sodium pyruvate	Gibco
Trypan blue solution, 0.4%	Sigma-Aldrich

3.1.7 Reagents for flow cytometry

DAPI (4'6-diamidino-2-phenylindole)	Invitrogen
FACS flow	BD Bioscience
Fluo4AM	Sigma-Aldrich
Live/dead fixable dead cell stain kit	Invitrogen
Lysing solution	BD Bioscience

3.1.8 Antibodies

Antigen	*Fluorochrome conjugate*	*Clone*	*Manufacturer*
CD3	PerCPCy5.5	OKT3	eBioscience
CD3	APC	-	-
CD4	Alexa Fluor 488	RPA-T4	Biolegend
CD4	APCCy7	RPA-T4	Biolegend
CD8	V500	RPA-T8	BD Bioscience
CD8	PECy7	SK1	Biolegend
CD8β	PE	2ST8.5H7	BD Bioscience
CD11c	PE	3.9	Biolegend
CD14	V450	MφP9	BD Bioscience
CD14	PerCPCy5.5	HCD14	Biolegend
CD16	APCCy7	3G8	Biolegend
CD19	PECy7	HIB 19	Biolegend
CD25	PE	2A4	BD Bioscience
CD25	BV 421	BC96	Biolegend
CD38	Alexa Fluor 488	HIT2	Biolegend
CD39	PECy7	A1	Biolegend
CD45	V500	HI30	BD Bioscience
CD45RA	BV421	HI100	Biolegend
CD56	PECy7	B159	BD Bioscience

CD62L	PE	DREG-56	BD Bioscience
CD80	PE	L307.4	BD Bioscience
CD123	PECy7	6H6	Biolegend
CD127	PerCPCy5.5	A019D5	Biolegend
CD161	BV421	HP-3G10	Biolegend
Lineage	FITC	UCHT1/HCD1	Biolegend
HLA-DR	FITC	G46-6	BD Bioscience
HLA-DR	PB	L243	Biolegend
IgG1	PerCPCy5.5	Oct-03	eBioscience
TCR γδ	PE	11F2	BD Bioscience
TCR Vα7.2	PE	-	BD Bioscience
TCR Vα24	FITC	6B11	Biolegend

3.1.9 Nanobodies

Antigen	*Fluorochrome conjugate*	*Clone*	*Format*	*Manufacturer*
P2X7	-	Dano1	rbFc	AG Nolte
P2X7	AF 647	Dano1	rbFc	AG Nolte
P2X7	-	Dano1	dimer	AG Nolte
P2X7	-	Dano3	rbFc	AG Nolte
P2X7	AF 647	Dano3	rbFc	AG Nolte
-	-	S+16	monomer	AG Nolte

3.1.10 ELISA

ELISA plate	Maxisorp
Human IL-1β ELISA Kit “Ready-SET-Go!”	eBioscience

3.1.11 Consumables

Cryo tubes	Greiner
Eppendorf tubes 0.5 ml, 1.5 ml	Eppendorf

FACS tubes	Sarstedt
Falcon tubes 15 ml, 50 ml	Falcon
Parafilm M	Pechiney
Pipette tips	Sarstedt
Polystyrene round bottom tubes	Sarstedt
Serological pipets	Falcon
Tissue culture flasks 15 ml, 75 ml	Sarstedt
Tissue culture plates 12 wells, 48 wells, 96 wells	Sarstedt

3.1.12 Equipment

Benchtop refrigerated centrifuges	Eppendorf, Heraeus Sepatech
ELISA-Reader, VICTOR³	Perkin Elmer
FACS CantoII	BD Bioscience
Freezers	Liebherr
Fridges	Liebherr
Incubator, Incu Safe Thermo Scientific	Sanyo
Microscopes	Zeiss
Neubauer chamber	Marienfeld
Pipets	Eppendorf, Gilson
Racks	Roth
Sterile bank, class II standard	Thermo Scientific
Vortex Genie 2, class II standard	Thermo scientific
Waterbath	GFL

3.1.13 Software

Adobe Photoshop CS2	Adobe Systems Incorporated
BD FACSDiva™6.0	BD Bioscience
GraphPad Prism 5.0	Graph Pad Software

3.2 Methods

3.2.1 Donors

Blood samples were obtained from 17 healthy Donors. Informed consent was obtained from all study subjects.

3.2.2 Isolation of human cells

3.2.2.1 Isolation of peripheral blood mononuclear cells

Isolation of human peripheral blood mononuclear cells (PBMCs) was achieved by density gradient centrifugation. 20 ml of lymphocyte separation medium were layered with 30 ml of human peripheral whole blood diluted 1:2 in PBS. The mononuclear cells were separated from the other blood components by 30 min. centrifugation at 400x g and 20 °C without brakes. On the basis of their size, shape and density the mononuclear cells migrate through the Lymphocyte separation medium and form a characteristic layer between the medium and the plasma. These cells were aspirated and washed two times in 50 ml of cold PBS (1. 10 min. at 1800 rpm, 2. 5 min. at 1500 rpm). Cell counting was performed in a Neubauer counting chamber and dead cells were excluded by staining using Trypan blue. The cells were either directly used for further experiments or frozen.

3.2.2.2 Isolation of monocytes from peripheral blood mononuclear cells

Monocytes were isolated from PBMCs by magnetic cell sorting (MACS). PBMCs were incubated with CD14 MicroBeads for 15 min. and flushed through a column placed in a magnetic field. Due to the magnetic pull between the magnetic field and the magnetic beads the CD14+ cells remain in the column and are separated from the rest of the cell suspension. After retraction of the column from the magnetic field and subsequent flushing the labeled cells were collected in a Falcon tube and used for further experiments.

3.2.3 Cell culture

3.2.3.1 Culture of human microglia cell line CHME-5

The microglial cells were sowed at a density of 1 x 10^6 cells /25 cm² in a tissue culture flask and cultured in 10 ml microglia culture medium. Passage of the cells was done every 4^{th} day as follows: The microglia culture medium was taken off by aspiration and the cells were

washed with 5 ml PBS. After taking off the PBS cells were incubated with 500 µl Trypsin for 10 min. at 37 °C. The detachment of the adherent microglial cells was controlled by light microscopy. Cells were washed again with 5 ml PBS, centrifuged for 5 min. at 1500 rpm and resuspended in 1 ml warm microglia culture medium. Cells were then counted with a Neubauer counting chamber and sowed again or used for experiments.

3.2.3.2 Culture of human neuroblastoma cell line SH-SY5Y

The neuronal cells were sowed at a density of 5 x 10^5 cells/25 cm^2 in a tissue culture flask and cultured in 10 ml neuron culture medium. Passage of the cells was done every 3rd day as follows: The neuron culture medium was taken off by aspiration and the cells were washed with 5 ml PBS. After taking off the PBS cells were incubated with 500 µl Trypsin for 10 min. at 37 °C. The detachment of the adherent neuronal cells was controlled by light microscopy. Cells were washed again with 5 ml PBS, centrifuged for 5 min. at 1500 rpm and resuspended in 1 ml warm Neuron culture medium. Cells were then counted with a Neubauer counting chamber and sawn again or used for experiments.

3.2.4 Cell stimulation

3.2.4.1 Activation of $CD4^+$, $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T cells with anti-CD3 and retinoic acid

2 x 10^6 PBMCs were incubated with 500 ng/ml soluble anti-CD3 at 37 °C for 48 hrs. After washing the cells were plated on a 24-well-plate at a density of 5 x 10^5 cells/well and incubated with 0, 10 or 50 nM retinoic acid (RA) and additional 20 U/ml Interleukin 2 (IL-2) for further 3 days. The preparation without RA served as negative control. Cells were stained as described in section 4.2.6.2 and analyzed by flow cytometry before the treatment and at day 5.

3.2.4.2 Stimulation of isolated monocytes with LPS and IFN-γ

Monocytes were isolated from PBMCs by MACS as described in section 4.2.2.2. 5 x 10^5 cells/tube (Polystyrene Round Bottom Tube) were incubated in 1 ml RPMI and stimulated with 0.5 µg/ml LPS or 0.5 µg/ml LPS + 50 ng/ml Interferon γ (IFN-γ) for 48 h at 37 °C. A preparation containing no stimuli served as negative control. The cells were stained as

described in section 4.2.6.2 and analyzed by flow cytometry before the treatment and after 48 h.

3.2.5 In vitro generation of monocyte-derived dendritic cells

Monocytes were isolated from PBMCs by MACS as described in section 4.2.2.2. The cells were sowed at a density of 1 x 10^5 cells/cm² and incubated with 0.1 µg/ml Granulocyte macrophage colony-stimulating factor (GM-CSF) + 50 ng/ml IL-4 for 7 days. A preparation containing no stimuli was used as control. The phenotype was analyzed by light microscopy and flow cytometry respectively at day 0 and 7.

3.2.6 Flow cytometry

Flow cytometry (also FACS, fluorescence-activated cell sorting) allows the sorting of cells by means of their size, granularity and unique cell markers. The cells pass through the cytometer as a hydrodynamically focused single-cell-stream. As they pass a laser beam the light is scattered and collected by detectors. The forward-scattered light (FSC) is indicative of the size, the sideward-scattered light (SSC) correlates with the granularity of the passing cell. Cell populations can be further specified by detection of unique cell surface and intracellular molecules using Fluorochrome conjugated antibodies. After excitation with light of an adequate wave length fluorescence is emitted from the fluorochrome proportional to the expression of the molecule the antibody is directed against. The fluorescence signal is transmitted by optical band pass and long filters and collected by optical detectors. After conversion into electrical impulses and subsequent transformation the digital information is visualized in plotted graphs.
In this project a FACS CantoII cytometer (BD Biosciences) was used for flow cytometry. For the analysis of the data BD FACSDiva™6.0 software was used.

3.2.6.1 Cell surface staining for flow cytometry

50 µl of whole blood or 90 µl of PBMCs were incubated with 50 µl or respectively 10 µl of the required antibody cocktail for 30 min. in the dark at room temperature (RT). In case whole blood was used, a 10 min. erythrocyte lysis with 1 ml lysing solution was performed after the staining. Finally the cells were washed with 1 ml PBS (centrifugation at 1500 rpm, 5 min.) and resuspended in 100 µl FACS buffer.

3.2.6.2 P2X7 staining for flow cytometry

P2X7 staining ("unblocked") and blocking analysis ("blocked") were performed in parallel. For the preparation "blocked" 50 µl whole blood or 90 µl PBMCs were incubated with 3 µl of each anti-P2X7 nb-Fc fusion protein of Dano1 (final concentration of 33 ng/µl) and Dano3 (final concentration of 33 ng/µl) for 30 min. in the dark at RT. For the preparation "unblocked" 50 µl whole blood or 90 µl PBMCs were incubated with 6 µl anti-mouse nb S+16 (final concentration of 7.92 ng/µl) for 30 min. in the dark at RT . An incubation with 50 µl antibody cocktail against different cell markers (described in section 4.2.6.1) was performed in parallel. Both preparations were subsequently incubated with 1 µl of each Alexa Fluor 647-conjugated anti-P2X7 nb-Fc fusion protein of Dano1 (final concentration: 2.5 ng/µl) and Dano3 (final concentration: 2.5 ng/µl) for further 30 min. in the dark at 4 °C. In case whole blood was used, a 10 min. erythrocyte lysis with 1 ml lysing solution was performed after the staining. Finally the cells were washed with 1 ml PBS and resuspended in 100 µl FACS buffer.

3.2.7 P2X7-mediated shedding of CD62L and DAPI uptake

The shedding of CD62L and the uptake of DAPI after P2X7 activation were analyzed in parallel. 90 µl whole blood were incubated with 2 µM DAPI and different concentrations of ATP for 30 min at 37 °C. Blocking was achieved by incubation with 500 ng/ml nb-dimer of Dano1 for 10 min. prior to the treatment with ATP and DAPI. After washing (centrifugation at 1800 rpm, 5 min.) the cells were stained with abs against CD62L and cell markers as described in section 4.2.6.1. The cells were analyzed by FACS.

3.2.8 P2X7-mediated IL-1β release

3.5 ml whole blood were incubated with 1 µg/ml LPS for 1.5 h at 37 °C. The blood was then divided into samples à 0.5 ml and incubated with different concentrations of ATP for 30 min. at 37 °C. One preparation without ATP served as negative control. Blocking was achieved by incubation with 500 ng/ml nb-dimer of Dano1 10 min. prior to the LPS treatment. The top ATP concentration was applied to this sample. All samples were centrifuged at 2800 rpm for 5 min. and the supernatant (blood plasma) aspirated. The plasma was diluted 1:10 in RPMI and directly used for further experiments or frozen. In case isolated monocytes, MoDCs or

microglia cells were used, the experiment was performed with 1 x 10^5 or 3 x 10^5 cells/sample. The cells were diluted in RPMI to a volume of 0.5 ml/sample.

3.2.9 IL-1β ELISA

The measurement of human IL-1β in the blood plasma was performed with a commercial sandwich-ELISA kit "Ready-SET-Go!" by eBioscience according to the manufactures protocol.

4 Results

4.1 P2X7 expression in primary human leukocytes

In order to determine the expression of P2X7 in the different leukocyte subsets we designed panels of antibodies containing lineage markers for the different immune cell types in combination with P2X7-specific nbs (Tab. 2). The cell types to be analyzed included cells of the innate immune system (neutrophils, eosinophils, basophils, monocytes, NK cells, mDCs and pDCs) and of the adaptive as well as the innate-like branch of the immune system (B cells and the different subtypes of T cells). To stain for P2X7 we used Alexa Fluor 647-conjugated anti-P2X7 nb-Fc fusion proteins of Dano1 and Dano3 recognizing different epitopes of the receptor. Combining nb-Fc fusion proteins with independent epitopes improves the detection sensitivity (Danquah 2012). As a control for specific staining, we used cold blocking with the same unconjugated nb-Fc fusion proteins. Analysis of the P2X7 expression was performed using FACS.

Seven donors were included in this study and peripheral whole blood was stained with all antibody panels (Tab. 2).

Fluorochrome	Subset	T cells invariant	DC	T cells
V450	CD14	CD161	HLA-DR	-
V500	CD45	-	CD45	CD8
AF488	CD4	Vα24	Lineage	HLA-DR
PE	TCRγδ	Vα 7.2	CD11c	CD25
PerCP-Cy5.5	CD3	CD3	-	CD127
PE-Cy7	CD19	CD56	CD123	CD39
AF647	P2X7	P2X7	P2X7	P2X7
APC-Cy7	CD16	-	-	CD4

Tab. 2: Antibody panels for determination of P2X7 expression in human peripheral blood leukocytes.

4.1.1 Gating strategy

The first step was the identification of all relevant immune cell subsets. For this, we eliminated cell debris and dead cells with a “cells” gate in the forward/side scatter (FSC/SSC) dot plot and subsequently selected the single cells according to their appearance in the FSC-A/FSC-H scatter diagonal; cells outside of the diagonal were considered as doublets.

Leukocytes were identified by the expression of the common leukocyte antigen CD45. In the "Subset" tube, we directly selected the eosinophil population on the basis of their high autofluorescence in the V450 channel and high side scatter.

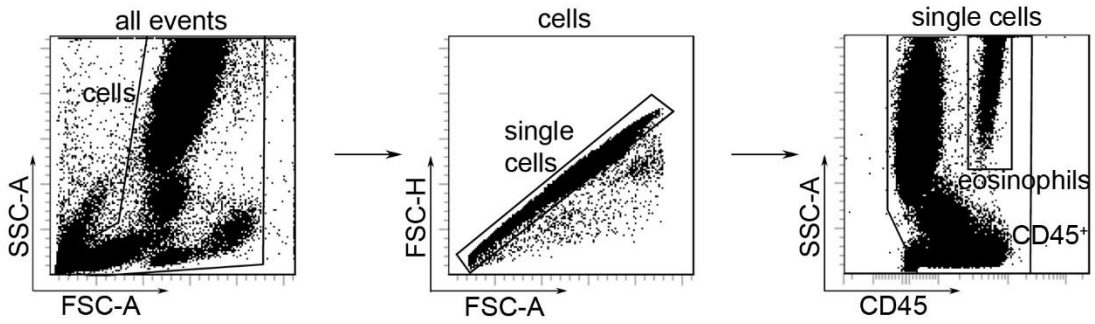


Fig. 5: Gating strategy for the identification of leucocytes and eosinophils in peripheral whole blood.

4.1.1.1 Identification of granulocytes, monocytes, B cells and T cells

On the $CD45^+$ cells we used the markers CD14 for the characterization of monocytes and high expression of CD16 for the neutrophils. The remaining cells ("rest of") were considered lymphocytes. In the lymphocyte gate, we further identified the B cells ($CD19^+$) and T cells ($CD3^+$). The T cells were subdivided into $TCR\gamma\delta^+$ cells and the rest of the cells not expressing this receptor, which were considered classical $TCR\alpha\beta^+$ lymphocytes.

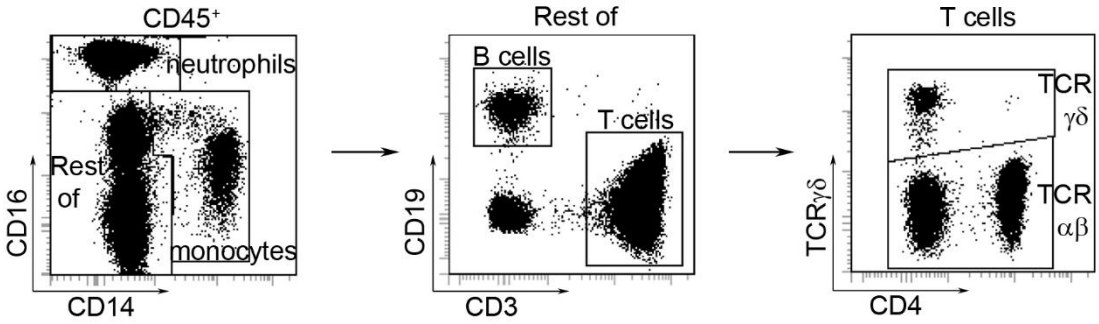


Fig. 6: Gating strategy for the identification of monocytes, neutrophil granulocytes, B cells, T cells and their subsets TCRαβ and TCRγδ cells.

4.1.1.2 Identification of innate-like lymphocytes and natural killer cells

The "T cells invariant" tube provided us with information about innate-like lymphocytes, such as MAIT cells and iNKT cells, and about NK cells. From the $CD3^+$ cells, we characterized the MAIT cells as $CD161^+V\alpha7.2^+$ and the iNKT cells as $V\alpha24^+$. For the NK cells we selected

those cells not expressing CD3 (“rest of”) and further identified them by the expression of CD56.

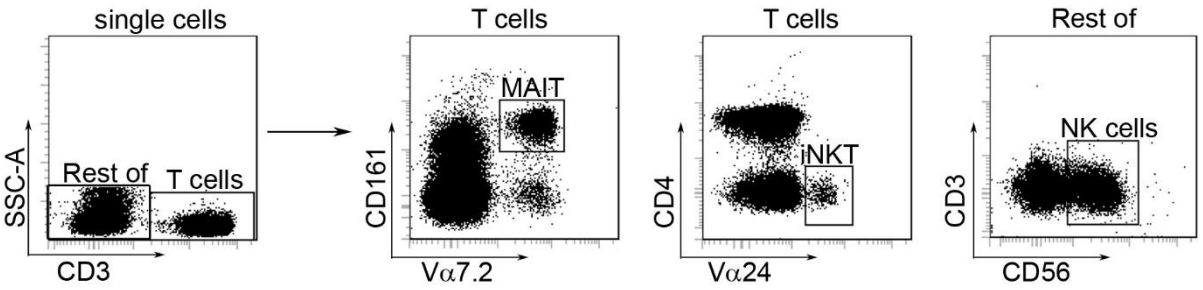


Fig. 7: Gating strategy for the identification of NK cells and the invariant T cell subsets.

4.1.1.3 Identification of dendritic cells and basophils

We used the ab panel “DC” for the identification of mDCs, pDCs and basophils. The component “lineage (lin)” contained antibodies against CD3, CD19, CD56 and CD14. After gating on the $CD45^+$ cells we selected the non-lineage cells that were positive for HLA-DR ($lin^-HLA^-DR^+$) as basis for the DC lineages mDC ($CD11c^+CD123^-$) and pDC ($CD11c^-CD123^+$). Within the $lin^-HLA^-DR^-$ cells we characterized the basophils as $CD123^+$.

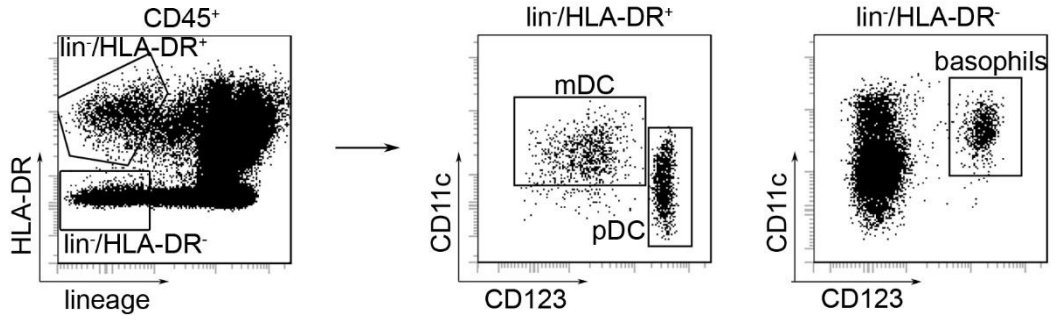


Fig. 8: Gating strategy for the identification of basophils, mDCs and pDCs.

4.1.1.4 Identification of cytotoxic T cells, T helper cells and regulatory T cells

In the “T cell” tube, we directly selected the lymphocytes according to their low forward/side scatter properties. From the single cells, we selected the cytotoxic T cell population ($CD8^+$) and the Th cells ($CD4^+$). From the $CD4^+$ cells we characterized the Tregs as $CD127^-CD25^+$.

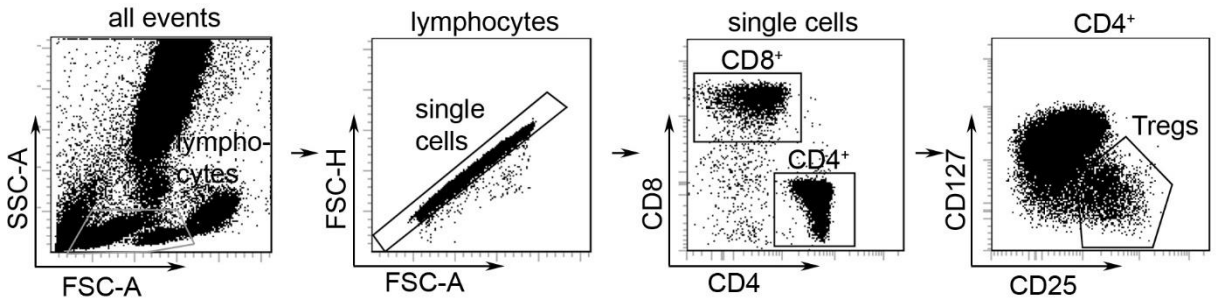


Fig. 9: Gating strategy for the identification of cytotoxic T cells ($CD8^+$), T helper cells ($CD4^+$) and regulatory T cells (Tregs).

4.1.2 Analysis of P2X7 expression levels

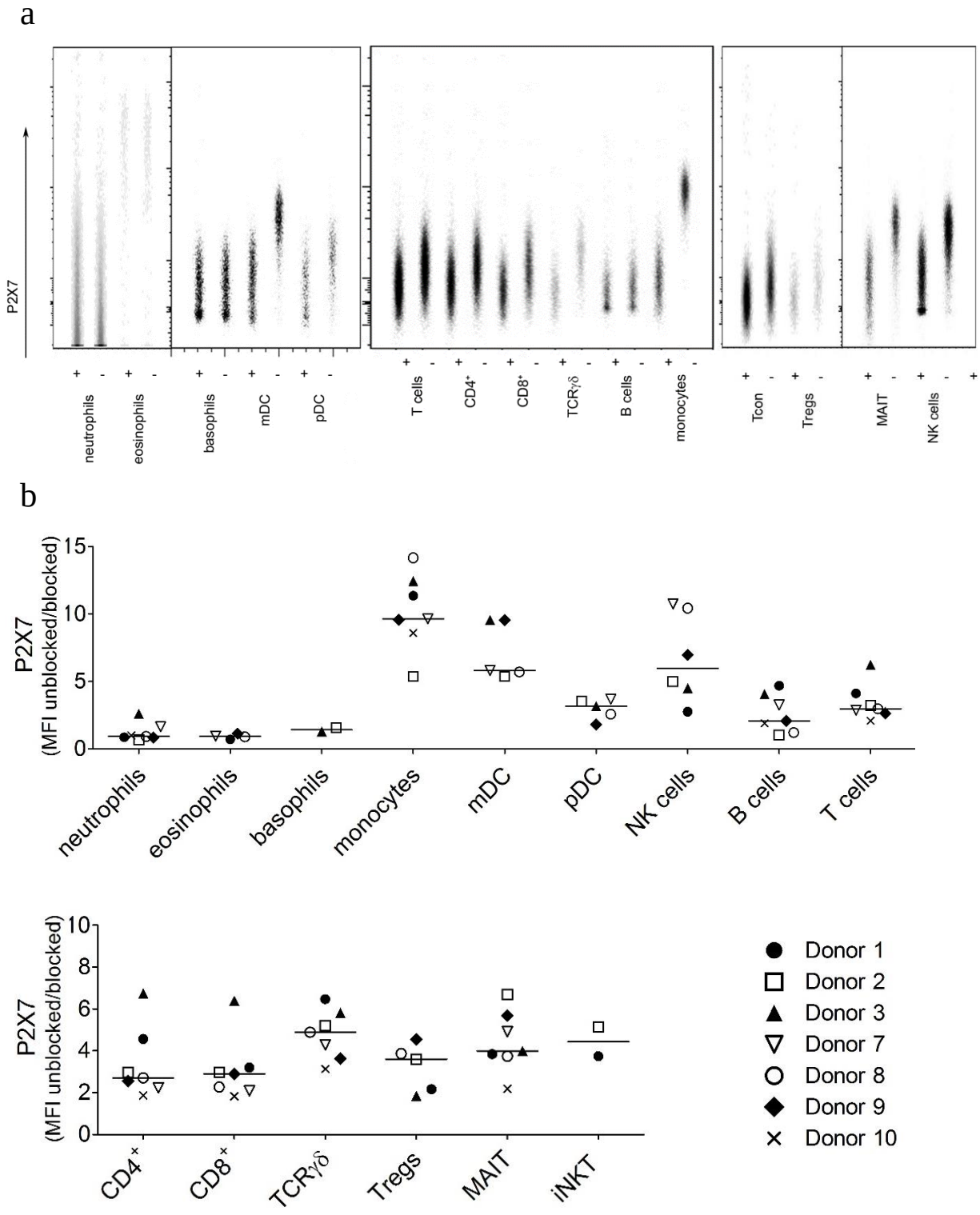
We assessed the extend of P2X7 specific staining by comparing the P2X7 fluorescence signal of the stained preparation ("unblocked") to the P2X7 fluorescence signal of the blocking analysis ("blocked"). In order to quantify the P2X7 expression levels we calculated median fluorescence intensity ratios (MFI ratios) as quotients of MFI "unblocked" versus MFI "blocked".

The cell types attributed to the innate immunity showed the strongest P2X7 expression: In comparison we observed the highest levels on the monocytes ($\tilde{x}$ = 9.64, range 5.38 - 14.16) followed by medium expression levels on the NK cells ($\tilde{x}$ = 5.98, range 2.75 – 10.76) and mDCs ($\tilde{x}$ = 5.82, range 5.37 – 9.56) and medium to low levels on the pDCs ($\tilde{x}$ = 3.17, range 1.81 – 3.69). The lymphocytes revealed relatively low P2X7 expression levels: We observed very low expression levels on the B cells ($\tilde{x}$ = 2.06, range 1.01 – 4.67), low levels on the $CD4^+$ ($\tilde{x}$ = 2.70, range 1.86 – 6.74) and $CD8^+$ T cells ($\tilde{x}$ = 2.89, range 1.82 – 6.38) and medium to low levels on the Tregs ($\tilde{x}$ = 3.60, range 1.84 – 4.54). Interestingly, the P2X7 expression levels of the innate-like cells were between those of the cells of the innate and the adaptive immunity: We found medium P2X7 expression levels on the TCRγδ cells ($\tilde{x}$ = 4.88, range 3.13 – 6.46) and medium to low levels on the MAIT cells ($\tilde{x}$ = 3.99, range 2.18 – 6.69) and the iNKT cells ($\tilde{x}$ = 4.44, range 3.74 – 5.13) The granulocytes did not show P2X7 expression: neutrophils ($\tilde{x}$ = 0.92, range 0.66 – 2.62), eosinophils ($\tilde{x}$ = 0.93, range 0.71 – 1.13), basophils ($\tilde{x}$ = 1.43, range 1.29 – 1.57).

The P2X7 MFI ratios are no absolute values but give us tendencies concerning the P2X7 expression on leukocytes. The expression levels for the different leucocyte populations can be ordered qualitatively as monocytes > NK cells ≥ mDCs > pDCs ≥ T cells > B cells > neutrophils/eosinophils/basophils. The expression levels for the T cell subsets can be ordered

qualitatively as TCRγδ cells > iNKT > MAIT > Tregs > T cytotoxic cells ($CD8^+$) ≥ Th cells ($CD4^+$).

The results reveal inter-individual differences in terms of the P2X7 expression levels. We also see that the degree of P2X7 expression varies among the cell populations of one individual, e.g. Donor 3 shows relatively high P2X7 expression levels on the monocytes, DCs, B cells and the majority of T cells, whereas showing relatively low levels on the NK cells and Tregs.



F**ig. 10: P2X7 expression levels in the different leukocyte subsets.** Whole blood (n = 7) was stained with a combination of Alexa Fluor 647-conjugated anti-P2X7 nb-Fc fusion proteins of Dano1 and Dano3 ("unblocked") and the same unconjugated nb-Fc fusion proteins ("blocked"). The cells were co-stained with lineage markers for the determination of the different immune cell types and analyzed by FACS. **(a)** Representation of P2X7 expression as 'raw data' in form of a concatenated analysis. The preparations "unblocked" and "blocked" are indicated by (+) and (–), respectively. Ratios of Median Fluorescence Intensity (MFI ratios) for P2X7 were calculated as quotients of MFI "unblocked" versus MFI "blocked" and the median values ($\tilde{x}$) within each leukocyte subset are shown for **(b)** the different main leukocyte populations and **(c)** the different T cell subsets in particular.

4.2 P2X7 expression in monocyte-derived dendritic cells

Since DCs are rare in human blood, we wanted to assess P2X7 expression in the most broadly used in vitro model for DCs, namely the monocyte-derived dendritic cell (MoDC).

For the generation of MoDCs we isolated monocytes from peripheral whole blood by MACS and incubated $1x10^5$ cells/ cm² with 0.1 µg/ml GM-CSF + 50 ng/ml IL-4 for 7 d. At d 0 and d 7 we assessed the cell morphology using light microscopy and the phenotype using FACS.

At d 0 we could observe characteristically big, round-shaped and uniform monocytes in the microscope. The cell population showed high expression of the monocyte marker CD14 as well as high expression of the P2X7 receptor in the FACS analysis. After 7 days of treatment, we could see a remarkable change in the cell morphology and phenotype. Microscopically the cell bodies were of diverse shapes and the cells showed finger-like processes, which are characteristic for dendritic cells. FACS analysis revealed down regulation of monocyte marker CD14 and simultaneous up regulation of the myeloid dendritic cell marker CD11c on the cell surface. The P2X7 expression on the MoDCs was lower than on the original monocytes and similar to the P2X7 expression levels we had assessed for mDCs before.

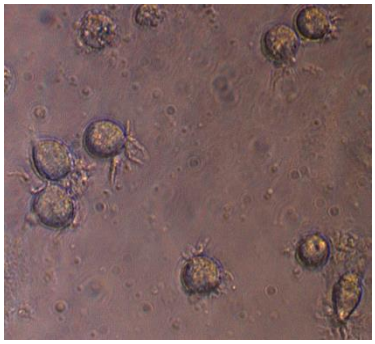


Fig. 11: Monocyte-derived dendritic cells (MoDCs) at day 7 of generation.

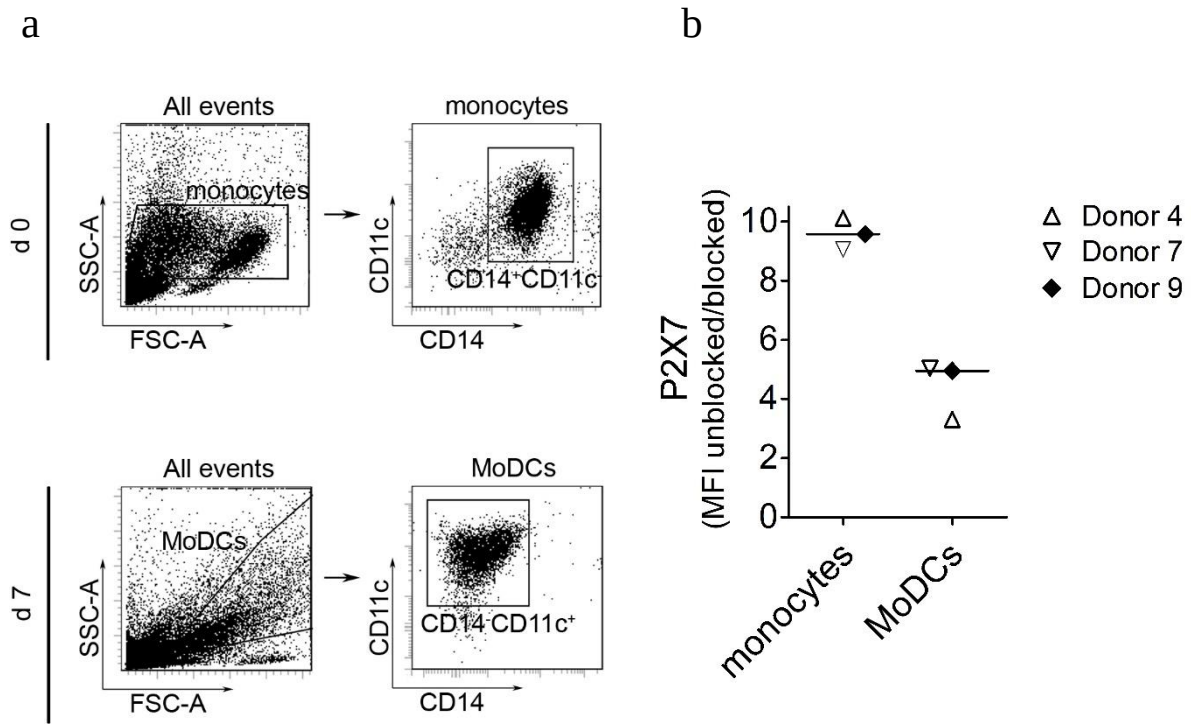


Fig. 12: Phenotype and P2X7 expression of monocyte-derived dendritic cells (MoDCs). In order to generate MoDCs monocytes were isolated from whole blood (n = 3) using MACS and incubated with GM-CSF and IL-4 for 7 days. **(a)** The expression of monocyte marker CD14 and dendritic cell marker CD11c was analyzed by FACS at day 0 and 7. **(b)** The P2X7 expression levels (MFI unblocked/MFI blocked) for the monocyte and MoDC population were compared.

4.3 P2X7 expression in human microglia and neuroblastoma cell lines

Microglia is assumed to play a critical role in P2X7-mediated diseases of the central nervous system (He, Taylor et al. 2017). Due to the difficult access to primary human microglia, we turned to a well-characterized cell line of human microglial origin (Janabi, Peudenier et al. 1995). Similar to the leukocyte staining we used Alexa Fluor 647-conjugated anti-P2X7 Nb-fc Fusion proteins Dano1 and Dano3 for P2X7 detection by FACS. In contrast to peripheral monocytes, we did not detect P2X7 expression on the cell surface of microglia cell line CHME-5.

Moreover P2X7 expression was reported on neuronal cells (Bernier, Ase et al. 2017). Therefore, we also analyzed P2X7 expression in cells of the human neuroblastoma cell line SH-SY5Y, but could not detect any signal.

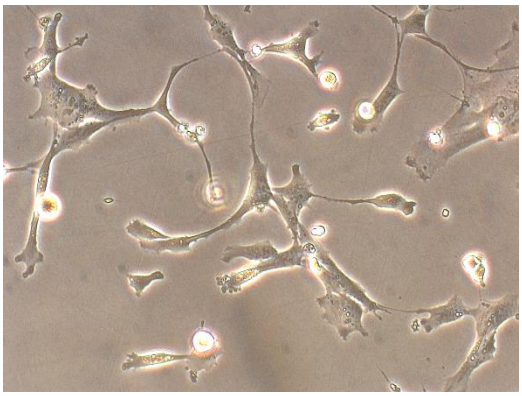


Fig. 13: Microglial CHME-5 cells 1 day after thawing.

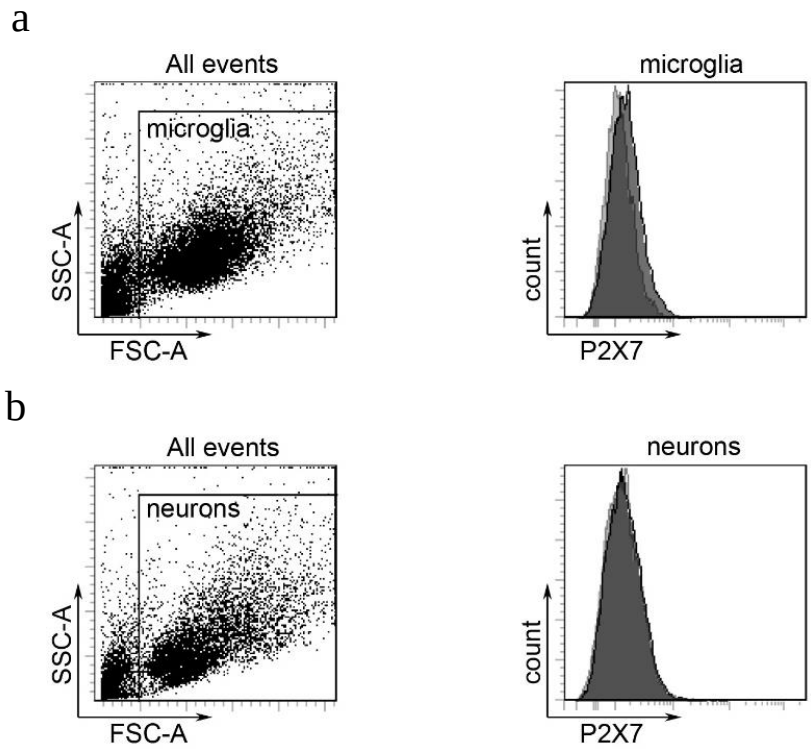


Fig. 14: P2X7 expression in human microglial CHME-5 and neuronal SH-SY5Y cells. The P2X7 expression was analyzed by FACS. P2X7 fluorescence signals for preparations "blocked" (shown in light grey) and "unblocked" (shown in dark grey) were compared for **(a)** microglial CHME-5 cells and **(b)** neuronal SH-SY5Y cells.

4.4 Modulation of P2X7 expression after cell stimulation

Whereas P2X7 expression in mouse peripheral $CD4^+$ and $CD8^+$ T cells is just marginally, the receptor is highly expressed in mouse intestinal T cells and suggested as a regulatory element in the control of T cell responses in the intestinal mucosa. In mice, activation of peripheral and intestinal $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T cells as well as intestinal $CD4^+$ T cells with anti-CD3 in the presence of retinoic acid (RA) results in upregulation of P2X7 on the cell surface (Heiss, Janner et al. 2008, Hashimoto-Hill, Friesen et al. 2017). We investigated whether this was also true in human peripheral $CD4^+$, $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T cells. Therefore, we activated PBMCs from three different donors with anti-CD3 for two days and with RA for further three days and assessed P2X7 expression levels using FACS. CD25 and CD38 were used as activation markers.

At baseline, only a marginal number of the $CD8\alpha\beta^+$ and $CD8\alpha\alpha^+$ cells were positive for the activation markers CD25 and CD38, while nearly one third of $CD4^+$ cells did already show expression of CD38. After five days, the majority of $CD8\alpha\beta^+$ and $CD4^+$ cells and 37.5 % of $CD8\alpha\alpha^+$ cells already showed up regulation of both activation markers, even in the absence of RA treatment. RA treatment enhanced the activation status so that 90.4 % of the $CD8\alpha\beta^+$, 49.8 % of the $CD8\alpha\alpha^+$ cells and 84.7 % of the $CD4^+$ cells showed expression of CD25 and CD38 after application of 50 nM RA. Remarkably, over 40 % of the $CD8\alpha\alpha^+$ cell population in the different preparations did not show any sign of activation.

P2X7 expression levels in the untreated $CD8\alpha\beta^+$, $CD8\alpha\alpha^+$ and $CD4^+$ T cells were relatively low and comparable to our previous results (Fig. 10c). Contrary to what is reported in mice, we did not observe significant change in P2X7 expression at any time point and in any of the cell types analyzed.

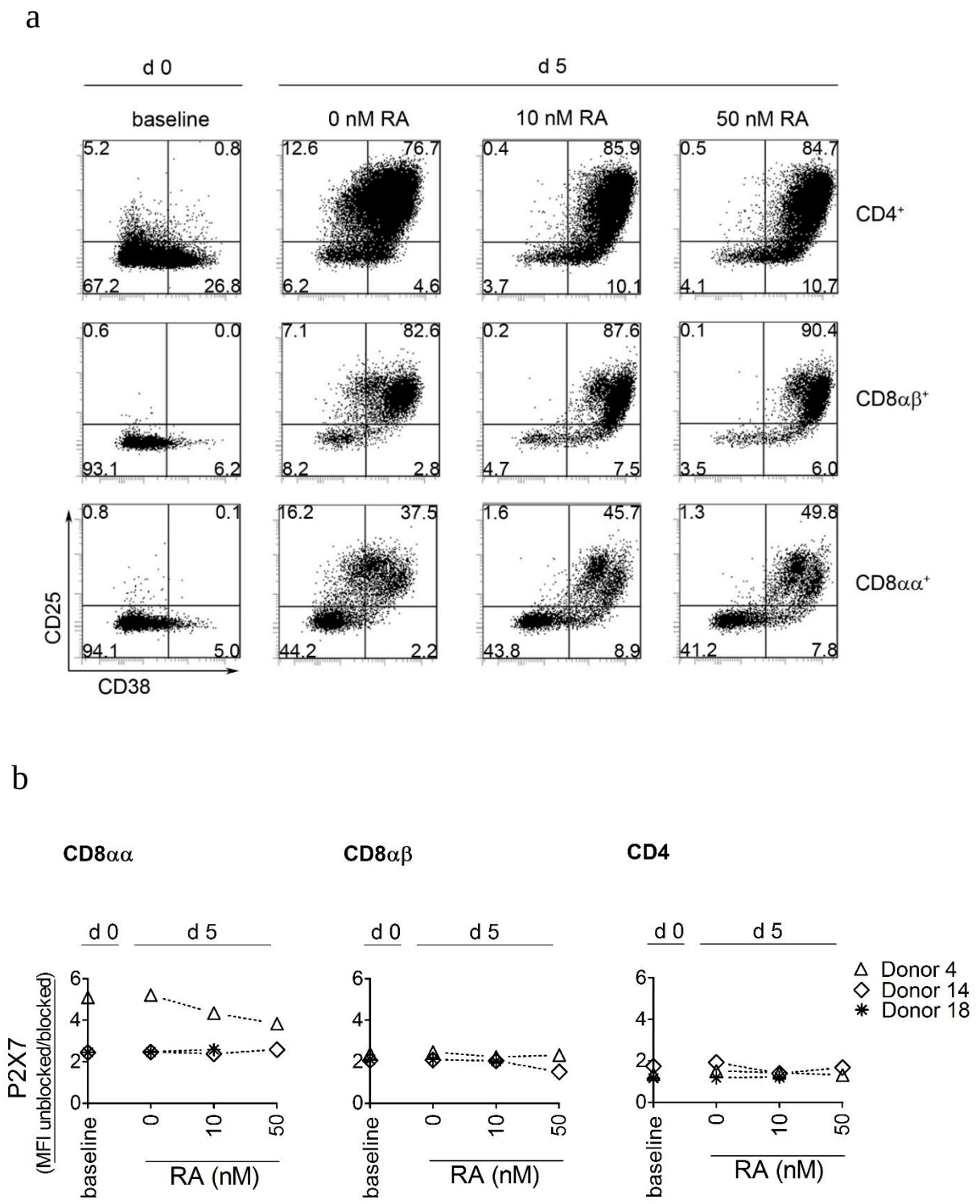


Fig. 15: P2X7 expression levels on CD4⁺, CD8αα+ and CD8αβ+ T cells after activation with anti-CD3 and Retinoic acid (RA). PBMCs (n = 3) were incubated with soluble anti-CD3 for 2 d and with different concentrations of RA for further 3 d. For FACS analysis, cells were stained with antibodies against T cell marker CD3, CD8αα, CD8αβ and CD4 and the activation markers CD25 and CD38. **(a)** The expression of the activation markers CD25 and CD38 on the CD4⁺, CD8αα+ and CD8αβ+ T cells was analyzed by FACS at d 0 and 5. **(b)** The P2X7 expression levels (MFI unblocked/MFI blocked) were assessed at d 0 and 5.

We furthermore assessed the effect of cell stimulation on the P2X7 expression in monocytes. To this end, we isolated monocytes from whole blood from three different donors and stimulated the cells with Lipopolysaccharide (LPS) or LPS + Interferon-γ (IFN-γ) for 24 h. Upregulation of the marker CD80 confirmed stimulation of the monocytes.

Before treatment, only 1.1 % of the monocytes were positive for CD80. 92.0 % of the cells treated with LPS only showed up regulation of the stimulation marker after 24 h. The degree of stimulation was similar for the cells treated with LPS + IFN-γ: After 24 h 92.3 % of the cells showed up regulation of CD80. Of note, 86.8% of the monocytes that were not treated also expressed the stimulation marker after 24 h.

In analogy to the observation on T cells, we did not find remarkable changes in the P2X7 expression levels on monocytes after *in vitro* activation.

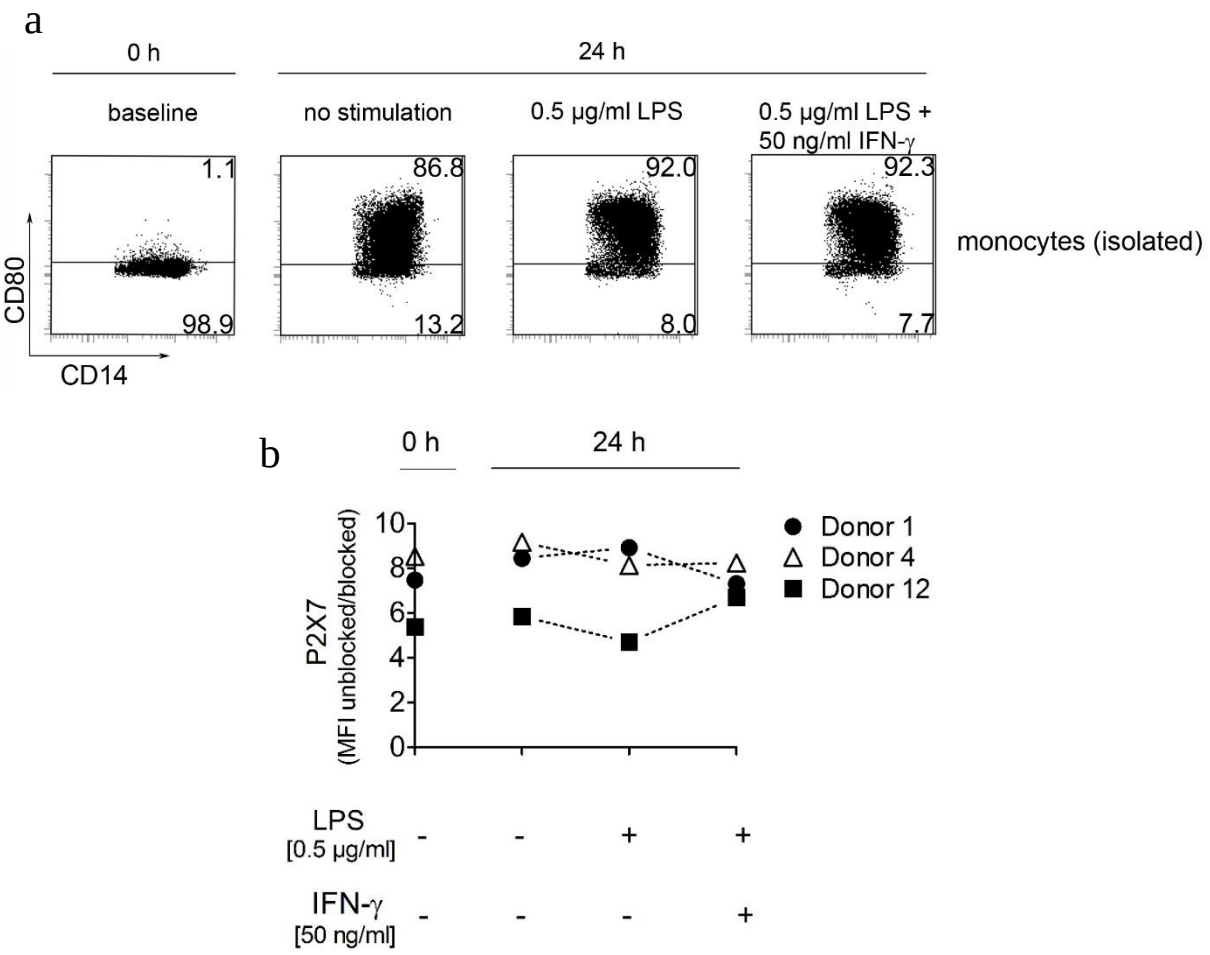


Fig. 16: P2X7 expression levels on isolated monocytes after stimulation with LPS or LPS+IFN-γ. Monocytes were isolated from PBMCs (n =3) by magnetic cell sorting (MACS) and incubated with LPS or LPS+IFN-γ for 24 h. For FACS analysis, the cells were stained with monocyte marker CD14 and stimulation marker CD80. **(a)** The expression of stimulation marker CD80 was analyzed by FACS at d 0 and 5 at 0 h and 24 h. **(b)** P2X7 expression levels (MFI unblocked/blocked) were determined at 0 h and 24 h.

In summary, our results show that P2X7 expression does not change upon in vitro cell activation or stimulation in human blood $CD4^+$, $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T cells and monocytes.

4.5 P2X7 function and modulatory capacity of P2X7-specific nanobodies

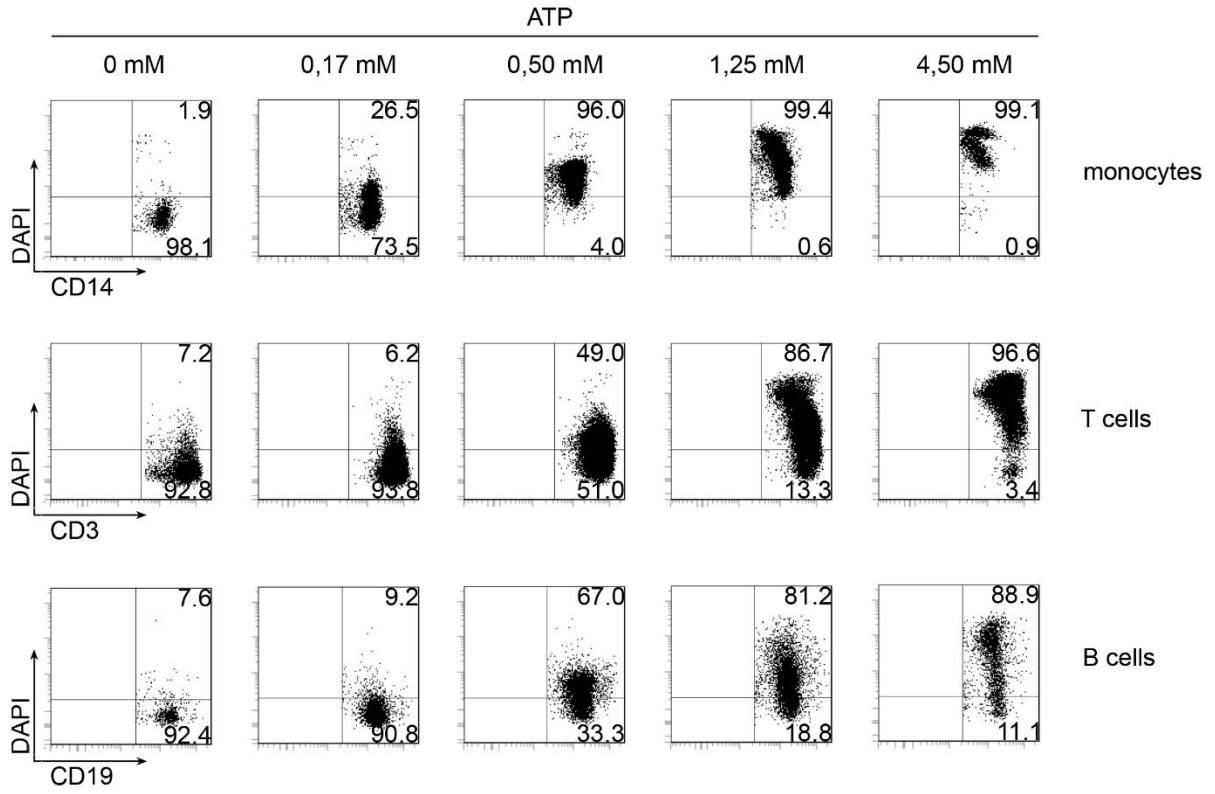
P2X7 engagement mediates a variety of effects on immune cells. To test the functional properties of the P2X7 nbs we used as readouts i) pore formation, ii) activation of proteases that cleave membrane proteins, and iii) production of IL-1β upon inflammasome activation in monocytes.

4.5.1 P2X7 engagement with ATP triggers DAPI uptake in T cells, B cells and monocytes

Upon interaction of ATP, the P2X7 receptor builds an ion channel that allows influx and efflux of ions and bigger molecules (Surprenant and North 2009, Idzko, Ferrari et al. 2014). The uptake of molecules is probably a result of P2X7-mediated Pannexin-1 pore formation in the cell membrane (Gulbransen, Bashashati et al. 2012). We used the fluorescent dye DAPI to test the pore forming capacity of P2X7 after engagement with ATP in monocytes, T cells and B cells. For this, we incubated whole blood from five different donors with different concentrations of ATP (0.17 mM, 0.5 mM, 1.25 mM, 4.5 mM) for 30 min and determined the DAPI uptake using FACS.

We observed P2X7-mediated DAPI uptake for all cell types tested. The uptake of the dye was depending on the applied ATP concentration. Comparing the cell types, monocytes showed the strongest DAPI uptake, T cells showed medium levels and the B cells showed the weakest uptake of the dye, which correlates with the expression of P2X7 in these cell types. The degree of ATP response varied among the different donors, e.g. the monocytes, T cells and B cells of Donor 3 showed noticeable stronger DAPI uptake than the cells of Donor 2 at the same ATP concentrations.

a



b

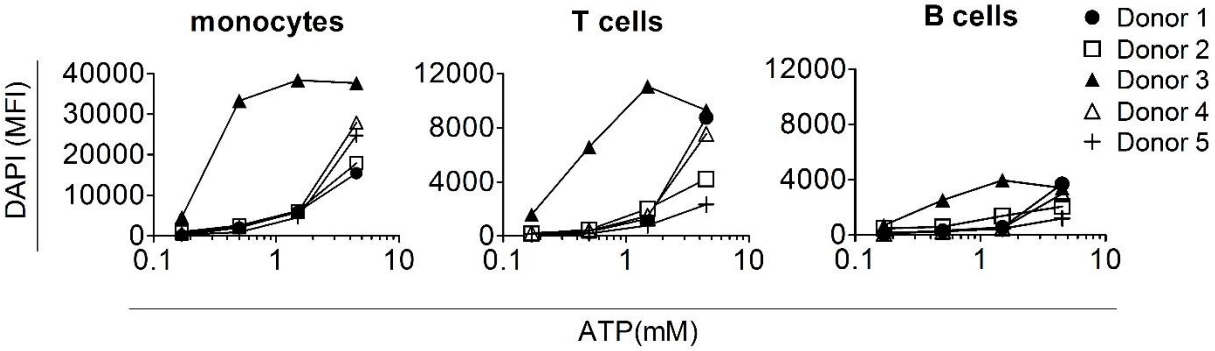


Fig. 17: Analysis of ATP-induced uptake of fluorescent dye DAPI by monocytes, T cells and B cells. Whole blood (n = 5) was incubated with fluorescent dye DAPI and different concentrations of ATP for 30 min. The cells were then stained with antibodies against CD14, CD3 and CD19 in order to detect monocytes, T cells and B cells and FACS analysis was performed. ATP-depending uptake of DAPI is shown by means of **(a)** the percentages of cells that are positive for DAPI and **(b)** the Mean fluorescence intensity (MFI) for DAPI.

4.5.2 The P2X7-specific nb-dimer of Dano1 is a potent inhibitor of P2X7-mediated DAPI uptake in T cells, B cells and monocytes

In order to determine the blocking potential of anti-P2X7 nbs we performed the P2X7-dependent pore formation assay as described in section 5.5.1 in the presence of the anti-P2X7 nb-dimer of Dano1. Whole blood from five different donors was treated with a relatively high concentration of ATP (4.5 mM) after 10 min. incubation with the anti-P2X7 Nb.

Our results show an almost complete prevention of ATP-induced DAPI uptake in all three cell populations, indicating that this is a P2X7-specific effect. The blockade was slightly weaker for the B cells and monocytes of donor 6 compared to donor 4 and 5. With this experiment, we could demonstrate that the anti-P2X7 nb-dimer of Dano1 efficiently blocks P2X7-mediated DAPI uptake.

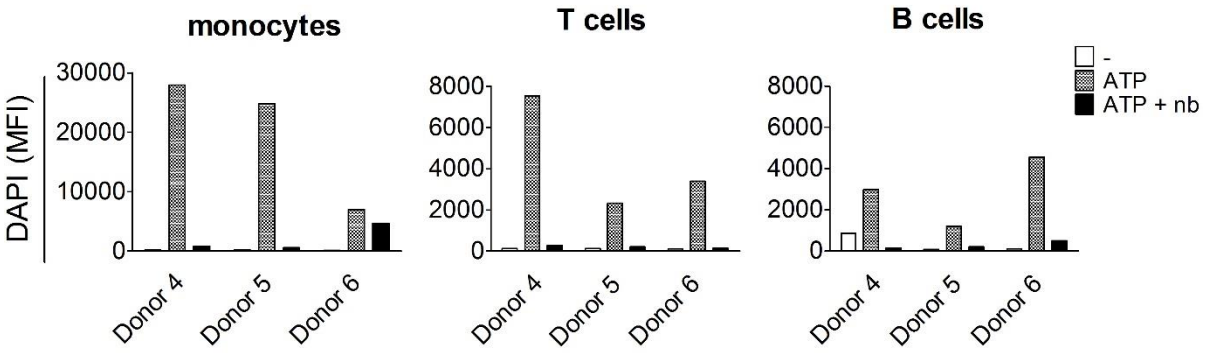


Fig. 18: Analysis of the inhibition of ATP-induced uptake of fluorescent dye DAPI by monocytes, T cells and B cells by anti-P2X7 nb-dimer of Dano1. Whole blood (n = 3) was incubated with anti-P2X7 nb-dimer of Dano1 10 min. prior to the 30 min. incubation with DAPI and ATP. The cells were then stained with antibodies against CD14, CD3 and CD19 in order to detect monocytes, T cells and B cells and FACS analysis was performed measuring the mean fluorescence intensities (MFI) for DAPI. The cell response in the absence (black bar) and presence of anti-P2X7 nb-dimer of Dano1 (dotted grey bar) is shown as well as a control without ATP and anti-P2X7 nb treatment (white bar).

4.5.3 P2X7 engagement with ATP triggers shedding of CD62L in T cells and B cells

CD62L (L-selectin) is an adhesion molecule found on lymphocytes. Engagement of P2X7 results in activation of the metalloprotease ADAM17, which in turn cleaves CD62L from the cell surface (Le Gall, Bobe et al. 2009, Scheuplein, Schwarz et al. 2009), therefore constituting a readout for P2X7-activation. We analyzed the shedding of CD62L for peripheral blood T cells and B cells in five donors after 30 min. incubation with different concentrations of ATP by FACS.

We observed ATP-depending shedding of CD62L in T cells and B cells. As it was the case for the P2X7-mediated DAPI uptake, the degree of the ATP response varied from donor to

donor. Whereas the majority of cells from Donor 1, Donor 2 and Donor 4 and the majority of T cells from Donor 3 responded to the ATP, just a small fraction of the T cells and B cells from donor 5 and the B cells from Donor 3 showed shedding of CD62L.

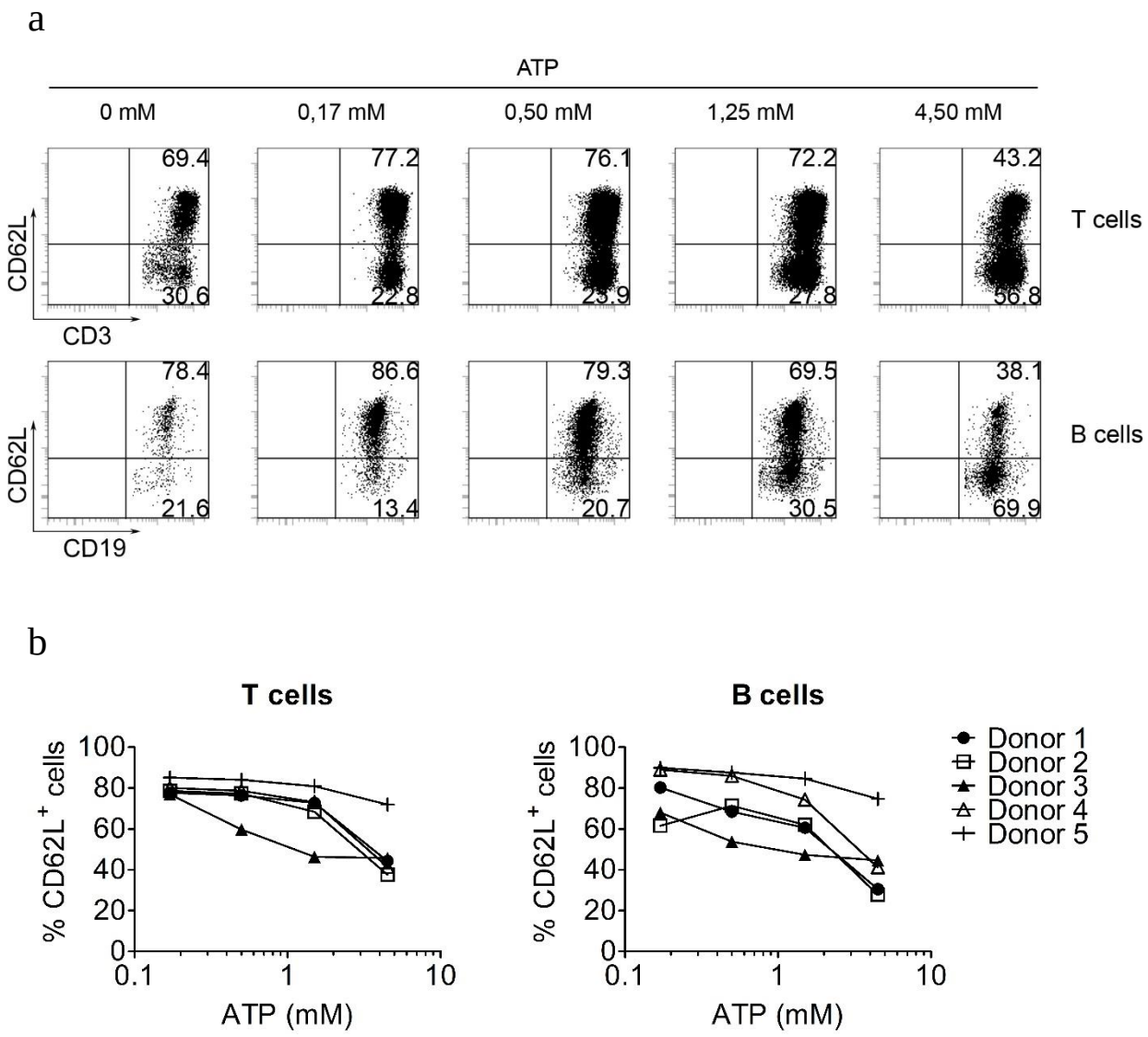


Fig. 19: Analysis of ATP-induced shedding of CD62L in T cells and B cells. Whole blood (n = 5) was incubated with different concentrations of ATP for 30 min. The cells were then stained with antibodies against CD62L, T cell marker CD3 and B cell marker CD19 and FACS analysis was performed. **(a)** The numbers in the right upper and lower quadrant of the dot plots indicate the changes in the percentage of cells responding to rising concentrations of ATP. **(b)** ATP-depending shedding of CD62L from the cell surface is shown via the percentages of $CD62L^+$ cells.

4.5.4 The P2X7-specific nb-dimer of Dano1 prevents P2X7-mediated shedding of CD62L in T cells and B cells

In this experiment, we evaluated the blocking effect of the anti-P2X7 nb-dimer of Dano1 in terms of P2X7-mediated CD62L shedding. Whole blood from three different donors was incubated with 500 pg/ml anti-P2X7 nb-dimer of Dano1 10 min. prior to the treatment with 4.5 mM ATP Afterwards, shedding of CD62L was analyzed using FACS.

Our results demonstrate an almost complete blockade of CD62L shedding for T cells and B cells. We conclude that the anti-P2X7 nb-dimer of Dano1 is a potent inhibitor of P2X7-mediated CD62L shedding in T cells and B cells.

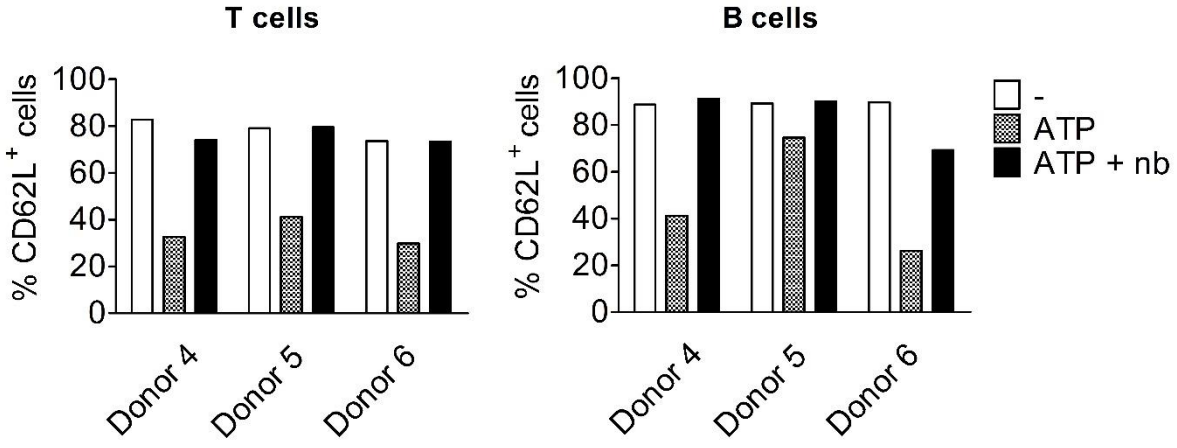


Fig. 20: Analysis of the inhibition of ATP-induced shedding of CD62L in T cells and B cells by the anti-P2X7 nb-dimer of Dano1. Whole blood (n = 3) was incubated with anti-P2X7 nb-dimer of Dano1 10 min. prior to the 30 min. incubation ATP. The cells were then stained with antibodies against CD62L, T cell marker CD3 and B cell marker CD19 and FACS analysis was performed. The cell response in the absence (black bar) and presence of the anti-P2X7 nb-dimer of Dano1 (grey bar) is shown as well as a control without ATP and anti-P2X7 nb treatment (white bar).

4.5.5 P2X7 engagement with ATP triggers release of LPS-induced IL-1β in monocytes

P2X7 activation promotes the release of pro-inflammatory cytokine IL-1β from monocytes/macrophages (Pelegrin and Surprenant 2006), DCs (Englezou, Rothwell et al. 2015) and microglial cells (Sanz, Chiozzi et al. 2009). PAMPs like LPS promote the transcription of the IL-1β gene upon binding to PRRs therefore triggering the production and storage of the inactive pro-IL-1β. P2X7-mediated efflux of K^+ triggers the assembly and activation of the inflammasome, facilitating the cleavage of pro-caspase 1 into active caspase 1. Caspase 1 in turn cleaves pro-IL1-β into the active IL1-β, which is finally released from the cell (Di Virgilio 2007, Giuliani, Sarti et al. 2017). In this experiment, we analyzed the P2X7-

mediated release of IL-1β in LPS-primed monocytes. Therefore, we incubated whole blood from three different donors with LPS for 1.5 h and treated the cells with different concentrations of ATP (0.17 mM, 0.5 mM, 1.25 mM, 4.5 mM) for another 30 min. The blood plasma was collected and IL-1β was measured using ELISA.

Analysis revealed an ATP dose-depending IL-1β release showing remarkable inter-individual differences. Donor 1 showed a comparatively rapid IL-1β release reaching high cytokine concentrations in the blood plasma whereas the cells from Donor 2 released lower amounts of IL-1β and only responded to higher ATP concentrations. The cells from Donor 3 just displayed marginal IL-1β release.

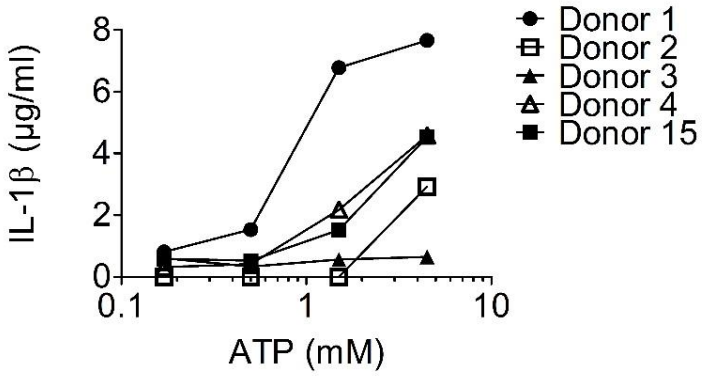


Fig. 21: Analysis of ATP-induced release of IL-1β in LPS-primed monocytes. Whole blood (n = 5) was incubated with LPS for 1.5 h and different concentrations of ATP for further 30 min. After centrifugation of all samples, the supernatants (blood plasma) were collected and released IL-1β was measured by ELISA.

In addition, we performed the same experiment with monocytes, isolated by MACS, and MoDCs of three different donors. Under these conditions, we did not detect any released IL-1β.

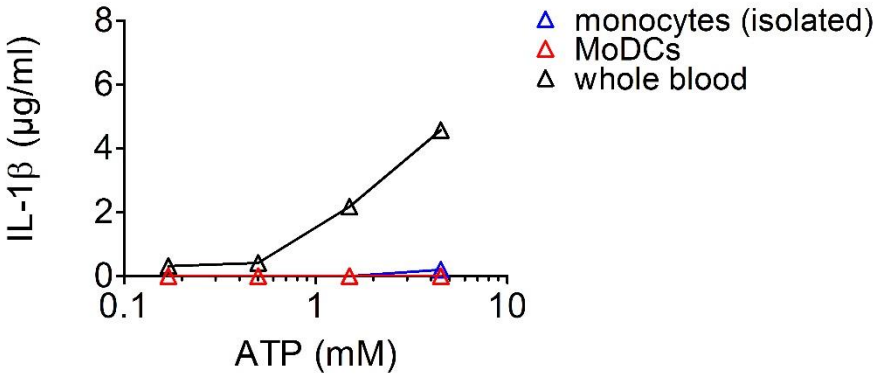


Fig. 22: Analysis of ATP-induced release of IL-1β in isolated monocytes and MoDCs. (a) Whole blood, MACS-isolated monocytes and MoDCs ($1x10^5$ or $3x10^5$ cells) from the same donor (n = 3) were incubated with LPS for 1.5 h and different concentrations of ATP for further 30 min. After centrifugation, the supernatants were collected and released IL-1β was measured by ELISA. A representative example (donor 4) is shown here.

Microglial cells are as well able to release IL-1β upon P2X7 engagement with ATP (He, Taylor et al. 2017). We evaluated ATP-induced IL-1β release from microglial CHME-5 cells according to the previous protocol.

In line with the lack of expression of P2X7 on this cell line, our results did not reveal ATP-induced IL-1β release from microglial CHME-5 cells.

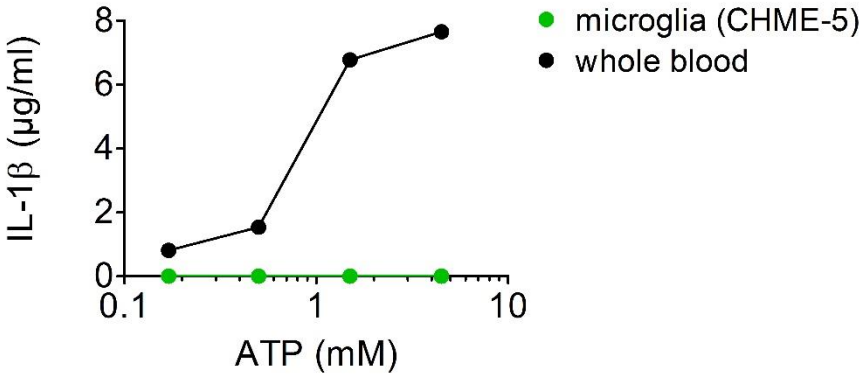


Fig. 23: Analysis of ATP-induced release of IL-1β in microglial CHME-5 cells. Microglial CHME-5 cells ($1x10^5$ or $3x10^5$ cells) and whole blood (Donor 1) were incubated with LPS for 1.5 h and different concentrations of ATP for further 30 min. After centrifugation of all samples the supernatants were collected and released IL-1β was measured by ELISA.

4.5.6 The P2X7-specific nb-dimer of Dano1 inhibits P2X7-mediated IL-1β release in monocytes

To assess the blocking potential of the anti-P2X7 nb-dimer of Dano1 regarding P2X7-mediated IL-1β release in LPS-primed monocytes we incubated whole blood from three different donors with 500 pg/ml anti-P2X7 nb-dimer of Dano1 for 10 min. prior to the 1,5 h treatment with LPS and subsequent 30 min. treatment with 4.5 mM ATP. The levels of IL-1β were measured in the blood plasma by ELISA.

Our data indicate an almost complete inhibition of IL-1β release in all three donors after incubation with P2X7-specific nbs. Once more, these findings show the excellent blocking effect of the anti-P2X7 nb-dimer of Dano1.

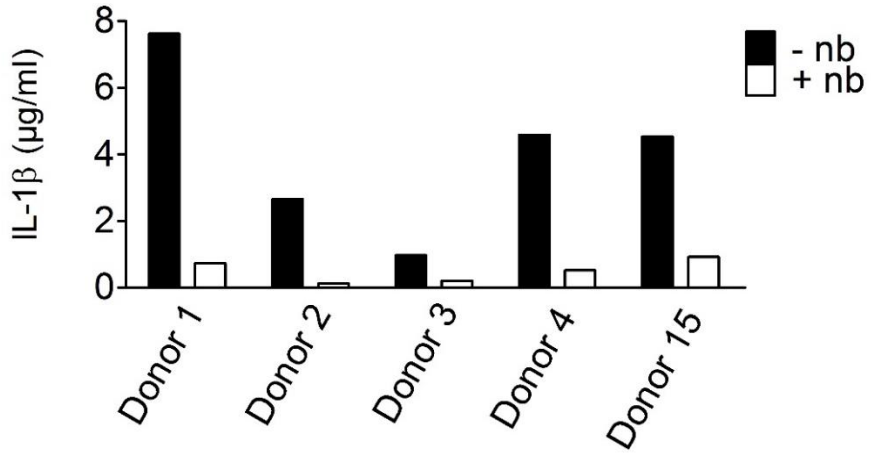


Fig. 24: Analysis of the inhibition of ATP-induced IL-1β release from monocytes by the anti-P2X7 nb-dimer of Dano1. Whole blood (n=3) was incubated with anti-P2X7 nb-dimer of Dano1 10 min. prior to the 1.5 h incubation with LPS and subsequent 30 min. incubation with ATP. IL-1β released into the blood plasma was measured by ELISA. Levels of IL-1β in the absence (black bar) or presence (white bar) of anti-P2X7 nb-dimer of Dano1 are shown.

Taken together, we demonstrated that P2X7 engagement with ATP triggers different downstream events in an ATP-dependent manner. We saw remarkable inter individual differences in the receptor function, since some donors responded rapidly to the presence of ATP while others showed weak responses. Interestingly we also noticed intra individual differences: The response of one cell population differed in intensity depending on the readout, e.g. monocytes from Donor 3 showed a strong DAPI uptake but weak IL-1β release. Moreover, our findings show the excellent blocking effect of the P2X7-specific nb-dimer of Dano1.

5 Discussion

The P2X7 receptor is a key player in the development of autoimmune, infectious and neoplastic diseases as it mediates various immunological reactions. Thus, it represents a promising therapeutic target.

In this study, we analyzed the expression of P2X7 in human immune cells and its reactions in response to ATP. We moreover assessed the potential of nbs directed against P2X7 for the inhibition of P2X7 function in human cells**.**

5.1 P2X7 expression in the different human immune cells

We found the highest P2X7 expression levels predominantly in the cells of the innate immunity, particularly in the monocytes followed by NK cells and DCs. The expression levels on the mDCs correlated with the expression levels in the cell types of myeloid origin, while the pDCs showed P2X7 expression levels comparable to those of the cells of the lymphoid lineage. Since DCs are rare in the human blood, in vitro generation of MoDCs is a broadly used model to facilitate research on this cell type. We successfully generated MoDCs from monocytes and identified the cells by their characteristic morphology and expression pattern $CD14^-/CD11c^+$. The assessed P2X7 expression was lower than on the original monocytes and similar to the one on the mDCs derived from human whole blood. Thus, MoDCs may be an applicable model for P2X7 research on DCs. In our experiments, cells of the innate-like immunity showed P2X7 expression levels between those of the cells of the innate and the adaptive immunity, which emphasizes their role as a connecting element between these immunity branches. T and B cells display rather low expression levels in our experiments, which also correlates with existing data (Gu, Zhang et al. 2000, Martel-Gallegos, Rosales-Saavedra et al. 2010, Rissiek, Baumann et al. 2015). We did not observe P2X7 expression in the granulocytes. This could either be due to actual missing expression of the receptor or extremely low expression levels. It is known, that microglial cells and neurons express P2X7 and the relation between P2X7 and diseases of the central nervous system are subjects of intensive research (Beamer, Fischer et al. 2017, He, Taylor et al. 2017). However, in our study we could not observe P2X7 expression on cells of human neuronal cell line SH-SY5Y and microglial cell line CHME-5. This may be due to internalization of the receptor from the cell surface into the intracellular space.

5.2 P2X7 expression in the intestine and the role of retinoic acid as P2X7 inductor

P2X7 expression is high on T cells of the intestine of mice. T cells of the intestine carry out the defense against pathogenic microorganisms and regulation of the intestinal flora, a complex task that requires nearly constant cell activation. Particularly stimulated effector and memory T cells and not the naïve T cells express those high levels of P2X7. To regulate the immune system and avoid exuberant immune responses excessive effector cells are sent to apoptosis, an essential procedure which is called contraction. It is assumed, that P2X7 mediates part of these apoptotic mechanisms (Hashimoto-Hill, Friesen et al. 2017). In this context, retinoic acid (RA) plays an important role. RA is a metabolite of Vitamin A and is in the gut and the associated lymphoid tissues produced by DCs, epithelial and stroma cells (Hall, Grainger et al. 2011). RA mediates the upregulation of P2X7 in conventional $CD8\alpha\beta^+$ and unconventional $CD8\alpha\alpha^+$ T cells of the intestine and the periphery as well as in intestinal $CD4^+$ T cells and increases their susceptibility to P2X7 apoptosis signaling (Heiss, Janner et al. 2008, Hashimoto-Hill, Friesen et al. 2017). We transferred this experiment to human peripheral blood lymphocytes but could not detect P2X7 upregulation for human $CD8\alpha\beta^+$ and $CD8\alpha\alpha^+$ T cells as well as $CD4^+$ T cells upon treatment with RA, tough the cells showed clear upregulation of the activation markers CD25 and CD38. This could on the one hand be due to unknown technical problems. On the other hand, it might be, that RA does not play the same role in the human intestinal T cell homeostasis as it plays in the intestine of the mouse. Also, the stimulation of peripheral blood monocytes with LPS and IFN-γ did not result in P2X7 upregulation, though the cells showed clear upregulation of stimulation marker CD80. To get proper information about the situation in the human it is necessary to examine these cell populations directly of the intestine. There are not many studies describing P2X7 in the human intestine so far, but one recent study indicates the association between P2X7 upregulation in the human gut and inflammatory bowel disease. Mucosal biopsies of patients with active Crohn's disease showed significantly stronger P2X7 expression than the tissue of healthy control patients. This was on the one hand due to increased migration of DCs, macrophages and T cells, on the other hand caused by increased receptor expression especially on the DCs (Neves, Castelo-Branco et al. 2014). Upregulation of P2X7 very likely plays a role in the development or maintenance of inflammatory bowel disease, which in general emphasizes the significance of P2X7 in the mechanisms of autoimmunity.

5.3 P2X7-mediated immune responses and the role of Il-1β as mediator of inflammation

P2X7 engagement with ATP triggers various immune responses as essential reactions to external threats like bacteria and parasites as well as internal threats like ischemia. However, exuberant receptor activation can result in autoimmune disorders and tumor growth. In general, P2X7 activation leads to initiation and maintenance of inflammatory conditions. Investigation of the P2X7 downstream effects is an important element in understanding the mechanisms of disease development. One effect is the gating of the receptor itself and influx of Ca^+ and Na^+ ions and efflux of K^+ ions as well as the uptake of larger molecules, which is probably also mediated through accessory membrane pores (Gulbransen, Bashashati et al. 2012, Idzko, Ferrari et al. 2014). The shift of ions can in turn trigger further events that finally result in the release of pro-inflammatory cytokines like IL-1β from monocytes (Danquah, Meyer-Schwesinger et al. 2016), DCs (Englezou, Rothwell et al. 2015) and microglia (He, Taylor et al. 2017), shedding of cell surface proteins including CD62L (Scheuplein, Schwarz et al. 2009), CD27 (Moon, Na et al. 2006), CD23 (Pupovac, Geraghty et al. 2015), IL-6 (Garbers, Janner et al. 2011) and TNF-α (Suzuki, Hide et al. 2004) as well as externalization of phosphatidylserine (Scheuplein, Schwarz et al. 2009) and induction of cell death (Di Virgilio, Dal Ben et al. 2017).

In our studies, we demonstrate the ATP triggered uptake of fluorescent dye DAPI into monocytes, T cells and B cells and shedding of CD62L from T and B cells. We furthermore show P2X7-mediated IL-1β release from LPS-primed monocytes in whole blood; however, we could not verify this finding for isolated monocytes. One reason for this might be that the blood plasma contains substances we do not know yet, which are required for proper P2X7 activation and would miss in case of isolation. This we could test by retransferring the isolated monocytes into the cell-free blood plasma and performing the activation this way. Receptor internalization during the treatment of the cells is most likely not the cause as we could find unchanged P2X7 expression levels after the isolation (data not shown). In our studies, we could as well not verify release of IL-1β from the MoDCs. This might be due to the same reasons. From the beginning, we did not detect P2X7 expression on microglial CHME-5 cells. Therefore, it is not surprising that we did not find any ATP triggered IL-1β release from these cells.

IL-1β is presumably one of the molecules, if not even the molecule, which is primarily responsible for P2X7 associated inflammation. The IL-1 family is a well-known family of

cytokines, which is predominantly released by immune cells of the myeloid origin, and promotes inflammatory conditions by effecting the migration and activation of neutrophils, T cells, B cells and fibroblasts as well as initiation of fever and prostaglandin synthesis (Keyel 2014). IL-1β is a key molecule not just in acute inflammation but as well in the development of chronic inflammatory diseases including atherosclerosis and Diabetes type 2 (Giuliani, Sarti et al. 2017). The production of IL-1β is linked to inflammasome activation. The inflammasome is a multi-protein complex consisting of the NACHT, LRR and PYD-containing protein 3 (NALP3), apoptosis-associated speck-like protein containing a Caspase Activation and Recruitment Domain (ASC) and pro-Caspase 1. Downstream events of P2X7 gating like efflux of K+ and influx of Ca^{2+} lead to inflammasome activation and autoproteolytic cleavage of pro-Caspase 1 into its active form Caspase 1, which in turn cleaves pro-IL-1β into IL-1β (Keyel 2014) . The production of pro-IL-1β is triggered by interaction of the TLR with components of invading microbes like lipopolysaccharides (LPS), which are components of bacterial cell walls (Giuliani, Sarti et al. 2017). In the past few years, studies have provided exciting new insights into the release mechanism of IL-1β. Cytosolic Gasdermin D (GSDMD) was demonstrated to regulate the package of mature IL-1β together with P2X7, active GSDMD and Caspase 1 into membrane coated microparticles (MP), which are released from the cell. Signaling by the microparticulate P2X7 then prompts the rupture of the MPs and subsequent release of the encapsulated IL-1β into the intercellular space (Mitra and Sarkar 2019). The P2X7-inflammasome-IL-1β-axis is crucial for the combat of infections. This mechanism is well investigated for intracellular bacteria like Chlamydia species (Santos, Rodrigues-Junior et al. 2013) and protozoa like toxoplasma gondii (Lees, Fuller et al. 2010). Many viruses produce inflammasome or Caspase 1 inhibitors (Keyel 2014). This suggests a participation of the P2X7-inflammasome-IL-1β-axis in the defense of viruses as well. Promotion of P2X7 activation could thus be an effective method to support the immune system in the fight against invading microorganisms and viruses. On the other hand, inhibition of the receptor activation could diminish the IL-1β-associated chronic inflammatory diseases.

5.4 ATP and NAD^+ as P2X7 activators

We observe that ATP-mediated activation of P2X7 requires relatively high concentrations of ATP in a millimolar range. Physiologically intracellular ATP is in the range of low millimolar concentrations while extracellular concentrations are much lower within the micromolar range

and clearly below the threshold of P2X7 activation. Thus, in vivo activation of the P2X7 receptor requires a significant increase of ATP in the extracellular compartment. There are different mechanisms in discussion: On the one hand damaged or dying cells release ATP due to cell lysis, on the other hand mounting evidence also indicates molecular pathways including ATP release via pannexins and connexin hemichannels in intact cells (Idzko, Ferrari et al. 2014). Probably there are many more ways of release, which have not been discovered yet. P2X7 activation is not restricted to binding of ATP. Nicotinamide adenine dinucleotide (NAD) is another known P2X7 activator and - analog to ATP - used as energy currency and signaling molecule not just in the human body but all kingdoms of life. It is released similarly to the ATP release mechanisms, triggers comparable downstream reactions and by that holds a similar pro-inflammatory potential. Intracellular levels are in the lower millimolar, whereas extracellular levels are only in the micromolar range. Interestingly, there are only micromolar concentrations of NAD^+ needed to achieve P2X7 activation. Another contrast to ATP is that NAD^+-mediated receptor activation does not work by direct binding of NAD^+ to P2X7. In this case Mono-ADP-ribosyltransferase 2.2 (ART2.2) transfers the ADP-ribose moiety of NAD^+ to a nucleotide binding site on the receptor (Scheuplein, Schwarz et al. 2009). This predestines ART2.2 as another target for the inhibition of P2X7 downstream effects. In this manner, blockade of ART2.2 by specific nanobody S+16a was demonstrated to prevent NAD-induced cell death of tissue-resident memory T cells in the liver of naïve mice in vivo (Rissiek, Lukowiak et al. 2018). The activation mechanism of ADP ribosylation is described mainly for murine T cells and not for human cells. It remains unclear which role NAD^+ plays in human P2X7 activation. There are five different ARTs (ART1-5) described in the human. ART1 is found on leukocytes including Tregs, monocytes and neutrophil granulocytes (Grahnert, Friedrich et al. 2002, Koch-Nolte, Adriouch et al. 2006, Cortes-Garcia, Lopez-Lopez et al. 2016) as well as on epithelial cells and striated and cardiac muscle (Zhao, Gruszczynska-Biegala et al. 2005). While ART2 is the key player in murine P2X7 gating, it is only a pseudogene in the human (Cortes-Garcia, Lopez-Lopez et al. 2016). ART3 is expressed in the embryo, muscle, brain and testis (Levy, Wu et al. 1996, Glowacki, Braren et al. 2002), ART4 can be found on erythrocytes and to a lower extend on monocytes and macrophages in the spleen. The ART5 expression is similar to ART4 expression, though ART5 just occurs in the secreted form (Koch-Nolte, Adriouch et al. 2006). ARTs might also mediate human P2X7 gating by NAD^+, in this regard especially ART1 got into focus (Cortes-Garcia, Lopez-Lopez et al. 2016). Though studies could not prove this presumption yet,

ARTs remain subject of interest as they could present an alternative way of P2X7 activation and hence a further target in the therapy of P2X7-associated diseases.

5.5 ATP activates all P2X receptors and members of the P2Y receptor family

ATP is not only the ligand for P2X7 but for all seven members (P2X1-7) of the P2X receptor family. P2X7 is the best studied of the P2X receptors, whereas surprisingly little is known about P2X1-6. P2X receptors are found throughout the human body where they are involved in a variety of inflammatory processes often acting in concert with one another. P2X1, P2X4, P2X5 and P2X6 are widely expressed on immune cells including neutrophils, monocytes/macrophages and T cells, on neurons, endothelial cells, smooth muscle cells and, except P2X5, in the kidney. P2X2 and P2X3 are expressed on neurons, endothelial cells and smooth muscle cells; P2X3 is additionally found in the kidney (de Rivero Vaccari, Bastien et al. 2012, Fiebich, Akter et al. 2014, Faas, Saez et al. 2017, Fabbretti 2017). A number of cell responses that were so far associated with P2X7 signaling have recently been demonstrated for other P2X receptors as well: In a mouse model of neuroinflammation, P2X4-mediated ion shift triggered the release of IL-1β in neurons of the spinal cord via the inflammasome-Caspase-1-pathway analogue to our findings for P2X7 in human peripheral blood monocytes (de Rivero Vaccari, Bastien et al. 2012). This has not been demonstrated in human cells so far, though P2X4-mediated influx of calcium was demonstrated for human monocytes and macrophages (Layhadi and Fountain 2017). Given these findings, we must assume P2X4 as a further activator of the inflammasome in the human. By contrast, we could show almost complete inhibition of IL-1β release in human peripheral blood monocytes after application of P2X7-specific nb-dimer of Dano1, which implicates P2X7 as the major player in ATP-induced inflammasome activation here. More research has to be done, to shed light on the role of P2X receptors in the inflammasome-IL-1β-axis. In human primary $CD4^+$ T cells, apparently not only P2X7 significantly contributes to T cell activation, but P2X4 and P2X1 as well. All three receptors mediate the influx of Ca^{2+} after TCR stimulation, therefore activating the transcription factor nuclear factor of activated T cells (NFAT), which leads to the expression of cytokine IL-2 (Woehrle, Yip et al. 2010). We know that P2X7 plays a crucial role in the pathomechanism of pain hypersensitivity (Chessell, Hatcher et al. 2005). More recent findings determine the involvement of further P2X receptors, here especially P2X3: homomeric P2X3 and heteromeric P2X2/3 located in the terminal nerve endings in peripheral tissue and the central terminals of dorsal root ganglia are key players in the transmission of

pain by sensitization of C-Fibers (Wang, Dai et al. 2012, Bele and Fabbretti 2016, Bernier, Ase et al. 2017). Besides, P2X4 triggers the release of Prostaglandin 2, a well-known mediator of inflammation, by macrophages in a mouse model of pain hypersensitivity (Ulmann, Hirbec et al. 2010). ATP does not just bind to P2X receptors but as well to receptors of the P2Y receptor family namely P2Y1, P2Y2 and P2Y11. Similar to P2X receptors these receptors are expressed in neutrophils, monocytes/macrophages, T cells, endothelial cells, smooth muscle cells and in the kidney (Faas, Saez et al. 2017) and act in concert with P2X7. For example, P2X7 and P2Y11 activation with ATP inhibits migration of tumor endothelial cells from human breast carcinoma (Avanzato, Genova et al. 2016). A variety of diseases is associated with P2X receptor signaling and we clearly need more research to understand the underlying mechanisms. Without question, P2X receptors are exciting targets of novel treatment strategies.

5.6 Single nucleotide polymorphisms and presence of ectoenzyme CD39 as causes of inter- and intraindividual differences in P2X7 expression and function

Our results reveal notable inter- and even intraindividual differences in P2X7 expression and function. We can in fact determine tendencies concerning P2X7 expression levels in the different cell populations though at the same time we see a considerable range of variation among the individuals. Interestingly, the response to ATP e.g. the amount of IL-1β release does not correlate with the extent of P2X7 expression. The strength of the response rather seems to depend on the functionality of the receptor than on its density on the cell surface (Fig. 10b, Fig. 21).

The human P2X7 gene is highly polymorphic and encloses a considerable number of non-synonymous single nucleotide polymorphisms (nsSNPs). nsSNPs can represent gain- or loss-of-function mutations, that alter the functionality of the P2X7 receptor and affect the disease susceptibility of an organism (Caseley, Muench et al. 2014). ATP-induced ion currents, calcium influx, and dye uptake are common readouts to assess the effect of nsSNPs on P2X7 functioning. The receptor mutations A348T and H155Y facilitate larger ion currents than the wildtype-P2X7 and the H155Y mutation moreover increases the expression of P2X7 on the cell surface in human embryonic kidney HEK293 cells (Bradley, Baldwin et al. 2011). Macrophages of patients with Systemic Lupus Erythematodes and pericarditis expressing the H155Y mutant show increased rates of ethidium dye uptake (Hu, Yu et al. 2019). The A76V mutation facilitates larger ion currents, a stronger influx of calcium and elevated uptake of

ethidium dye in HEK293 cells (Oyanguren-Desez, Rodriguez-Antiguedad et al. 2011). The H270R mutation elevates the ethidium dye uptake in HEK293 cells (Stokes, Fuller et al. 2010). Notably, coexpression of the wild-type P2X7 with the E460R mutant in HEK293 cells results in reduced ion currents and calcium influx following physical interaction of the receptors, whereas single expression of the E460R mutant does not lead to a diminished receptor function (Aprile-Garcia, Metzger et al. 2016). Human peripheral blood lymphocytes and monocytes expressing the T357S mutation show impaired ethidium dye uptake (Shemon, Sluyter et al. 2006). The individual variations in P2X7 functionality we see in our results can be based on nsSNPs. For verification, we could perform genotype analysis for our donors.

In the past few years, a number of studies provided evidence for the association of P2X7 specific nsSNPs and susceptibility to diseases. In case-control-studies, the E460R variant of P2X7 was associated with affective mood disorders including bipolar affective disorder (Barden, Harvey et al. 2006, McQuillin, Bass et al. 2009), major depressive disorder (Hejjas, Szekely et al. 2009) and depression in diabetic patients (Vereczkei, Abdul-Rahman et al. 2019). The E496A mutation is associated with increased susceptibility to infectious diseases including Tuberculosis (Wu, Zhao et al. 2014) and Toxoplasmosis (Lees, Fuller et al. 2010). Moreover, this receptor variant was demonstrated to increase the risk of stress fracture injuries (Varley, Greeves et al. 2016). Interestingly, this P2X7 variation appears to be protective against Alzheimer's disease (Sanz, Falzoni et al. 2014). Various other P2X7 nsSNPs were shown play an important role in disease development. Regarding this data, P2X7 nsSNPs might function as biomarkers for diagnostic purposes as well as for the designing of specific disease treatments (Caseley, Muench et al. 2014).

One further cause for inter- and intraindividual differences in P2X7 responses to ATP could be the presence of ectoenzyme CD39. CD39 (ectonucleoside triphosphate diphosphohydrolase 1, ENTPD1) is expressed by a range of immune cells including T cells, B cells, DCs, monocytes and NK cells. It takes part in the regulation of the extracellular ATP concentration as it has the ability to hydrolase ATP and also Adenosinediphosphate (ADP) into Adenosine monophosphate (AMP) (Antonioli, Pacher et al. 2013). Since AMP does not bind to P2X7, it reduces the supply of P2X7 agonists and diminishes the activation of the receptor. CD39 is therefore eminent in the regulation of pro-inflammatory signals. It even provides anti-inflammatory signals in interaction with CD73 (ecto-5'-nucleotidase, NT5E), another ectoenzyme expressed by lymphocytes. CD73 degrades AMP to Adenosine, which binds to adenosine receptors on T cells and mediates immunosuppressive signals (Zhang 2010). The expression level of CD39 differs depending on the cell type and is subject to great

interindividual fluctuations. Tregs exhibit a relatively strong CD39 expression whereas expression levels are low on conventional Th cells. At the same time, the expression on the Tregs varies from donor to donor ranging from 2 – 70% of the cell populations (Rissiek, Baumann et al. 2015). This means, P2X7 function might vary inter- and even intra-individually depending on the expression of CD39 and the availability of ATP linked to it.
To determine the association of inter- and intraindividual variations in P2X7 function observed in our experiments and the expression of CD39, we should assess the CD39 expression levels in the cells of our donors.

5.7 Nanobodies and other P2X7 modulators as tools for P2X7 research and potential therapeutic drugs

In our studies, we identify the nbs Dano1 and Dano3 - in this case labelled with a fluorochrome - as an effective tool for P2X7 imaging by FACS. Determination of the MFI ratios gives us tendencies regarding P2X7 expression levels and allows the comparison of the different cell types and the individuals. One limitation of this method is that it does not enable a statement on absolute expression levels. However, it is perfectly suitable to relate the P2X7 expression to an approximate magnitude. We moreover demonstrate P2X7-specific nb Dano1 as a potent inhibitor of ATP triggered P2X7 activation. We achieved effective blocking of ATP triggered uptake of fluorescence dye DAPI in monocytes, T cells and B cells, shedding of CD62L in T cells and B cells and release of IL-1β in monocytes. These findings emphasize the great potential of nbs directed against P2X7 for the therapy of P2X7-mediated diseases in the future. Danquah et al. could already demonstrate the benefit of anti-P2X7 nb application in vivo: Systemic injection of nb 13A7 ameliorated allergic contact dermatitis and antibody-induced glomerulonephritis in mouse models of chronic inflammatory disease. Injection of nb 14D5-HLE in contrast enhanced the disease progression. This effect could be useful for the treatment of cancer or infection. Besides, nb Dano1 blocks the release of IL-1β in our in vitro inflammation model to a 1000-fold higher potency than small-molecule P2X7 antagonists that are currently in development for inflammatory and neurological diseases (Danquah, Meyer-Schwesinger et al. 2016). Our studies show that the reaction to ATP varies from human to human. We assume that the sensitivity of the receptor is decisively determined by individual genetic polymorphisms (see chapter 6.6). It is conceivable that the clinical effect of P2X7 nbs could also be subject of considerable fluctuations depending on the sensitivity of the receptor. According to this, patients with highly sensitive receptor variants would respond better to a nb

treatment than patients with less sensitive variants. In that case it might be a necessity to genotype the patients before treatment and to adjust the therapy individually. In our in vitro studies, however, the nb shows a great efficacy for all donors apparently independent of the respective level of P2X7 response to ATP.

There is a broad spectrum of further P2X7 antagonists, which greatly differ in their properties like their chemical structure or selectivity. Of the first generation of P2X7 antagonists, which was manufactured in the 1980'ies and 1990'ies, particularly Brilliant Blue G (BBG), pyridoxal phosphate-6-azophenyl-2-4-disulfonic acid (PPDAS) and periodate-oxidized ATP (oATP) have proven effectiveness until today. Yet, their usefulness in vivo is restricted by poor pharmacokinetics and interfering degradation processes (Bartlett, Stokes et al. 2014). The next generations of P2X7 antagonists were rather tailored for the in-vivo use with regard to a potential clinical application in the future. Examples include the cyclic imide (AZ11645373) (Stokes, Jiang et al. 2006) and the nicotinamide derivative JNJ-47965567 (Bhattacharya, Wang et al. 2013). At the moment, there are several compounds in human clinical trials, benefit was just proven for one compound yet. AZD9056 by AstraZeneca, was demonstrated to be effective in the treatment of Crohn's disease, though the effect is rather assumed to be symptomatic as it ameliorates chronic abdominal pain, but does not effect change in inflammatory biomarkers (Eser, Colombel et al. 2015). However, AZD9056 did not show benefit in the treatment of rheumatoid arthritis in phase II clinical trials, treatment of osteoarthritis or treatment chronic obstructive pulmonary disease (Keystone, Wang et al. 2012, Danquah, Meyer-Schwesinger et al. 2016). Compound CE-224,535 by Pfizer was as well not beneficial in phase II clinical trials with patients with rheumatoid arthritis (Stock, Bloom et al. 2012). There are many further P2X7 inhibitors described including phenothiazine-class antipsychotic drugs prochlorperazine and trifluoperazine (Hempel, Norenberg et al. 2013), enzyme inhibitors like protein kinase C (Shemon, Sluyter et al. 2006) or even particular cations including Mg^{2+} and Ca^{2+} (Chessell 1998). The previous clinical studies with P2X7 antagonists have not achieved remarkable results yet. Insufficient selectivity, short in-vivo half-life or small therapeutic windows are just a few of the current limitations. Nbs directed against P2X7 feature various advantageous properties including simpler pharmacodynamics and higher selectivity compared to the current small-molecule P2X7 antagonists (Danquah, Meyer-Schwesinger et al. 2016). Nbs targeting P2X7 have to be tested in human clinical trials now in order to verify the promising present results for the clinical use. All in all, nbs represent a highly potent new generation of biological P2X7 antagonists that hold a great potential as therapy for P2X7-mediated diseases.

6 Summary

In this study, we investigated the expression and function of the P2X7 receptor in human immune cells and assessed the potential of nanobodies (nb) directed against human P2X7 for receptor staining and modulation.

We found the highest P2X7 expression levels in innate immune cells: P2X7 expression was particularly strong in the monocytes, followed by NK cells, mDCs and pDCs. Cells of the adaptive immune system showed rather weak P2X7 expression, with medium to low levels on the Tregs, followed by $CD4^+$ T cells, $CD8^+$ T cells and B cells. In mice, Retinoic acid mediates upregulation of P2X7 on conventional $CD8\alpha\beta^+$ and unconventional $CD8\alpha\alpha^+$ T cells of the intestine and periphery as well as in intestinal $CD4^+$ T cells. We did not observe upregulation of human P2X7 in stimulated peripheral $CD8\alpha\alpha^+$, $CD8\alpha\beta^+$ and $CD4^+$ T cells or isolated monocytes. The expression levels of the innate-like cells including MAIT cells, TCRγδ cells and iNKT were in between those of the cells of the innate and the adaptive immunity. We could not detect P2X7 in the granulocytes. P2X7 signaling is associated with diseases of the central nervous system and due to difficult access to human primary microglia, we investigated P2X7 expression in a cell line of human microglial origin. We did not detect P2X7 expression in the microglial cell-line CHME-5 or the neuroblastoma cell line SH-SY5Y.

We demonstrated the ATP-induced uptake of fluorescent dye DAPI into monocytes, T cells and B cells, shedding of CD62L from the cell surface of T and B cells and release of pro-inflammatory cytokine IL-1β in LPS-primed monocytes. Nb-dimer of Dano1 was a potent inhibitor in all cases. We could not show IL-1β release in isolated monocytes, MoDCs or human microglia cell line CHME-5. We observed remarkable inter- and even intra-individual differences in P2X7 expression and functionality.

Our study demonstrates the presence of human P2X7 in various immune cell types and highlights its pro-inflammatory potential. Nbs prove as excellent tools for P2X7 research. Since studies indicate P2X7 as a key player in inflammatory diseases, nbs hold promise as a novel therapeutic strategy.

7 Zusammenfassung

In dieser Arbeit untersuchten wir die Expression und Funktion des P2X7-Rezeptors in humanen Immunzellen und evaluierten das Potential P2X7-spezifischer Nanobodies (Nb) bezüglich Anfärbung und funktioneller Modulation des Rezeptors.

Die höchsten Expressionslevel fanden sich auf Zellen des angeborenen Immunsystems: Die P2X7-Expression war insbesondere ausgeprägt auf den Monozyten, gefolgt von den NK Zellen, den myeloiden dendritischen Zellen und den plasmazytoiden dendritischen Zellen. Zellen des adaptiven Immunsystems zeigten eine eher geringe P2X7-Expression mit mittelgradigem bis niedrigem Expressionslevel auf den regulatorischen T-Zellen gefolgt von $CD4^+$ T-Zellen, $CD8^+$ T-Zellen und B-Zellen. In Mäusen bewirkt Retinsäure eine Hochregulation von P2X7 auf konventionellen $CD8\alpha\beta^+$ und unkonventionellen $CD8\alpha\alpha^+$ T-Zellen der Peripherie und des Intestinums sowie auch auf intestinalen $CD4^+$ T-Zellen. Wir konnten keine Hochregulation von humanem P2X7 auf peripheren stimulierten $CD8\alpha\alpha^+$, $CD8\alpha\beta^+$ und $CD4^+$ T-Zellen oder isolierten Monozyten beobachten. Die Expressionslevel der *innate-like* Zellen einschließlich MAIT-Zellen, TCRγδ-Zellen und iNKT-Zellen lagen zwischen denen der Zellen des angeborenen und des adaptiven Immunsystems. Auf den Granulozyten ließ sich P2X7 nicht nachweisen. P2X7-vermittelte Immunreaktionen sind assoziiert mit Erkrankungen des Zentralnervensystems und aufgrund des erschwerten Zuganges zu primärer humaner Mikroglia untersuchten wir die P2X7-Espression auf einer Zelllinie humanen mikroglialen Ursprungs. Wir konnten auf Zellen der Mikroglia-Zelllinie CHME-5 sowie der Neuroblastom-Zelllinie SH-SY5Y keine P2X7-Expression nachweisen.

Wir zeigten die ATP-induzierte Aufnahme von Fluoreszenzfarbstoff DAPI in Monozyten, T-Zellen und B-Zellen, Abspaltung von CD62L von der Zelloberfläche von T-Zellen und B-Zellen und die Freisetzung des pro-inflammatorischen Zytokins IL-1β aus LPS-geprimten Monozyten. Nb-Dimer Dano1 erwies sich in allen Fällen als potenter Inhibitor. Wir konnten keine Freisetzung von IL-1β in isolierten Monozyten, von Monozyten abgeleiteten dendritischen Zellen (MoDC) oder Zellen der humanen Mikroglia-Zelllinie CHME-5 nachweisen. Wir stellten bemerkenswerte inter- und sogar intraindividuelle Unterschiede in der Expression und Funktionalität des P2X7-Rezeptors fest.

Diese Arbeit zeigt die Präsenz des humanen P2X7-Rezeptors auf einer Vielzahl unterschiedlicher Immunzelltypen und beleuchtet sein pro-inflammatorisches Potential. Nbs erweisen sich als exzellentes Werkzeug für die Erforschung von P2X7. Aktuelle Studien

deuten auf eine Schlüsselrolle von P2X7 bei der Entstehung entzündlicher Erkrankungen hin, folglich stellen Nbs eine vielversprechende neuartige Therapiestrategie dar.

8 Abbreviations

Ab	Antibody
ADAM	A disintegrin and metalloprotease
ADP	Adenosine diphosphate
AF	Alexa Fluor
AMP	Adenosine monophosphate
APC	Allophycocyanin
ART	ADP-ribosyl transferase
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BBG	Brilliant Blue G
BCR	B cell receptor
BSA	Bovine serum albumin
BV	Brilliant violet
cAb	Classical antibody
Calm	Calmodulin
CD	Cluster of differentiation
C_H	Constant domain of the light chain
C_L	Constant domain of the light chain
Cy	Cyanine
d	Day
DAMP	Danger associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
dim	Dimer
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide cell culture grade
ELISA	Enzyme-linked immunosorbent assay
ENTPD	Ectonucleoside triphosphate diphosphohydrolase
FACS	Fluorescence-activated cell sorting
Fc	Fragment of crystallization
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
FSC	Forward scatter light
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSDMD	Gasdermin D
hcAb	Heavy chain only ab
HLA-DR	Human leukocyte antigen-antigen D related
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant natural killer T
Lin	Lineage
LPS	Lipopolysaccharide
MACS	Magnetic cell sorting
MAIT	Mucosa associated innate-like T
M-CSF	Macrophage colony-stimulating factor
mDC	Myeloid dendritic cell

MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MoDC	Monocyte-derived dendritic cell
MP	Microparticle
NAD	Nicotinamide adenine dinucleotide
NALP3	NACHT, LRR and PYD-containing protein 3
Nb	Nanobody
NFAT	Nuclear factor of activated T cells
NK cells	Natural killer cells
NOD	Nucleotide-binding oligomerization domain
nsSNP	Non-synonymous single nucleotide polymorphism
NT5E	Ecto-5'-nucleotidase
oATP	periodate-oxidized ATP
PBMC	Peripheral blood mononuclear cell
PBS	Dulbecco's Phosphate Buffered Saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PPDAS	Pyridoxal phosphate-6-azophenyl-2-4-disulfonic acid
PS	Phosphatidylserine
RA	Retinoic acid
rbFc	Rabbit Fragment of crystallization
rh	Recombinant human
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute (medium)
RT	Room temperature
sCD62L	soluble CD62L
SNP	Single nucleotide polymorphism
SSC	Side scatter light
Tc	Cytotoxic T
TCR	T cell receptor
Tfh cell	T follicular helper cells
TGF	Transforming growth factor
Th cell	T helper cell
TLR	Toll-like-receptor
TNF	Tumor necrosis factor
Treg	Regulatory T
UTP	Uridine triphosphate
V	Violet
V_H	Variable domain of the heavy chain
V_HH	Variable domain of the heavy chain only antibody
V_L	Variable domain of the light chain

9 Bibliography

Ablynx website. URL: https://clinicaltrials.gov/ct2/results?term=ablynx [Stand: 06.10.2019]

Adinolfi, E., L. Raffaghello, A. L. Giuliani, L. Cavazzini, M. Capece, P. Chiozzi, G. Bianchi, G. Kroemer, V. Pistoia and F. Di Virgilio (2012). "Expression of P2X7 receptor increases in vivo tumor growth." Cancer Res **72**(12): 2957-2969.

Agrati, C., G. D'Offizi, P. Narciso, S. Abrignani, G. Ippolito, V. Colizzi and F. Poccia (2001). "Vdelta1 T lymphocytes expressing a Th1 phenotype are the major gammadelta T cell subset infiltrating the liver of HCV-infected persons." Mol Med **7**(1): 11-19.

Amoroso, F., M. Capece, A. Rotondo, D. Cangelosi, M. Ferracin, A. Franceschini, L. Raffaghello, V. Pistoia, L. Varesio and E. Adinolfi (2015). "The P2X7 receptor is a key modulator of the PI3K/GSK3beta/VEGF signaling network: evidence in experimental neuroblastoma." Oncogene **34**(41): 5240-5251.

Andrei, C., P. Margiocco, A. Poggi, L. V. Lotti, M. R. Torrisi and A. Rubartelli (2004). "Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: Implications for inflammatory processes." Proc Natl Acad Sci U S A **101**(26): 9745-9750.

Antonioli, L., P. Pacher, E. S. Vizi and G. Hasko (2013). "CD39 and CD73 in immunity and inflammation." Trends Mol Med **19**(6): 355-367.

Apolloni, S., S. Amadio, C. Parisi, A. Matteucci, R. L. Potenza, M. Armida, P. Popoli, N. D'Ambrosi and C. Volonte (2014). "Spinal cord pathology is ameliorated by P2X7 antagonism in a SOD1-mutant mouse model of amyotrophic lateral sclerosis." Dis Model Mech **7**(9): 1101-1109.

Aprile-Garcia, F., M. W. Metzger, M. Paez-Pereda, H. Stadler, M. Acuna, A. C. Liberman, S. A. Senin, J. Gerez, E. Hoijman, D. Refojo, M. Mitkovski, M. Panhuysen, W. Stuhmer, F. Holsboer, J. M. Deussing and E. Arzt (2016). "Co-Expression of Wild-Type P2X7R with Gln460Arg Variant Alters Receptor Function." PLoS One **11**(3): e0151862.

Avanzato, D., T. Genova, A. Fiorio Pla, M. Bernardini, S. Bianco, B. Bussolati, D. Mancardi, E. Giraudo, F. Maione, P. Cassoni, I. Castellano and L. Munaron (2016). "Activation of P2X7 and P2Y11 purinergic receptors inhibits migration and normalizes tumor-derived endothelial cells via cAMP signaling." Sci Rep **6**: 32602.

Bannas, P., L. Well, A. Lenz, B. Rissiek, F. Haag, J. Schmid, K. Hochgrafe, M. Trepel, G. Adam, H. Ittrich and F. Koch-Nolte (2014). "In vivo near-infrared fluorescence targeting of T cells: comparison of nanobodies and conventional monoclonal antibodies." Contrast Media Mol Imaging **9**(2): 135-142.

Barden, N., M. Harvey, B. Gagne, E. Shink, M. Tremblay, C. Raymond, M. Labbe, A. Villeneuve, D. Rochette, L. Bordeleau, H. Stadler, F. Holsboer and B. Muller-Myhsok (2006). "Analysis of single nucleotide polymorphisms in genes in the chromosome 12Q24.31 region points to P2RX7 as a susceptibility gene to bipolar affective disorder." Am J Med Genet B Neuropsychiatr Genet **141B**(4): 374-382.

Bartlett, R., L. Stokes and R. Sluyter (2014). "The P2X7 receptor channel: recent developments and the use of P2X7 antagonists in models of disease." Pharmacol Rev **66**(3): 638-675.

Beamer, E., W. Fischer and T. Engel (2017). "The ATP-Gated P2X7 Receptor As a Target for the Treatment of Drug-Resistant Epilepsy." Front Neurosci **11**: 21.

Becker, D., R. Woltersdorf, W. Boldt, S. Schmitz, U. Braam, G. Schmalzing and F. Markwardt (2008). "The P2X7 carboxyl tail is a regulatory module of P2X7 receptor channel activity." J Biol Chem **283**(37): 25725-25734.

Bele, T. and E. Fabbretti (2016). "The scaffold protein calcium/calmodulin-dependent serine protein kinase controls ATP release in sensory ganglia upon P2X3 receptor activation and is part of an ATP keeper complex." J Neurochem **138**(4): 587-597.

Bernier, L. P., A. R. Ase and P. Seguela (2017). "P2X receptor channels in chronic pain pathways." Br J Pharmacol.

Bhattacharya, A., Q. Wang, H. Ao, J. R. Shoblock, B. Lord, L. Aluisio, I. Fraser, D. Nepomuceno, R. A. Neff, N. Welty, T. W. Lovenberg, P. Bonaventure, A. D. Wickenden and M. A. Letavic (2013). "Pharmacological characterization of a novel centrally permeable P2X7 receptor antagonist: JNJ-47965567." Br J Pharmacol **170**(3): 624-640.

Biedler, J. L., S. Roffler-Tarlov, M. Schachner and L. S. Freedman (1978). "Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones." Cancer Res **38**(11 Pt 1): 3751-3757.

Blobel, C. P. (2005). "ADAMs: key components in EGFR signalling and development." Nat Rev Mol Cell Biol **6**(1): 32-43.

Booth, J. S., R. Salerno-Goncalves, T. G. Blanchard, S. A. Patil, H. A. Kader, A. M. Safta, L. M. Morningstar, S. J. Czinn, B. D. Greenwald and M. B. Sztein (2015). "Mucosal-Associated Invariant T Cells in the Human Gastric Mucosa and Blood: Role in Helicobacter pylori Infection." Front Immunol **6**: 466.

Bradley, H. J., J. M. Baldwin, G. R. Goli, B. Johnson, J. Zou, A. Sivaprasadarao, S. A. Baldwin and L. H. Jiang (2011). "Residues 155 and 348 contribute to the determination of P2X7 receptor function via distinct mechanisms revealed by single-nucleotide polymorphisms." J Biol Chem **286**(10): 8176-8187.

Bumbaca, D., C. A. Boswell, P. J. Fielder and L. A. Khawli (2012). "Physiochemical and biochemical factors influencing the pharmacokinetics of antibody therapeutics." AAPS J **14**(3): 554-558.

Burnstock, G. (2007). "Purine and pyrimidine receptors." Cell Mol Life Sci **64**(12): 1471-1483.

Caseley, E. A., S. P. Muench, S. Roger, H. J. Mao, S. A. Baldwin and L. H. Jiang (2014). "Non-synonymous single nucleotide polymorphisms in the P2X receptor genes: association with diseases, impact on receptor functions and potential use as diagnosis biomarkers." Int J Mol Sci **15**(8): 13344-13371.

Chen, S., Z. Zhu, D. Klebe, H. Bian, P. R. Krafft, J. Tang, J. Zhang and J. H. Zhang (2014). "Role of P2X purinoceptor 7 in neurogenic pulmonary edema after subarachnoid hemorrhage in rats." PLoS One **9**(2): e89042.

Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady and S. M. Wahl (2003). "Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3." J Exp Med **198**(12): 1875-1886.

Chen, Z., H. Wang, C. D'Souza, S. Sun, L. Kostenko, S. B. Eckle, B. S. Meehan, D. C. Jackson, R. A. Strugnell, H. Cao, N. Wang, D. P. Fairlie, L. Liu, D. I. Godfrey, J. Rossjohn, J. McCluskey and A. J. Corbett (2017). "Mucosal-associated invariant T-cell activation and accumulation after in vivo infection depends on microbial riboflavin synthesis and co-stimulatory signals." Mucosal Immunol **10**(1): 58-68.

Chessell, I. P., J. P. Hatcher, C. Bountra, A. D. Michel, J. P. Hughes, P. Green, J. Egerton, M. Murfin, J. Richardson, W. L. Peck, C. B. Grahames, M. A. Casula, Y. Yiangou, R. Birch, P. Anand and G. N. Buell (2005). "Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain." Pain **114**(3): 386-396.

Ciccarelli, B. T., G. Yang, E. Hatjiharissi, L. Ioakimidis, C. J. Patterson, R. J. Manning, L. Xu, X. Liu, H. Tseng, P. Gong, J. Sun, Y. Zhou and S. P. Treon (2009). "Soluble CD27 is a faithful marker of disease burden and is unaffected by the rituximab-induced IgM flare, as well as by plasmapheresis, in patients with Waldenstrom's macroglobulinemia." Clin Lymphoma Myeloma **9**(1): 56-58.

Cisneros-Mejorado, A., M. Gottlieb, F. Cavaliere, T. Magnus, F. Koch-Nolte, E. Scemes, A. Perez-Samartin and C. Matute (2015). "Blockade of P2X7 receptors or pannexin-1 channels similarly attenuates postischemic damage." J Cereb Blood Flow Metab **35**(5): 843-850.

Cooper, A. M., P. S. Hobson, M. R. Jutton, M. W. Kao, B. Drung, B. Schmidt, D. J. Fear, A. J. Beavil, J. M. McDonnell, B. J. Sutton and H. J. Gould (2012). "Soluble CD23 controls IgE synthesis and homeostasis in human B cells." J Immunol **188**(7): 3199-3207.

Correia, D. V., M. Fogli, K. Hudspeth, M. G. da Silva, D. Mavilio and B. Silva-Santos (2011). "Differentiation of human peripheral blood Vdelta1+ T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells." Blood **118**(4): 992-1001.

Cortes-Garcia, J. D., C. Lopez-Lopez, N. Cortez-Espinosa, M. H. Garcia-Hernandez, J. M. Guzman-Flores, E. Layseca-Espinosa, L. Portales-Cervantes and D. P. Portales-Perez (2016). "Evaluation of the expression and function of the P2X7 receptor and ART1 in human regulatory T-cell subsets." Immunobiology **221**(1): 84-93.

Coutinho-Silva, R., L. Stahl, M. N. Raymond, T. Jungas, P. Verbeke, G. Burnstock, T. Darville and D. M. Ojcius (2003). "Inhibition of chlamydial infectious activity due to P2X7R-dependent phospholipase D activation." Immunity **19**(3): 403-412.

D'Alimonte, I., E. Nargi, M. Zuccarini, P. Lanuti, P. Di Iorio, P. Giuliani, L. Ricci-Vitiani, R. Pallini, F. Caciagli and R. Ciccarelli (2015). "Potentiation of temozolomide antitumor effect by purine receptor ligands able to restrain the in vitro growth of human glioblastoma stem cells." Purinergic Signal **11**(3): 331-346.

Dang, L. V., A. Nilsson, H. Ingelman-Sundberg, A. Cagigi, L. B. Gelinck, K. Titanji, A. De Milito, S. Grutzmeier, J. Hedlund, F. P. Kroon and F. Chiodi (2012). "Soluble CD27 induces IgG production through activation of antigen-primed B cells." J Intern Med **271**(3): 282-293.

Danquah, W. (2012). "Selection and characterization of llama-derived anti-P2X7 single domain antibodies." Scientific dissertation. University of Hamburg

Danquah, W., C. Meyer-Schwesinger, B. Rissiek, C. Pinto, A. Serracant-Prat, M. Amadi, D. Iacenda, J. H. Knop, A. Hammel, P. Bergmann, N. Schwarz, J. Assuncao, W. Rotthier, F. Haag, E. Tolosa, P. Bannas, E. Boue-Grabot, T. Magnus, T. Laeremans, C. Stortelers and F. Koch-Nolte (2016). "Nanobodies that block gating of the P2X7 ion channel ameliorate inflammation." Sci Transl Med **8**(366): 366ra162.

Darville, T., L. Welter-Stahl, C. Cruz, A. A. Sater, C. W. Andrews, Jr. and D. M. Ojcius (2007). "Effect of the purinergic receptor P2X7 on Chlamydia infection in cervical epithelial cells and vaginally infected mice." J Immunol **179**(6): 3707-3714.

Das, S., R. K. Seth, A. Kumar, M. B. Kadiiska, G. Michelotti, A. M. Diehl and S. Chatterjee (2013). "Purinergic receptor X7 is a key modulator of metabolic oxidative stress-mediated autophagy and inflammation in experimental nonalcoholic steatohepatitis." Am J Physiol Gastrointest Liver Physiol **305**(12): G950-963.

De Milito, A., S. Aleman, R. Marenzi, A. Sonnerborg, D. Fuchs, M. Zazzi and F. Chiodi (2002). "Plasma levels of soluble CD27: a simple marker to monitor immune activation during potent antiretroviral therapy in HIV-1-infected subjects." Clin Exp Immunol **127**(3): 486-494.

de Rivero Vaccari, J. P., D. Bastien, G. Yurcisin, I. Pineau, W. D. Dietrich, Y. De Koninck, R. W. Keane and S. Lacroix (2012). "P2X4 receptors influence inflammasome activation after spinal cord injury." J Neurosci **32**(9): 3058-3066.

DeLeo, F. R. (2007). "Attractive shedding." Blood **110**(6): 1711-1712.

Di Virgilio, F. (2007). "Liaisons dangereuses: P2X(7) and the inflammasome." Trends Pharmacol Sci **28**(9): 465-472.

Di Virgilio, F., D. Dal Ben, A. C. Sarti, A. L. Giuliani and S. Falzoni (2017). "The P2X7 Receptor in Infection and Inflammation." Immunity **47**(1): 15-31.

Diaz-Hernandez, M., M. Diez-Zaera, J. Sanchez-Nogueiro, R. Gomez-Villafuertes, J. M. Canals, J. Alberch, M. T. Miras-Portugal and J. J. Lucas (2009). "Altered P2X7-receptor level and function in mouse models of Huntington's disease and therapeutic efficacy of antagonist administration." FASEB J **23**(6): 1893-1906.

Ding, J., K. Wang, W. Liu, Y. She, Q. Sun, J. Shi, H. Sun, D. C. Wang and F. Shao (2016). "Pore-forming activity and structural autoinhibition of the gasdermin family." Nature **535**(7610): 111-116.

Dominitzki, S., M. C. Fantini, C. Neufert, A. Nikolaev, P. R. Galle, J. Scheller, G. Monteleone, S. Rose-John, M. F. Neurath and C. Becker (2007). "Cutting edge: trans-signaling via the soluble IL-6R abrogates the induction of FoxP3 in naive CD4+CD25 T cells." J Immunol **179**(4): 2041-2045.

Englezou, P. C., S. W. Rothwell, J. S. Ainscough, D. Brough, R. Landsiedel, A. Verkhratsky, I. Kimber and R. J. Dearman (2015). "P2X7R activation drives distinct IL-1 responses in dendritic cells compared to macrophages." Cytokine **74**(2): 293-304.

Eser, A., J. F. Colombel, P. Rutgeerts, S. Vermeire, H. Vogelsang, M. Braddock, T. Persson and W. Reinisch (2015). "Safety and Efficacy of an Oral Inhibitor of the Purinergic Receptor P2X7 in Adult Patients with Moderately to Severely Active Crohn's Disease: A Randomized Placebo-controlled, Double-blind, Phase IIa Study." Inflamm Bowel Dis **21**(10): 2247-2253.

Faas, M. M., T. Saez and P. de Vos (2017). "Extracellular ATP and adenosine: The Yin and Yang in immune responses?" Mol Aspects Med **55**: 9-19.

Fabbretti, E. (2017). "ATP-Gated P2X3 Receptors Are Specialised Sensors of the Extracellular Environment." Adv Exp Med Biol.

Fadok, V. A., D. L. Bratton, D. M. Rose, A. Pearson, R. A. Ezekewitz and P. M. Henson (2000). "A receptor for phosphatidylserine-specific clearance of apoptotic cells." Nature **405**(6782): 85-90.

Fenoglio, D., A. Poggi, S. Catellani, F. Battaglia, A. Ferrera, M. Setti, G. Murdaca and M. R. Zocchi (2009). "Vdelta1 T lymphocytes producing IFN-gamma and IL-17 are expanded in HIV-1-infected patients and respond to Candida albicans." Blood **113**(26): 6611-6618.

Fiebich, B. L., S. Akter and R. S. Akundi (2014). "The two-hit hypothesis for neuroinflammation: role of exogenous ATP in modulating inflammation in the brain." Front Cell Neurosci **8**: 260.

Figliuolo, V. R., S. P. Chaves, L. E. B. Savio, M. L. P. Thorstenberg, E. Machado Salles, C. M. Takiya, M. R. D'Imperio-Lima, H. L. de Matos Guedes, B. Rossi-Bergmann and R. Coutinho-Silva (2017). "The role of the P2X7 receptor in murine cutaneous leishmaniasis: aspects of inflammation and parasite control." Purinergic Signal **13**(2): 143-152.

Font, J., L. Pallares, J. Martorell, E. Martinez, A. Gaya, J. Vives and M. Ingelmo (1996). "Elevated soluble CD27 levels in serum of patients with systemic lupus erythematosus." Clin Immunol Immunopathol **81**(3): 239-243.

Fredholm, B. B., I. J. AP, K. A. Jacobson and J. M. Linden, C. E. (2011). "International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors--an update." Pharmacol Rev **63**(1): 1-34.

Garbers, C., N. Janner, A. Chalaris, M. L. Moss, D. M. Floss, D. Meyer, F. Koch-Nolte, S. Rose-John and J. Scheller (2011). "Species specificity of ADAM10 and ADAM17 proteins in interleukin-6 (IL-6) trans-signaling and novel role of ADAM10 in inducible IL-6 receptor shedding." J Biol Chem **286**(17): 14804-14811.

Geissmann, F., M. G. Manz, S. Jung, M. H. Sieweke, M. Merad and K. Ley (2010). "Development of monocytes, macrophages, and dendritic cells." Science **327**(5966): 656-661.

Giannuzzo, A., M. Saccomano, J. Napp, M. Ellegaard, F. Alves and I. Novak (2016). "Targeting of the P2X7 receptor in pancreatic cancer and stellate cells." Int J Cancer **139**(11): 2540-2552.

Ginhoux, F. and S. Jung (2014). "Monocytes and macrophages: developmental pathways and tissue homeostasis." Nat Rev Immunol **14**(6): 392-404.

Giuliani, A. L., A. C. Sarti, S. Falzoni and F. Di Virgilio (2017). "The P2X7 Receptor-Interleukin-1 Liaison." Front Pharmacol **8**: 123.

Glowacki, G., R. Braren, K. Firner, M. Nissen, M. Kuhl, P. Reche, F. Bazan, M. Cetkovic-Cvrlje, E. Leiter, F. Haag and F. Koch-Nolte (2002). "The family of toxin-related ecto-ADP-ribosyltransferases in humans and the mouse." Protein Sci **11**(7): 1657-1670.

Grahnert, A., M. Friedrich, M. Pfister, F. Haag, F. Koch-Nolte and S. Hauschildt (2002). "Mono-ADP-ribosyltransferases in human monocytes: regulation by lipopolysaccharide." Biochem J **362**(Pt 3): 717-723.

Granado, M., S. Amor, J. J. Montoya, L. Monge, N. Fernandez and A. L. Garcia-Villalon (2015). "Altered expression of P2Y2 and P2X7 purinergic receptors in the isolated rat heart mediates ischemia-reperfusion injury." Vascul Pharmacol **73**: 96-103.

Grygorowicz, T., M. Welniak-Kaminska and L. Struzynska (2016). "Early P2X7R-related astrogliosis in autoimmune encephalomyelitis." Mol Cell Neurosci **74**: 1-9.

Gu, B. J., W. Y. Zhang, L. J. Bendall, I. P. Chessell, G. N. Buell and J. S. Wiley (2000). "Expression of P2X(7) purinoceptors on human lymphocytes and monocytes: evidence for nonfunctional P2X(7) receptors." Am J Physiol Cell Physiol **279**(4): C1189-1197.

Gulbransen, B. D., M. Bashashati, S. A. Hirota, X. Gui, J. A. Roberts, J. A. MacDonald, D. A. Muruve, D. M. McKay, P. L. Beck, G. M. Mawe, R. J. Thompson and K. A. Sharkey (2012). "Activation of neuronal P2X7 receptor-pannexin-1 mediates death of enteric neurons during colitis." Nat Med **18**(4): 600-604.

Hall, J. A., J. R. Grainger, S. P. Spencer and Y. Belkaid (2011). "The role of retinoic acid in tolerance and immunity." Immunity **35**(1): 13-22.

Hashimoto-Hill, S., L. Friesen, M. Kim and C. H. Kim (2017). "Contraction of intestinal effector T cells by retinoic acid-induced purinergic receptor P2X7." Mucosal Immunol **10**(4): 912-923.

He, Y., N. Taylor, L. Fourgeaud and A. Bhattacharya (2017). "The role of microglial P2X7: modulation of cell death and cytokine release." J Neuroinflammation **14**(1): 135.

Heiss, K., N. Janner, B. Mahnss, V. Schumacher, F. Koch-Nolte, F. Haag and H. W. Mittrucker (2008). "High sensitivity of intestinal CD8+ T cells to nucleotides indicates P2X7 as a regulator for intestinal T cell responses." J Immunol **181**(6): 3861-3869.

Hejjas, K., A. Szekely, E. Domotor, Z. Halmai, G. Balogh, B. Schilling, A. Sarosi, G. Faludi, M. Sasvari-Szekely and Z. Nemoda (2009). "Association between depression and the Gln460Arg polymorphism of P2RX7 gene: a dimensional approach." Am J Med Genet B Neuropsychiatr Genet **150B**(2): 295-299.

Hempel, C., W. Norenberg, H. Sobottka, N. Urban, A. Nicke, W. Fischer and M. Schaefer (2013). "The phenothiazine-class antipsychotic drugs prochlorperazine and trifluoperazine are potent allosteric modulators of the human P2X7 receptor." Neuropharmacology **75**: 365-379.

Hori, S., T. Nomura and S. Sakaguchi (2003). "Control of regulatory T cell development by the transcription factor Foxp3." Science **299**(5609): 1057-1061.

Hu, H., B. Yang, Y. Li, S. Zhang and Z. Li (2016). "Blocking of the P2X7 receptor inhibits the activation of the MMP-13 and NF-kappaB pathways in the cartilage tissue of rats with osteoarthritis." Int J Mol Med **38**(6): 1922-1932.

Hu, S., F. Yu, C. Ye, X. Huang, X. Lei, Y. Dai, H. Xu, Y. Wang and Y. Yu (2019). "The presence of P2RX7 single nuclear polymorphism is associated with a gain of function in P2X7 receptor and inflammasome activation in SLE complicated with pericarditis." Clin Exp Rheumatol.

Huang, J., C. Jochems, A. M. Anderson, T. Talaie, A. Jales, R. A. Madan, J. W. Hodge, K. Y. Tsang, D. J. Liewehr, S. M. Steinberg, J. L. Gulley and J. Schlom (2013). "Soluble CD27-pool in humans may contribute to T cell activation and tumor immunity." J Immunol **190**(12): 6250-6258.

Hudspeth, K., M. Fogli, D. V. Correia, J. Mikulak, A. Roberto, S. Della Bella, B. Silva-Santos and D. Mavilio (2012). "Engagement of NKp30 on Vdelta1 T cells induces the production of CCL3, CCL4, and CCL5 and suppresses HIV-1 replication." Blood **119**(17): 4013-4016.

Idzko, M., D. Ferrari and H. K. Eltzschig (2014). "Nucleotide signalling during inflammation." Nature **509**(7500): 310-317.

Ito, T., R. Amakawa, T. Kaisho, H. Hemmi, K. Tajima, K. Uehira, Y. Ozaki, H. Tomizawa, S. Akira and S. Fukuhara (2002). "Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets." J Exp Med **195**(11): 1507-1512.

Janabi, N., S. Peudenier, B. Heron, K. H. Ng and M. Tardieu (1995). "Establishment of human microglial cell lines after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen." Neurosci Lett **195**(2): 105-108.

Ji, X., Y. Naito, H. Weng, K. Endo, X. Ma and N. Iwai (2012). "P2X7 deficiency attenuates hypertension and renal injury in deoxycorticosterone acetate-salt hypertension." Am J Physiol Renal Physiol **303**(8): F1207-1215.

Jimenez-Pacheco, A., M. Diaz-Hernandez, M. Arribas-Blazquez, A. Sanz-Rodriguez, L. A. Olivos-Ore, A. R. Artalejo, M. Alves, M. Letavic, M. T. Miras-Portugal, R. M. Conroy, N. Delanty, M. A. Farrell, D. F. O'Brien, A. Bhattacharya, T. Engel and D. C. Henshall (2016). "Transient P2X7 Receptor Antagonism Produces Lasting Reductions in Spontaneous Seizures and Gliosis in Experimental Temporal Lobe Epilepsy." J Neurosci **36**(22): 5920-5932.

Kaczmarek-Hajek, K., E. Lorinczi, R. Hausmann and A. Nicke (2012). "Molecular and functional properties of P2X receptors--recent progress and persisting challenges." Purinergic Signal **8**(3): 375-417.

Karasawa, A. and T. Kawate (2016). "Structural basis for subtype-specific inhibition of the P2X7 receptor." Elife **5**.

Keyel, P. A. (2014). "How is inflammation initiated? Individual influences of IL-1, IL-18 and HMGB1." Cytokine **69**(1): 136-145.

Keystone, E. C., M. M. Wang, M. Layton, S. Hollis, I. B. McInnes and D. C. S. Team (2012). "Clinical evaluation of the efficacy of the P2X7 purinergic receptor antagonist AZD9056 on the signs and symptoms of rheumatoid arthritis in patients with active disease despite treatment with methotrexate or sulphasalazine." Ann Rheum Dis **71**(10): 1630-1635.

Khalafalla, M. G., L. T. Woods, J. M. Camden, A. A. Khan, K. H. Limesand, M. J. Petris, L. Erb and G. A. Weisman (2017). "P2X7 receptor antagonism prevents IL-1beta release from salivary epithelial cells and reduces inflammation in a mouse model of autoimmune exocrinopathy." J Biol Chem **292**(40): 16626-16637.

Kinjo, Y., N. Kitano and M. Kronenberg (2013). "The role of invariant natural killer T cells in microbial immunity." J Infect Chemother **19**(4): 560-570.

Knight, A., A. J. Madrigal, S. Grace, J. Sivakumaran, P. Kottaridis, S. Mackinnon, P. J. Travers and M. W. Lowdell (2010). "The role of Vdelta2-negative gammadelta T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation." Blood **116**(12): 2164-2172.

Koch-Nolte, F., S. Adriouch, P. Bannas, C. Krebs, F. Scheuplein, M. Seman and F. Haag (2006). "ADP-ribosylation of membrane proteins: unveiling the secrets of a crucial regulatory mechanism in mammalian cells." Ann Med **38**(3): 188-199.

Layhadi, J. A. and S. J. Fountain (2017). "P2X4 Receptor-Dependent Ca(2+) Influx in Model Human Monocytes and Macrophages." Int J Mol Sci **18**(11).

Le Bourhis, L., E. Martin, I. Peguillet, A. Guihot, N. Froux, M. Core, E. Levy, M. Dusseaux, V. Meyssonnier, V. Premel, C. Ngo, B. Riteau, L. Duban, D. Robert, S. Huang, M. Rottman, C. Soudais and O. Lantz (2010). "Antimicrobial activity of mucosal-associated invariant T cells." Nat Immunol **11**(8): 701-708.

Le Gall, S. M., P. Bobe, K. Reiss, K. Horiuchi, X. D. Niu, D. Lundell, D. R. Gibb, D. Conrad, P. Saftig and C. P. Blobel (2009). "ADAMs 10 and 17 represent differentially regulated components of a general shedding machinery for membrane proteins such as transforming growth factor alpha, L-selectin, and tumor necrosis factor alpha." Mol Biol Cell **20**(6): 1785-1794.

Lees, M. P., S. J. Fuller, R. McLeod, N. R. Boulter, C. M. Miller, A. M. Zakrzewski, E. J. Mui, W. H. Witola, J. J. Coyne, A. C. Hargrave, S. E. Jamieson, J. M. Blackwell, J. S. Wiley and N. C. Smith (2010). "P2X7 receptor-

mediated killing of an intracellular parasite, Toxoplasma gondii, by human and murine macrophages." J Immunol **184**(12): 7040-7046.

Leon, B. and C. Ardavin (2008). "Monocyte-derived dendritic cells in innate and adaptive immunity." Immunol Cell Biol **86**(4): 320-324.

Levy, I., Y. Q. Wu, N. Roeckel, F. Bulle, A. Pawlak, S. Siegrist, M. G. Mattei and G. Guellaen (1996). "Human testis specifically expresses a homologue of the rodent T lymphocytes RT6 mRNA." FEBS Lett **382**(3): 276-280.

Lucattelli, M., S. Cicko, T. Muller, M. Lommatzsch, G. De Cunto, S. Cardini, W. Sundas, M. Grimm, R. Zeiser, T. Durk, G. Zissel, S. Sorichter, D. Ferrari, F. Di Virgilio, J. C. Virchow, G. Lungarella and M. Idzko (2011). "P2X7 receptor signaling in the pathogenesis of smoke-induced lung inflammation and emphysema." Am J Respir Cell Mol Biol **44**(3): 423-429.

MacKenzie, A., H. L. Wilson, E. Kiss-Toth, S. K. Dower, R. A. North and A. Surprenant (2001). "Rapid secretion of interleukin-1beta by microvesicle shedding." Immunity **15**(5): 825-835.

Mackenzie, A. B., M. T. Young, E. Adinolfi and A. Surprenant (2005). "Pseudoapoptosis induced by brief activation of ATP-gated P2X7 receptors." J Biol Chem **280**(40): 33968-33976.

Martel-Gallegos, G., M. T. Rosales-Saavedra, J. P. Reyes, G. Casas-Pruneda, C. Toro-Castillo, P. Perez-Cornejo and J. Arreola (2010). "Human neutrophils do not express purinergic P2X7 receptors." Purinergic Signal **6**(3): 297-306.

Mayadas, T. N., X. Cullere and C. A. Lowell (2014). "The multifaceted functions of neutrophils." Annu Rev Pathol **9**: 181-218.

McDonald, B. D., J. J. Bunker, S. A. Erickson, M. Oh-Hora and A. Bendelac (2015). "Crossreactive alphabeta T Cell Receptors Are the Predominant Targets of Thymocyte Negative Selection." Immunity **43**(5): 859-869.

McQuillin, A., N. J. Bass, K. Choudhury, V. Puri, M. Kosmin, J. Lawrence, D. Curtis and H. M. Gurling (2009). "Case-control studies show that a non-conservative amino-acid change from a glutamine to arginine in the P2RX7 purinergic receptor protein is associated with both bipolar- and unipolar-affective disorders." Mol Psychiatry **14**(6): 614-620.

Meierovics, A., W. J. Yankelevich and S. C. Cowley (2013). "MAIT cells are critical for optimal mucosal immune responses during in vivo pulmonary bacterial infection." Proc Natl Acad Sci U S A **110**(33): E3119-3128.

Min, B., M. A. Brown and G. Legros (2012). "Understanding the roles of basophils: breaking dawn." Immunology **135**(3): 192-197.

Miras-Portugal, M. T., J. I. Diaz-Hernandez, R. Gomez-Villafuertes, M. Diaz-Hernandez, A. R. Artalejo and J. Gualix (2015). "Role of P2X7 and P2Y2 receptors on alpha-secretase-dependent APP processing: Control of amyloid plaques formation "in vivo" by P2X7 receptor." Comput Struct Biotechnol J **13**: 176-181.

Mitra, S. and A. Sarkar (2019). "Microparticulate P2X7 and GSDM-D mediated regulation of functional IL-1beta release." Purinergic Signal **15**(1): 119-123.

Moon, H., H. Y. Na, K. H. Chong and T. J. Kim (2006). "P2X7 receptor-dependent ATP-induced shedding of CD27 in mouse lymphocytes." Immunol Lett **102**(1): 98-105.

Morita, C. T., C. Jin, G. Sarikonda and H. Wang (2007). "Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens." Immunol Rev **215**: 59-76.

Muller, T., R. P. Vieira, M. Grimm, T. Durk, S. Cicko, R. Zeiser, T. Jakob, S. F. Martin, B. Blumenthal, S. Sorichter, D. Ferrari, F. Di Virgillio and M. Idzko (2011). "A potential role for P2X7R in allergic airway inflammation in mice and humans." Am J Respir Cell Mol Biol **44**(4): 456-464.

Nadi, E., M. Hajilooi, S. Pajouhan and M. Haidari (2015). "Soluble L-Selectin as an Independent Biomarker of Bronchial Asthma." J Clin Lab Anal **29**(3): 191-197.

Neves, A. R., M. T. Castelo-Branco, V. R. Figliuolo, C. Bernardazzi, F. Buongusto, A. Yoshimoto, H. F. Nanini, C. M. Coutinho, A. J. Carneiro, R. Coutinho-Silva and H. S. de Souza (2014). "Overexpression of ATP-activated P2X7 receptors in the intestinal mucosa is implicated in the pathogenesis of Crohn's disease." Inflamm Bowel Dis **20**(3): 444-457.

Olejnik, I. (1999). "Serum soluble L-selectin in childhood acute lymphoblastic leukemia." Pediatr Int **41**(3): 246-248.

Oyanguren-Desez, O., A. Rodriguez-Antiguedad, P. Villoslada, M. Domercq, E. Alberdi and C. Matute (2011). "Gain-of-function of P2X7 receptor gene variants in multiple sclerosis." Cell Calcium **50**(5): 468-472.

Papayannopoulos, V. and A. Zychlinsky (2009). "NETs: a new strategy for using old weapons." Trends Immunol **30**(11): 513-521.

Pelegrin, P. and A. Surprenant (2006). "Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor." EMBO J **25**(21): 5071-5082.

Pobezinsky, L. A., G. S. Angelov, X. Tai, S. Jeurling, F. Van Laethem, L. Feigenbaum, J. H. Park and A. Singer (2012). "Clonal deletion and the fate of autoreactive thymocytes that survive negative selection." Nat Immunol **13**(6): 569-578.

Pupovac, A., N. J. Geraghty, D. Watson and R. Sluyter (2015). "Activation of the P2X7 receptor induces the rapid shedding of CD23 from human and murine B cells." Immunol Cell Biol **93**(1): 77-85.

Qu, Y. and G. R. Dubyak (2009). "P2X7 receptors regulate multiple types of membrane trafficking responses and non-classical secretion pathways." Purinergic Signal **5**(2): 163-173.

Ransohoff, R. M. and A. E. Cardona (2010). "The myeloid cells of the central nervous system parenchyma." Nature **468**(7321): 253-262.

Rissiek, A., I. Baumann, A. Cuapio, A. Mautner, M. Kolster, P. C. Arck, A. Dodge-Khatami, H. W. Mittrucker, F. Koch-Nolte, F. Haag and E. Tolosa (2015). "The expression of CD39 on regulatory T cells is genetically driven and further upregulated at sites of inflammation." J Autoimmun **58**: 12-20.

Rissiek, B., M. Lukowiak, F. Raczkowski, T. Magnus, H. W. Mittrucker and F. Koch-Nolte (2018). "In Vivo Blockade of Murine ARTC2.2 During Cell Preparation Preserves the Vitality and Function of Liver Tissue-Resident Memory T Cells." Front Immunol **9**: 1580.

Riteau, N., P. Gasse, L. Fauconnier, A. Gombault, M. Couegnat, L. Fick, J. Kanellopoulos, V. F. Quesniaux, S. Marchand-Adam, B. Crestani, B. Ryffel and I. Couillin (2010). "Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis." Am J Respir Crit Care Med **182**(6): 774-783.

Rose-John, S. (2012). "IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6." Int J Biol Sci **8**(9): 1237-1247.

Sahoo, A., S. Wali and R. Nurieva (2016). "T helper 2 and T follicular helper cells: Regulation and function of interleukin-4." Cytokine Growth Factor Rev **30**: 29-37.

Santos, A. A., Jr., V. Rodrigues-Junior, R. F. Zanin, T. J. Borges, C. Bonorino, R. Coutinho-Silva, C. M. Takyia, D. S. Santos, M. M. Campos and F. B. Morrone (2013). "Implication of purinergic P2X7 receptor in M. tuberculosis infection and host interaction mechanisms: a mouse model study." Immunobiology **218**(8): 1104-1112.

Sanz, J. M., P. Chiozzi, D. Ferrari, M. Colaianna, M. Idzko, S. Falzoni, R. Fellin, L. Trabace and F. Di Virgilio (2009). "Activation of microglia by amyloid {beta} requires P2X7 receptor expression." J Immunol **182**(7): 4378-4385.

Sanz, J. M., S. Falzoni, R. Rizzo, F. Cipollone, G. Zuliani and F. Di Virgilio (2014). "Possible protective role of the 489C>T P2X7R polymorphism in Alzheimer's disease." Exp Gerontol **60**: 117-119.

Schenten, D. and R. Medzhitov (2011). "The control of adaptive immune responses by the innate immune system." Adv Immunol **109**: 87-124.

Scheuplein, F., B. Rissiek, J. P. Driver, Y. G. Chen, F. Koch-Nolte and D. V. Serreze (2010). "A recombinant heavy chain antibody approach blocks ART2 mediated deletion of an iNKT cell population that upon activation inhibits autoimmune diabetes." J Autoimmun **34**(2): 145-154.

Scheuplein, F., N. Schwarz, S. Adriouch, C. Krebs, P. Bannas, B. Rissiek, M. Seman, F. Haag and F. Koch-Nolte (2009). "NAD+ and ATP released from injured cells induce P2X7-dependent shedding of CD62L and externalization of phosphatidylserine by murine T cells." J Immunol **182**(5): 2898-2908.

Schilbach, K., K. Frommer, S. Meier, R. Handgretinger and M. Eyrich (2008). "Immune response of human propagated gammadelta-T-cells to neuroblastoma recommend the Vdelta1+ subset for gammadelta-T-cell-based immunotherapy." J Immunother **31**(9): 896-905.

Sharma, S. K. and G. Naidu (2016). "The role of danger-associated molecular patterns (DAMPs) in trauma and infections." J Thorac Dis **8**(7): 1406-1409.

Shemon, A. N., R. Sluyter, S. L. Fernando, A. L. Clarke, L. P. Dao-Ung, K. K. Skarratt, B. M. Saunders, K. S. Tan, B. J. Gu, S. J. Fuller, W. J. Britton, S. Petrou and J. S. Wiley (2006). "A Thr357 to Ser polymorphism in homozygous and compound heterozygous subjects causes absent or reduced P2X7 function and impairs ATP-induced mycobacterial killing by macrophages." J Biol Chem **281**(4): 2079-2086.

Siegers, G. M. and L. S. Lamb, Jr. (2014). "Cytotoxic and regulatory properties of circulating Vdelta1+ gammadelta T cells: a new player on the cell therapy field?" Mol Ther **22**(8): 1416-1422.

Sinadinos, A., C. N. Young, R. Al-Khalidi, A. Teti, P. Kalinski, S. Mohamad, L. Floriot, T. Henry, G. Tozzi, T. Jiang, O. Wurtz, A. Lefebvre, M. Shugay, J. Tong, D. Vaudry, S. Arkle, J. C. doRego and D. C. Gorecki (2015). "P2RX7 purinoceptor: a therapeutic target for ameliorating the symptoms of duchenne muscular dystrophy." PLoS Med **12**(10): e1001888.

Siontorou, C. G. (2013). "Nanobodies as novel agents for disease diagnosis and therapy." Int J Nanomedicine **8**: 4215-4227.

Sluyter, R. and L. Stokes (2011). "Significance of P2X7 receptor variants to human health and disease." Recent Pat DNA Gene Seq **5**(1): 41-54.

Sopper, S., S. Mustjoki, D. White, T. Hughes, P. Valent, A. Burchert, B. T. Gjertsen, G. Gastl, M. Baldauf, Z. Trajanoski, F. Giles, A. Hochhaus, T. Ernst, T. Schenk, J. J. Janssen, G. J. Ossenkoppele, K. Porkka and D. Wolf (2017). "Reduced CD62L Expression on T Cells and Increased Soluble CD62L Levels Predict Molecular Response to Tyrosine Kinase Inhibitor Therapy in Early Chronic-Phase Chronic Myelogenous Leukemia." J Clin Oncol **35**(2): 175-184.

Stachon, P., A. Heidenreich, J. Merz, I. Hilgendorf, D. Wolf, F. Willecke, S. von Garlen, P. Albrecht, C. Hardtner, N. Ehrat, N. Hoppe, J. Reinohl, C. von Zur Muhlen, C. Bode, M. Idzko and A. Zirlik (2017). "P2X7 Deficiency Blocks Lesional Inflammasome Activity and Ameliorates Atherosclerosis in Mice." Circulation **135**(25): 2524-2533.

Stavnezer, J. and C. E. Schrader (2014). "IgH chain class switch recombination: mechanism and regulation." J Immunol **193**(11): 5370-5378.

Stock, T. C., B. J. Bloom, N. Wei, S. Ishaq, W. Park, X. Wang, P. Gupta and C. A. Mebus (2012). "Efficacy and safety of CE-224,535, an antagonist of P2X7 receptor, in treatment of patients with rheumatoid arthritis inadequately controlled by methotrexate." J Rheumatol **39**(4): 720-727.

Stokes, L., S. J. Fuller, R. Sluyter, K. K. Skarratt, B. J. Gu and J. S. Wiley (2010). "Two haplotypes of the P2X(7) receptor containing the Ala-348 to Thr polymorphism exhibit a gain-of-function effect and enhanced interleukin-1beta secretion." FASEB J **24**(8): 2916-2927.

Stokes, L., L. H. Jiang, L. Alcaraz, J. Bent, K. Bowers, M. Fagura, M. Furber, M. Mortimore, M. Lawson, J. Theaker, C. Laurent, M. Braddock and A. Surprenant (2006). "Characterization of a selective and potent antagonist of human P2X(7) receptors, AZ11645373." Br J Pharmacol **149**(7): 880-887.

Surprenant, A. and R. A. North (2009). "Signaling at purinergic P2X receptors." Annu Rev Physiol **71**: 333-359.
Suzuki, T., I. Hide, K. Ido, S. Kohsaka, K. Inoue and Y. Nakata (2004). "Production and release of neuroprotective tumor necrosis factor by P2X7 receptor-activated microglia." J Neurosci **24**(1): 1-7.

Takai, E., M. Tsukimoto, H. Harada and S. Kojima (2014). "Autocrine signaling via release of ATP and activation of P2X7 receptor influences motile activity of human lung cancer cells." Purinergic Signal **10**(3): 487-497.

Ulmann, L., H. Hirbec and F. Rassendren (2010). "P2X4 receptors mediate PGE2 release by tissue-resident macrophages and initiate inflammatory pain." EMBO J **29**(14): 2290-2300.

Unciti-Broceta, J. D., T. Del Castillo, M. Soriano, S. Magez and J. A. Garcia-Salcedo (2013). "Novel therapy based on camelid nanobodies." Ther Deliv **4**(10): 1321-1336.

van Wilgenburg, B., I. Scherwitzl, E. C. Hutchinson, T. Leng, A. Kurioka, C. Kulicke, C. de Lara, S. Cole, S. Vasanawathana, W. Limpitikul, P. Malasit, D. Young, L. Denney, S.-H. consortium, M. D. Moore, P. Fabris, M. T. Giordani, Y. H. Oo, S. M. Laidlaw, L. B. Dustin, L. P. Ho, F. M. Thompson, N. Ramamurthy, J. Mongkolsapaya, C. B. Willberg, G. R. Screaton and P. Klenerman (2016). "MAIT cells are activated during human viral infections." Nat Commun **7**: 11653.

Vantourout, P. and A. Hayday (2013). "Six-of-the-best: unique contributions of gammadelta T cells to immunology." Nat Rev Immunol **13**(2): 88-100.

Varley, I., J. P. Greeves, C. Sale, E. Friedman, D. S. Moran, R. Yanovich, P. J. Wilson, A. Gartland, D. C. Hughes, T. Stellingwerff, C. Ranson, W. D. Fraser and J. A. Gallagher (2016). "Functional polymorphisms in the P2X7 receptor gene are associated with stress fracture injury." Purinergic Signal **12**(1): 103-113.

Vereczkei, A., O. Abdul-Rahman, Z. Halmai, G. Nagy, A. Szekely, A. Somogyi, G. Faludi and Z. Nemoda (2019). "Association of purinergic receptor P2RX7 gene polymorphisms with depression symptoms." Prog Neuropsychopharmacol Biol Psychiatry **92**: 207-216.

Vergani, A., S. Tezza, F. D'Addio, C. Fotino, K. Liu, M. Niewczas, R. Bassi, R. D. Molano, S. Kleffel, A. Petrelli, A. Soleti, E. Ammirati, M. Frigerio, G. Visner, F. Grassi, M. E. Ferrero, D. Corradi, R. Abdi, C. Ricordi, M. H. Sayegh, A. Pileggi and P. Fiorina (2013). "Long-term heart transplant survival by targeting the ionotropic purinergic receptor P2X7." Circulation **127**(4): 463-475.

Verstichel, G., D. Vermijlen, L. Martens, G. Goetgeluk, M. Brouwer, N. Thiault, Y. Van Caeneghem, S. De Munter, K. Weening, S. Bonte, G. Leclercq, T. Taghon, T. Kerre, Y. Saeys, J. Van Dorpe, H. Cheroutre and B. Vandekerckhove (2017). "The checkpoint for agonist selection precedes conventional selection in human thymus." Sci Immunol **2**(8).

Vieira, F. S., H. F. Nanini, C. M. Takiya and R. Coutinho-Silva (2016). "P2X7 receptor knockout prevents streptozotocin-induced type 1 diabetes in mice." Mol Cell Endocrinol **419**: 148-157.

Vignali, D. A., L. W. Collison and C. J. Workman (2008). "How regulatory T cells work." Nat Rev Immunol **8**(7): 523-532.

Vivier, E., E. Tomasello, M. Baratin, T. Walzer and S. Ugolini (2008). "Functions of natural killer cells." Nat Immunol **9**(5): 503-510.

Wan, P., X. Liu, Y. Xiong, Y. Ren, J. Chen, N. Lu, Y. Guo and A. Bai (2016). "Extracellular ATP mediates inflammatory responses in colitis via P2 x 7 receptor signaling." Sci Rep **6**: 19108.

Wang, S., Y. Dai, K. Kobayashi, W. Zhu, Y. Kogure, H. Yamanaka, Y. Wan, W. Zhang and K. Noguchi (2012). "Potentiation of the P2X3 ATP receptor by PAR-2 in rat dorsal root ganglia neurons, through protein kinase-dependent mechanisms, contributes to inflammatory pain." Eur J Neurosci **36**(3): 2293-2301.

Wang, X. H., X. Xie, X. G. Luo, H. Shang and Z. Y. He (2017). "Inhibiting purinergic P2X7 receptors with the antagonist brilliant blue G is neuroprotective in an intranigral lipopolysaccharide animal model of Parkinson's disease." Mol Med Rep **15**(2): 768-776.

Wen, T. and M. E. Rothenberg (2016). "The Regulatory Function of Eosinophils." Microbiol Spectr **4**(5).

Wesolowski, J., V. Alzogaray, J. Reyelt, M. Unger, K. Juarez, M. Urrutia, A. Cauerhff, W. Danquah, B. Rissiek, F. Scheuplein, N. Schwarz, S. Adriouch, O. Boyer, M. Seman, A. Licea, D. V. Serreze, F. A. Goldbaum, F. Haag and F. Koch-Nolte (2009). "Single domain antibodies: promising experimental and therapeutic tools in infection and immunity." Med Microbiol Immunol **198**(3): 157-174.

Woehrle, T., L. Yip, A. Elkhal, Y. Sumi, Y. Chen, Y. Yao, P. A. Insel and W. G. Junger (2010). "Pannexin-1 hemichannel-mediated ATP release together with P2X1 and P2X4 receptors regulate T-cell activation at the immune synapse." Blood **116**(18): 3475-3484.

Wu, D., P. Wu, X. Wu, J. Ye, Z. Wang, S. Zhao, C. Ni, G. Hu, J. Xu, Y. Han, T. Zhang, F. Qiu, J. Yan and J. Huang (2015). "Ex vivo expanded human circulating Vdelta1 gammadeltaT cells exhibit favorable therapeutic potential for colon cancer." Oncoimmunology **4**(3): e992749.

Wu, G., M. Zhao, X. Gu, Y. Yao, H. Liu and Y. Song (2014). "The effect of P2X7 receptor 1513 polymorphism on susceptibility to tuberculosis: A meta-analysis." Infect Genet Evol **24**: 82-91.

Xia, J., X. Yu, L. Tang, G. Li and T. He (2015). "P2X7 receptor stimulates breast cancer cell invasion and migration via the AKT pathway." Oncol Rep **34**(1): 103-110.

Yan, Y., J. Bai, X. Zhou, J. Tang, C. Jiang, E. Tolbert, G. Bayliss, R. Gong, T. C. Zhao and S. Zhuang (2015). "P2X7 receptor inhibition protects against ischemic acute kidney injury in mice." Am J Physiol Cell Physiol **308**(6): C463-472.

Ye, Z., J. Liu, J. Zheng, J. Zhang and R. Huang (2017). "L-selectin Promotes the Maturation of Dendritic Cells via Up-regulation the Expression of TLR4 in vitro." Ann Clin Lab Sci **47**(4): 389-394.

Yip, L., T. Woehrle, R. Corriden, M. Hirsh, Y. Chen, Y. Inoue, V. Ferrari, P. A. Insel and W. G. Junger (2009). "Autocrine regulation of T-cell activation by ATP release and P2X7 receptors." FASEB J **23**(6): 1685-1693.

Zempo, H., Y. Sugita, M. Ogawa, R. Watanabe, J. Suzuki and M. Isobe (2015). "A P2X7 receptor antagonist attenuates experimental autoimmune myocarditis via suppressed myocardial CD4+ T and macrophage infiltration and NADPH oxidase 2/4 expression in mice." Heart Vessels **30**(4): 527-533.

Zhang, B. (2010). "CD73: a novel target for cancer immunotherapy." Cancer Res **70**(16): 6407-6411.
Zhao, J., H. Wang, C. Dai, H. Wang, H. Zhang, Y. Huang, S. Wang, F. Gaskin, N. Yang and S. M. Fu (2013). "P2X7 blockade attenuates murine lupus nephritis by inhibiting activation of the NLRP3/ASC/caspase 1 pathway." Arthritis Rheum **65**(12): 3176-3185.

Zhao, Z., J. Gruszczynska-Biegala and A. Zolkiewska (2005). "ADP-ribosylation of integrin alpha7 modulates the binding of integrin alpha7beta1 to laminin." Biochem J 385(Pt 1): 309-317.

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11 Curriculum vitae

Entfällt aus datenschutzrechtlichen Gründen

Publications

Danquah, W., C. Meyer-Schwesinger, B. Rissiek, C. Pinto, A. Serracant-Prat, M. Amadi, D. Iacenda, J. H. Knop, A. Hammel, P. Bergmann, N. Schwarz, J. Assuncao, W. Rotthier, F. Haag, E. Tolosa, P. Bannas, E. Boue-Grabot, T. Magnus, T. Laeremans, C. Stortelers and F. Koch-Nolte (2016). "Nanobodies that block gating of the P2X7 ion channel ameliorate inflammation." Sci Transl Med **8**(366): 366ra162.

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