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The effect of extracellular nucleotides on cytokine production and phagocytosis of macrophages

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Abbreviations

ADP –Adenosine 5’-diphosphate

ATCC® – American Type Culture Collection

ATP – Adenosine 5’-triphosphate

ATP γ S – Adenosin 5’-(gamma-thio)-triphospate

BP – bio particles = BioParticles®; E. coli fluorescent conjugate

BzATP – 2'(3')-*O*-(4-Benzoylbenzoyl)adenosine-5'-triphosphate

CD14 – Cluster of differentiation 14

DAMPs – Danger-associated molecular patterns

E. coli – Escherichia coli

EGTA – Ethylene glycol tetra acetic acid

ELISA – Enzyme Linked Immunosorbent Assay

FBS – Fetal Bovine Serum

FITC – Fluoresceinisothiocyanate

G – Gauge (needle size)

ICU – Intensive Care Unit

I κ B – Inhibitor of NF- κ B

IL-1, 6 – Interleukin 1, 6

IL-1R – Interleukin-1-Receptor

IMC – Intermediate Care Unit

KC – Kupffer cells

LBP – LPS-binding protein

LOS – Length of Stay

LPS – Lipopolysaccharides

M – molar (mol/liter)

Mal – MyD88 adaptor-like protein

MD-2 – Myeloid Differentiation-2

Min – minute(s)

MyD88 – Myeloid differentiation factor 88

NF- κ B – Nuclear factor kappa-light-chain-enhancer of activated B cells

PAMPs – Pathogen-associated molecular patterns

PBS – Phosphate buffered saline

PRRs – Pattern-recognition receptors

TIR – intracellular Toll/IL-1R domain

TIRAP – Toll-interleukin 1 receptor domain containing adaptor protein

TLR(s) – Toll-like-Receptor(s)

TRAM – TRIF-related adapter molecule

TRIF – TIR-domain-containing adapter-inducing interferon-β

TNF(α) – Tumor Necrosis Factor (alpha)

US – United States (of America)

UTP – Uridine 5'-triphosphate

µM – micro molar = µmol/liter

1 Background

The goal of the experiments described below was to show the impact of extracellular nucleotides on immunological effector functions of macrophages in experimental models of sepsis.

In this first chapter, I am going to introduce the reader shortly into the general topic and the specific ideas of this work. The clinical and cellular background of this basic research project are explained. Further, the clinical problem of sepsis, a clinical situation that is difficult to handle, its social importance and its mechanisms are introduced. Subsequently I am introducing into the mechanisms of purinergic signaling, and signaling induced by endotoxins, which are the basic principles of this work. Thereby, I am focusing on the interactions of purinergic signaling, macrophages, and bacterial infection. Furthermore, I am going to explain the questions, ideas and designs of our experimental approach with their expected effects – whether or not they were observed.

At the end of this chapter, the reader should be able to follow the reasons and designs of this work, and the series of experiments described afterwards.

1.1 Sepsis

1.1.1 Sepsis as a medical challenge

Bacterial sepsis is, due to the very complex and self-energizing signaling cascades later described in more detail, a very severe clinical condition, which can finally lead to severe sepsis, septic shock, and death. The complex pathophysiology of sepsis is not yet fully understood because of very complex interactions between microorganisms, the immune system and the diverse organs (Castelheim, 2009) (Kumar, 2007). All this makes bacterial sepsis and endotoxemia very difficult to treat.

Furthermore sepsis is a possible and potentially lethal complication. In surgery, where infections are an omnipresent clinical problem to be prevented and treated, abdominal sepsis represents a frequent indication for abdominal exploration. In particular in visceral surgery that represents surgery in a contaminated area further insight into pathophysiological mechanisms of sepsis may have clinical implications.

Furthermore, sepsis is a common problem in hospitals and in intensive care units associated with significant socio-economic consequences (Angus, 2001) (Vincent, 2006).

The very general motivation for this work therefore is trying to further explore one of the mechanisms of sepsis, in particular the role of extracellular nucleotides.

1.1.2 General mechanisms of sepsis

Lipopolysaccharides (LPS) are endotoxins, a toxic component of the outer cell membrane of gram-negative bacteria (Castelheim, 2009) (Kumar, 2007). When gram-negative bacteria are destroyed, LPS are released. This normally happens when gram-negative bacteria are fought by the immune response of the host. Bacterial infections by gram-negative bacteria can therefore lead to a release of LPS into the blood, called endotoxemia.

LPS lead to an activation of the membrane bound Toll-like-Receptors (TLRs) on various cell types, macrophages amongst others. The activation of TLR on

macrophages directly and indirectly leads to a release of diverse cytokines (e.g. TNF, IL-6). These cytokines act as modulators/promoters of the immune system and many other organs such as the vasculature. Together these cytokines are able to induce a vicious circle. An extensive release of such cytokines – a cytokine storm – can lead to systemic inflammation, sepsis, septic shock, and dead.

One difficulty in the clinical treatment of sepsis is to manage the dangerous effect of a cytokine release in response to endotoxemia, as described above. If it is possible in a one-cell-type-model (e.g. as described in the last passage) to diminish or even abolish – using extracellular nucleotides – the cytokine release provoked by LPS, this may also be possible in the entire organisms. The first we describe in our experiments with Kupffer cells. The latter, on the other hand could be possible either by applying nucleotides directly – for example in the systemic circulation by intravenous injection – or by finding a mechanism to let the organism do the same thing itself.

1.1.3 Social importance of sepsis

Two studies (Vincent, 2006) (Angus, 2001) in the United States and Europe underline that sepsis is a serious issue in health care concerning both mortality and costs. Furthermore, the study in the USA shows that sepsis is a problem of the elderly, and with the demographic change therefore of increasing importance, as shown by some selected data of these studies.

1.1.3.1 SOAP (Sepsis Occurrence in Acutely Ill Patients) study (Europe) (Vincent, 2006):

- 37% of ICU patients had identified infection with a high variability between countries (18-73%).
- ICU mortality rate in patients with sepsis was greater than in those without sepsis (27 vs. 14%).
- In patients with severe sepsis the ICU mortality rate was 32.2% and in those with septic shock 54.1%.
- There was a correlation between ICU mortality rate and sepsis rate in ICU patients in different countries.
- There is an inverse correlation between the number of organs failing and mortality in severe sepsis.

1.1.3.2 Epidemiology of severe sepsis (US): (Angus, 2001)

- 2.1-4.3% of hospital admissions had severe sepsis with a mean age of 64.8 years.
- 58% of patients with severe sepsis were admitted to ICU.
- Half of the patients with severe sepsis were 65 years or older.
- The incidence rate increased in the elderly aged 85 years or more.
- The overall hospital mortality rate was 28.6% but 38.4% in those 85 years or older.
- The average length of stay and costs were 19.6 days and $22100.
- If these numbers are extrapolated for the entire US, and adjusted for age and gender, it results in a yearly incidence of 3.0 cases per 1000 population with a total number of 751000 cases per year in the US, 215000 deaths and costs of $16.7 billion, thereof $8.7 billion (52.3%) for patients 65 and older and $5.1 billion (30.8%) for those 75 and older.

- The study estimated an annual 1.5% increase of cases because of the demographic change in population.

1.2 Macrophages

1.2.1 Function and derivation of macrophages

The mononuclear phagocyte system is a subgroup of leukocytes, derived from bone marrow (Geissmann, 2010) (Murray & Wynn, 2011). Macrophages circulate in the blood as monocytes and within a few days they populate the tissue where they become macrophages.

Monocytes are less specialized cells. Under inflammatory stimuli they are able to migrate into tissue, and to differentiate into macrophages or other cells. But they fulfill some effector functions themselves, too.

Macrophages are phagocytic cells in the tissue, able to recognize pathogens and induce inflammation by the production of cytokines.

1.2.2 Kupffer cells

Kupffer cells (KC) represent 20% of all non-parenchymal cells in the liver and are the largest population of macrophages in the body (Racanelli & Rehermann, 2006). They are located predominantly in the periportal area of the sinusoidal vascular space. With their localization they can get into close contact with passing blood lymphocytes. Also they can pass between the liver sinusoidal endothelial cells into the space of Dissé where they interact with hepatocytes.

Kupffer cells have an important regulatory role in the local immune system of the liver. For example they are involved in the presentation of antigens and the development of tolerance to antigens in the portal vein, the initiation of immune responses to infections, and they are in a close cytokine-mediated interaction with their neighboring cells.

The release of cytokines – TNFα among them – by macrophages and especially Kupffer cells after stimulation with LPS is a topic of research since many years. The fact that LPS finally leads to a release of TNFα is widely accepted and known for a long time (Lin, 2005) (Decker, 1990).

1.2.2.1 Role of Kupffer cells in sepsis

Due to their function, their localization in the liver and their high number, Kupffer cells are thought to be an important player in bacterial infection and sepsis (Kono , 2006) (Gregory, 2002) (Seki, 2000). However, their exact role in this complex pathology is not yet clear.

1.2.3 Purinergic receptor expression by macrophages

Macrophages express various purinergic receptors, as virtually every circulating cell is responsive to nucleotides (Di Virgilio, Nucleotide receptors: an emerging family of regulatory molecules in blood cells, 2001). The specific subtypes expressed are not yet entirely cleared, but some are known:

Murine macrophage lines express P2Y receptors (Di Virgilio, Nucleotide receptors: an emerging family of regulatory molecules in blood cells, 2001). RAW 264.7 macrophages express P2Y and a P2X7-like subtype. U-937 macrophages express P2Y2, P2Y4 and P2Y6 receptors. Human macrophages express P2X7.

There is little data about P2 receptor expressions by Kupffer cells (Beldi, 2008). On rat liver Kupffer cells $P2X_4$ and $P2X_6$ have been demonstrated (Xiang, 2006). There is no current literature about P2Y receptor expression in Kupffer cells.

Kupffer cells are known to express Toll like receptors, TLR 4 among them (Chen Y. , 2011)

1.3 Thrombocytes/platelets

1.3.1 Formation of thrombocytes/platelets

Thrombocytes, or platelets, originate from megakaryocytes (Beaulieu, 2010). Megakaryocytes are found in bone marrow. Upon stimulation, megakaryocytes adhere to the endothelium of the bone marrow and release pro-platelets into circulation. These undergo further maturation into platelets.

1.3.2 Properties of platelets

Platelets are mainly known for their hemostatic properties (Kumar, 2007). They are known to carry ADP and ATP in their dense bodies, which are released upon activation and contribute to and boost platelet adhesion and clot formation (Oury, 2002).

Based on these findings, we hypothesized, that platelets could be relevant for ATP release in a context of inflammation and thereby mediate inflammatory processes, for example in settings such as bacteremia or bacterial sepsis. If this were the case, the nucleotide secretion of platelets would not only have autocrine, but also paracrine and maybe even endocrine effects.

1.3.3 Effect of nucleotides on platelets

Platelets are activated by Nucleotides, i.e. ATP and ADP, via three P2 receptors. (Hechler, The P2 Receptors in Platelet Function, 2005) By P2X1, a ligand-gated cation channel activated by ATP, and by P2Y1 and P2Y12, two G-protein-coupled ADP receptors (Hechler, P2 receptors and platelet function, 2011). All of these receptors seem to play substantial roles in platelet activation, but P2Y12 appearing to be particularly important in the function of ADP as a cofactor in platelet activation.

The importance of P2Y12 in platelet aggregation is underlined by the wide clinical use of its antagonist clopidogrel and further developments in cardiovascular diseases such as acute coronary syndrome. (Cayla, 2012)

It has been shown, that ATP contributes to platelet activation via P2X1 receptor, when platelets are stimulated with only low concentrations of collagen (Oury, 2002). But if platelets are exposed to higher collagen concentrations, they are not dependent on this activation pathway anymore.

1.3.4 Effect of LPS on platelets

Furthermore, it has been demonstrated, that LPS can activate platelets together with subthreshold levels of thrombin or collagen and leads to ATP release of platelets (Zhang G. , 2009). But the activation of platelets via LPS is much weaker than by normal platelet agonists, such as collagen or thrombin. LPS alone does not activate platelets.

1.3.5 Receptor expressions

In primary clot formation of platelets, the GPIb complex (binding to von Willebrand factor) and platelet integrins such as $\alpha 2\beta 1$ $\alpha IIb\beta 3$ play central roles (Clemetson, 2012). In the secondary activation of platelets, involved receptors include the P2 receptors P2X1, P2Y1, and P2Y12, the thrombin receptors PAR1 and PAR4, and the thromboxane receptor for TXA2 are particularly important.

Platelets further express TLR-1-9 and may therefore be activated by pathogen associated molecular pattern (Semple, 2011). Besides these, they express many other receptors and molecules with effects on the immune system, such as CD40 and CD154 (also known as CD40L), important molecules in promoting interactions between lymphocytes and antigen-presenting cells, and all the components of the LPS receptor signaling complex (including TLR4, CD14, MD2) (Semple, 2011), and others. .

1.3.6 Role of platelets in inflammation and infection

Within the last years a lot of evidence was found, that platelets interact with the immune system in the context of infections. For instance they can play a role in early immune surveillance, and they can localize and aggregate around bacteria and promote clearance, and thereby interact with immune responses (Beaulieu, 2010).

LPS, which is an important factor in bacterial infections, has also been shown to have diverse effects on platelets such as increasing effects on platelet production, on circulating platelet numbers, and on platelet activation.

The connecting element between platelets and the immune system could be TLR2 and TLR4 receptors that are expressed on platelets (Beaulieu, 2010).

We think, that another connecting element between platelets and the immune system could be the release of ATP by platelets. Thereby platelets would activate purinergic receptors on other cells, such as macrophages or other immune cells. If these signals were strong enough to activate or at least modulate immune reactions, it could be an important link between platelets and the immune system.

1.4 Endotoxin/lipopolysaccharides signaling

Endotoxin is the lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria. It consists of the biologically active component lipid A, and the two carrier parts named core and O antigen (Barbieri, 2007).

1.4.1 Innate immune responses to danger signals: pathogen-associated molecular patterns (PAMPs) and pattern-recognition receptors (PRRs)

Anything that causes tissue stress or damage is viewed as a "danger" by the immune system (Matzinger, 2002). Molecules associated with such stress or damage are called Danger-Associated Molecular Patterns (DAMPs), and are signaling such danger to the immune system. (Castelheim, 2009). These molecular patterns (DAMPS) are either produced by stressed or damaged tissues of the body (then they are called alarmins) or they are exogenous molecules derived from microorganisms (in this case they are called PAMPs).

The latter exogenous molecules have in common that they are both essential for the microorganism's surviving and its infective potential. They are called pathogen-associated molecular patterns (PAMPs). LPS therefore is a PAMP.

In order to recognize and react to these DAMPs (PAMPs and alarmins), the innate immune system developed a series of pattern-recognition receptors (PRRs). Toll-like Receptors (TLRs, see below) are an important member of the PRR group. Among these, TLR-4 recognizes LPS.

1.4.2 Toll-like receptor-4

Toll-like receptors (TLRs) are a family of transmembrane receptor proteins (Castelheim, 2009). They are important pattern-recognition receptors (PRRs) and interact with the innate immune system. There are 13 TLRs in mammals, and 10 in human. With their recognition of danger signals, TLRs regulate the expression of various inflammatory cytokines. The precise composition of the secreted cytokine set depends on ligand specificity to the different receptors, the receptor expression patterns on the cell surface, and the downstream signaling pathways.

TLR-4 is the classic and first example of a TLR described. It has been established to be the receptor of LPS, but also recognizes other molecules, structurally both similar and different to LPS. Even if TLR-4 is called to be "the" LPS receptor, it is not the only molecule involved in LPS signaling.

1.4.2.1 Signaling pathways of TLR-4.

TLR-4 forms a complex with myeloid differentiation-2 (MD-2) on the cell surface (Akashi-Takamura , 2008). MD-2 is a soluble protein associated to TLR-4. MD-2 seems to be the LPS recognition molecule while TLR-4 is responsible for the signaling; together they serve as the main LPS-binding component.

LPS first binds to LPS-binding protein (LBP). LBP is an acute-phase protein, produced in the liver and circulating in the blood (Pålsson-McDermott & O'Neill, 2004) (Kawai, 2010) (Akira , 2006) (Castelheim, 2009). LBP delivers LPS to either soluble or membrane bound CD14. CD14 binds LPS and transfers the LPS-LBP complex to the

TLR-4-MD-2 complex. There is a soluble CD14 (sCD14) and a membrane bound (mCD14) form, which can both be involved in LPS signaling.

Hence the first complex, which initiates the TLR-4 signaling, consists of LPS, LBP, CD14, MD-2, and TLR-4. Multiple TLR-4 then oligomerize for the further signal transduction. The specific structure of LPS, changing with the bacterial species, is essential for the cluster of TLR oligomerization and therefore also the resulting secondary messaging and inflammatory response.

The cytoplasmic signaling domain of Toll-like receptors is homologous to that of the Interleukin-1 receptor (IL-1R) (Castelheim, 2009) (Akira , 2006). It is named Toll/IL-1R domain (TIR domain). Different TIR domain-associated molecules mediate the further signaling. Different TLRs activate different sets of TIR domain-associated molecules and thereby trigger different cellular responses (Kawai, 2010) (Akira , 2006). MyD88, TIRAP (Mal), TRIF, and TRAM are such associated molecules. Two main ways of TLR-4 mediated intracellular response are known: an early MyD88-dependant, and a delayed TRIF-dependent response. TLR signaling pathways can be largely classified as either MyD88-dependent pathways, which drive the induction of inflammatory cytokines, or TRIF-dependent pathways, which are responsible for the induction of type I interferon as well as inflammatory cytokines (Kawai, 2010) [sic] (Akira , 2006). TLR-4 is the only TLR using both signaling pathways. (Figure 1)

MyD88 dependent pathway

TIRAP (Mal = MyD88 adaptor-like protein) (toll-interleukin 1 receptor domain containing adaptor protein) recruits MyD88 (Myeloid differentiation factor 88) to TLR-4. (Kawai, 2010) (Akira , 2006). MyD88 then recruits different IL-1 receptor–associated kinases (IRAKs), which are required for the activation of NF- κ B. Briefly, by interaction with a series of regulatory proteins such as TRAF6, TAK1, NEMO, and others, finally this cascade leads to activation of MAPK kinase, and degradation of I κ B and thereby activation of NF- κ B. NF- κ B in its inactive form is bound to inhibitor of NF- κ B (I κ B) and regularly present in the cytoplasm. Phosphorylation of I κ B by I κ B kinase leads to a release of NF- κ B, which then translocates into the nucleus and acts as a transcription factor (Pålsson-McDermott & O'Neill, 2004). MAPK kinase, NF-κB, and other factors activated by this pathway lead to production of proinflammatory cytokines.

TRIF dependent pathway

TRAM (TRIF-related adapter molecule) recruits TRIF (TIR-domain-containing adapter-inducing interferon- β) to TLR-4 (Kawai, 2010) (Akira , 2006). TRIF also recruits TRAF6 and via TAK1 and other proteins it leads to activation of NF-κB. Additionally TRIF recruits TRAF3 and a signaling complex involving TBK1, IKKi, and others. The activation of these proteins finally leads to IRF3 activation and interferon-β transcription.

Interestingly, various proteins, e.g. TRAF3 are involved in the regulation of both the MyD88 dependent and the TRIF dependent pathways with oppositional effects. Thereby they may have essential roles in balancing the inflammatory stimulations (Kawai, 2010).

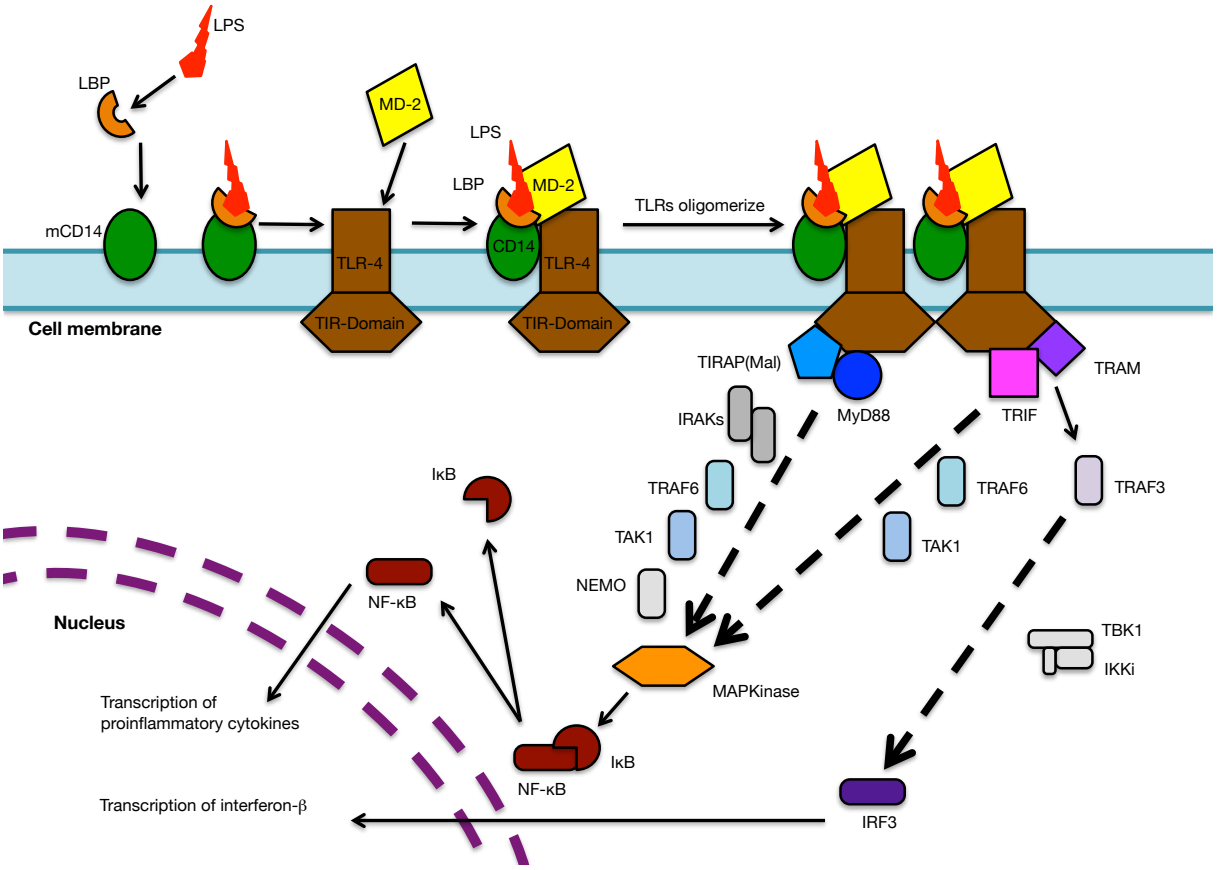

Figure 1: Simplified diagram of TLR-4 activation; Abbreviations: LPS – Lipopolysaccharides, LBP – LPS-binding protein; (m)CD14 – (membrane bound) Cluster of Differentiation 14; TLR-4 – Toll-like Receptor 4; TIR-Domain – Intracellular Toll/IL-1R domain; TIRAP(Mal) – Toll-Interleukin 1 Receptor Domain containing Adaptor Protein (MyD88 adaptor-like protein) ; MyD88 – Myeloid differentiation factor 88; TRAM – TRIF-related adapter molecule; TRIF – TIR-domain-containing adapter-inducing interferon- β ; IRAKs – IL-1 Receptor–Associated Kinases; NF- κ B – Nuclear Factor kappa-light-chain-enhancer of activated B cells; I κ B – Inhibitor of NF- κ B

1.5 Purinergic/nucleotide signaling

Purinergic signaling consists of purines (i.e. primarily adenine), the purine nucleoside adenosine, purinergic nucleotides (i.e. mainly ATP and ADP), and pyrimidinergic nucleotides (i.e. manly UTP), their release into the extracellular space, their modulation by enzymes, and the stimulation of purinergic receptors (Burnstock, Purinergic signalling: Its unpopular beginning, its acceptance and its exciting future , 2012) (Graubardt, 2012). Purinergic signaling was initially discovered as a novel neurotransmitting pathway, but since then many other effects in various cell types have been discovered. Purinergic signaling is involved in both short-term and long-term signaling with a wide range of different effects within the organism.

1.5.1 Purines/nucleotides

Nucleotides consist of a purine- or pyrimidine base, a ribose (or deoxyribose respectively), and a phosphoric acid group (Löffler, Purin- und Pyrimidinstoffwechsel, 2005). The most important purine in purinergic signaling is adenosine (Bours, 2006) (Graubardt, 2012). The most important nucleotides are adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), and uridine 5'-triphosphate.

Classically, ATP is known as an energy-carrier in cell metabolism (Löffler, Chemische Grundlagen des Stoffwechsels, 2005) but it has also been established as a signaling molecule, first in neuronal tissue, and later in non-neuronal cells, too (Burnstock, Purine and pyrimidine receptors, 2007).

Intracellular ATP in epithelia is under basal conditions present in concentrations of 3-8 mM (Eltzschig, Sitkovsky, & Robson, 2012) (Fitz, 2007), while its concentration is much lower in the extracellular plasma (400-700nM) (Bours, 2006) (Graubardt, 2012).

1.5.2 Nucleotide release

Different conditions such as cell death or inflammatory states and different pathways of nucleotide release are known (Eltzschig, Sitkovsky, & Robson, 2012). Necrosis and apoptosis lead to an uncontrolled ATP egress from intracellular stores, and a controlled ATP release through pannexin hemichannels, respectively. Activated immune and other cells release ATP through connexin hemichannels, and platelets release ADP from intracellular granules. Thereby released nucleotides can act as chemotactic signaling molecules.

For a half-maximal activation of purinergic receptors, ATP is typically required in concentrations of 3-500 nM (Fitz, 2007). Nucleotide signaling therefore does not lead to alterations in the intracellular energy stores.

1.5.3 Extracellular metabolism of nucleotides

Due to various extracellular nucleotidases and other hydrolytic effects, ATP outside the cell has a very short half-life in the range of seconds (Fitz, 2007) (Bours, 2006) (Graubardt, 2012). The extracellular hydrolysis cascade leads to a sequential degradation of nucleotides into their respective nucleosides and free phosphate (Zimmermann, 2000).

There are four families of ectonucleotidases responsible for nucleotide hydrolysis (Zimmermann, 2000): E-NTPDase family (ecto-nucleoside triphosphate diphosphohydrolase family), E-NPP family (ectonucleotide pyrophophatase/phosphodiesterase family), Alkaline phosphatases, and Ecto-5'-

nucleotidase. The ectonucleotidases are normally membrane-bound enzymes, but soluble variants of various ectonucleotidases exist through cleavage from their anchor. It is noteworthy, that ectonucleotidases generally have broad individual substrate specificities and can hydrolyze triphosphate, diphosphate, and monophosphate variants of different nucleotides with different preferences.

NTPDase1, also named CD39, is a member of the E-NTPDase family. Ecto-5'-nucleotidase is also named CD73.

1.5.4 Purinergic receptors

Two families of purinergic receptors are known, named the P1 and P2 receptors.

1.5.4.1 P1 receptors

P1 receptors are G protein-coupled receptors and are divided into four subtypes (A_1, A_{2A}, A_{2B}, A_3). P1 receptors bind extracellular Adenosine (Burnstock, Purine and pyrimidine receptors, 2007).

1.5.4.2 P2 receptors

P2 receptors have two subfamilies, P2X and P2Y (Burnstock, Purine and pyrimidine receptors, 2007).

P2X receptors are ligand-gated ion channel receptors. Seven subtypes of P2X have been characterized (P2X1-7).

P2Y receptors are G protein-coupled receptors. Depending on the subtype they are coupling to different G proteins with either a stimulating or an inhibiting effect, or even both. Eight human subtypes of have been identified so far and they are divided into two subgroups (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11; and P2Y12, P2Y13, P2Y14).

All P2X and P2Y receptors respond to extracellular ATP with different affinities, and some to other natural and synthetic nucleotides, too (Jacobsson, 2012) (Graubardt, 2012). For some of the receptors, more specific agonists and antagonists have been found (Burnstock, Purinergic signalling: Its unpopular beginning, its acceptance and its exciting future , 2012) (IUPHAR, 2012): E.g. BzATP is agonist for P2X1, P2X3, P2X7, P2Y11; ATPγS is agonist for P2X2, P2X5, P2Y1, P2Y11, P2Y12, P2Y13; and suramin is antagonist for P2X2, P2X5, P2Y2, P2Y4, P2Y11. ADP is agonist for P2Y1, P2Y6, P2Y12, and P2Y13. UTP is agonist for P2Y2, P2Y4, P2Y6, and P2Y11.

1.5.4.3 Expression and interactions of purinergic receptors

Purinergic receptors are expressed on many cells throughout the body, both neuronal, and non-neuronal cells, such as immune cells (Bours, 2006). The specific expression patterns of purinergic receptors on immune cells vary between cell types and depend on inflammatory states. However most immune cells express both subtypes, i.e. P1 and P2 receptors.

Monocytes and Macrophages express various P1 and P2 receptor subtypes (Bours, 2006). Specific expression of the P2 receptor may depend on maturation stage, and nature of cellular activation.

In addition to different and mixed expression patterns, purincergic receptors show receptor dimerization (both homodimers and heterodimers) and receptor cross talk (Burnstock, Purine and pyrimidine receptors, 2007) (Jacobsson, 2012). Therefore they show

very complex physiological interactions both on the level of the receptors themselves, and within the downstream signaling.

1.5.5 Effects of purinergic/nucleotide signaling on the immune system and repair mechanisms

It is widely recognized that purinergic signaling is involved in many neuronal and non-neuronal mechanisms (Burnstock, Purine and pyrimidine receptors, 2007).

Bours (Bours, 2006) concludes, that because of these multiple observation, there is overwhelming evidence that extracellular nucleotides and purinergic receptors play a major role in immunity and inflammation. However, the role of extracellular nucleotides is extremely complex and interdependent and is not yet fully understood (Bours, 2006), (Graubardt, 2012).

Adenosine and purine nucleotides often act in a reciprocal way, and therefore the balance and metabolism between Adenosine and purine nucleotides (which can be degraded into Adenosine) are an especially important factor in the effect of purinergic signaling (Eltzschig, Sitkovsky, & Robson, 2012).

Furthermore, it has been shown, that purinergic signaling (i.e. ATP and Adenosine) is a key mediator in various disease states of the liver and other organs, such as in liver repair after injury or in hypoxic-reperfusion injuries as they occur during liver transplantation (Vaughn, Robson, & Burnstock, 2012) (Beldi, 2008) (Gonzales, 2010).

In vivo, ATP decreased the circulating concentration of TNF and IL-6 after trauma-hemorrhage and resuscitation, a condition, which normally increases these cytokines (Wang P. , 1992).

In the following explanations I am focusing on the immune system and Monocytes/Macrophages.

1.5.6 Effects of purinergic/nucleotide signaling on monocytes/macrophages

Many factors affect purinergic signaling, such as type and concentration of the extracellular nucleotide, extracellular metabolism of the nucleotides through ectonucleoside kinases, and receptors expressed on the different cells.

Monocytes express P2Y1,2,4,6,11,12,13, and P2X1,4,5,7 receptors [sic (Bours, 2006)] (Hickmann, 1994) (Falzoni, 1995) (Clifford, 1997) . (Martin, 1997) (Buell, 1998) (Humphreys, 1998) (Jin, 1998) (Koziak, 1999) (Adrian, 2000) (Gu, 2000) (Chessel, 2001) (Mehta, 2001) (Moore, 2001) (Warny, 2001) (Aga, 2002) (Into, 2002) (Zhang F. L., 2002) (Derks, 2004) (Sluyter, 2004) (Wang L. , 2004) (Kaufmann, 2005).

Macrophages express the same receptor subtypes except for P2Y13 [sic (Bours, 2006)] (Hickmann, 1994) (Falzoni, 1995) (Martin, 1997) (Schmid-Antomarchi, 1997) (Buell, 1998) (Adrian, 2000) (Chen B.-C. , 2000) (Moore, 2001) (Stober, 2001) (Adinolfi, 2002) (Eschke, 2002) (Bowler, 2003) (Hanley, 2004) (Coutinho-Silva, 2005).

Monocytes/Macrophages exhibit CD39 as well as CD73 activity, and are capable of releasing both ATP and Adenosine upon activation, rendering them susceptible to autocrine and paracrine purinergic regulation by extracellular ATP and Adenosine [sic (Bours, 2006)].

Extracellular nucleotides have been described to have various effects on monocytes/macrophages and may even affect their inflammatory state (Bours, 2006).

The specific effects depend on many factors such as the nucleotide itself, purinergic receptor expression, nucleotide concentration, and the downstream activation pathway of the cells. Possible effects appear to be for example: a contribution to the adhesion of inflammatory monocytes to vascular endothelium (Ventura, 1995) (von Albertini, 1998) (Goepfert, CD39 modulates endothelial cell activation and apoptosis., 2000); recruitment and chemotaxis of monocytes/macrophages (McCloskey, 1999) (Goepfert, Disordered cellular migration and angiogenesis in cd39-null mice., 2001) (Honda, 2001); both up- and downregulation of production and release of cytokines (such as e.g. TNFα, IL-6) (Tonetti, 1995) and other inflammatory mediators; bi-directional modulation of phagocytosis; and apoptotic as well as necrotic cell death (Bours, 2006).

Even though many effects have been observed, the exact mechanisms seem not yet to be fully understood and foreseeable.

1.5.7 Effects of extracellular nucleotides on the phagocytic properties of macrophages

Marques-da-Silva (Marques-da-Silva, 2011) could produce a significantly increased phagocytosis of latex beads by murine peritoneal macrophages when they were pre-treated with ATP, ADP or UDP. Furthermore he could abolish these effects with pre-treatments with various P2-receptor antagonists. Based on these and other experiments P2-receptors seem to be involved in the modulation of phagocytosis.

On the other hand Elliott (Elliott, 2009) concentrated his work on the recruitment of macrophages towards apoptotic cells and could also show an effect of nucleotides. In two different in vivo models he demonstrated a more efficient recruitment of macrophages due to the presence of ATP and UTP. This effect could be abolished by depletion of nucleotides and decreased in P2Y2 -/- mice. Because of these results ATP and UTP were identified as find-me signals released by apoptotic cells and P2Y2-receptors on monocytes and macrophages as a critical sensor of the find-me signal.

1.5.8 Effects of extracellular nucleotides on platelets

The effects of extracellular nucleotides are established in the clotting properties of platelets and were described above in this work. The broad clinical implementation of this knowledge is the use of clopidogrel as an anticoagulant drug in coronary heart disease.

1.6 Experimental approach

To investigate the role of extracellular nucleotides in the context of sepsis, we focused on primary and cultured monocytes/macrophages in vitro, which were exposed to lipopolysaccharides. In these models we administered different extracellular nucleotides to observe its effect on cytokine expression and phagocytosis of macrophages.

Furthermore we planned to expose human thrombocytes to extracellular lipopolysaccharides and measure their effect on the nucleotide release of platelets.

1.6.1 A) Do extracellular nucleotides modulate cytokine secretion of primary murine Kupffer cells after stimulation with LPS?

To evaluate effects of hepatic macrophages in vitro we used a model with primary Kupffer cells (KCs) from mice. Primary Kupffer cells are easily gained in a sufficient amount using specific experimental techniques. Kupffer cells represent a large and relevant subpopulation of all macrophages in the body – also regarding to their immunological functions with effects possibly on the entire organism. As a laboratory associated with visceral surgery we are especially interested in Kupffer cells because of their localization in the liver. Being inflammatory cells Kupffer cells may be involved in regeneration processes, e.g. after liver resection, liver transplantation, hypoxia-reperfusion damages, and others (Beldi, 2008) (Seki, 2000).

Using a model with primary murine Kupffer cells we wanted to show a dose dependent effect of extracellular nucleotides on the cytokine release of LPS-stimulated Kupffer cells. Briefly, we stimulated Kupffer cells with lipopolysaccharides and administered nucleotides (ATP, ADP, ATP γ S, UTP, Bz-ATP) and a known P2-receptor antagonist (Suramin) in different concentrations, and measured TNFα-release using an ELISA assay. The goal was, based on preliminary data and previous publications to show dose-dependent difference of TNFα-release under the administration of nucleotides and the modulation of this effect with the P2-receptor antagonist.

1.6.2 B) Do (human) thrombocytes/platelets release ATP after stimulation and/or co-stimulation with LPS?

To search for a possible systemic effect of ATP, we focused on platelets, because they are circulating in the blood when compared to Kupffer cells.

This activation of platelets by LPS and its release of nucleotides may play a role in systemic or local inflammatory or immune responses. In our experimental approach we initially planned to combine the effect of LPS on nucleotide release by platelets and the effect of nucleotides on cytokine release by macrophages.

As previously described, activated platelets release and sense extracellular ATP and – predominantly – ADP to produce a positive feedback loop for an efficient primary clot formation during hemostasis (Kumar, 2007) (Oury, 2002). Platelets have also been shown to release ATP and ADP in response to LPS (Zhang G. , 2009).

Our initial idea was to reproduce this effect first in human and then in murine platelets. If we had a reliable ATP/ADP release by platelets we aimed to combine the two experiments in order to explore an interaction of platelets and KC in response to LPS. Therefore we planned to use the supernatant of the activated platelets to put in

the media of Kupffer cells. LPS should activate platelets and make them release ATP/ADP. The supernatant with these released nucleotides should then replace the added nucleotides in the first series of experiments, and therefore deplete the effect of LPS on cytokine release by Kupffer cells.

Briefly, we wanted to explore if LPS-activated platelets reduce the cytokine release by LPS-activated Kupffer cells and thereby reduce inflammatory reactions.

1.6.3 C) Do extracellular Nucleotides influence phagocytosis by cell culture macrophages?

An effect of extracellular nucleotides on phagocytosis by macrophages has been shown (Marques-da-Silva, 2011), (Elliott, 2009).

For our work, we adapted the protocol from one of the authors referred to (Marques-da-Silva, 2011). Instead of primary peritoneal macrophages we used macrophages from murine (RAW 264.7) and human (U-937) cell lines. With these macrophages we aimed to reproduce an increase of phagocytosis of bio particles (instead of latex beads) in response to stimulation with extracellular nucleotides. Based on such data, the further plan was to search the responsible receptor pathways for this effect. For that purpose possible means could be the use of various specific agonists and antagonists (of P2 receptors), and e.g. short interfering RNAs to specifically knock down single receptors responsible for the effect.

To measure phagocytosis under the effect of extracellular nucleotides we used fluorescein-labeled bio particles. We incubated macrophages from cell cultures together with marked bio particles and stimulated with different concentrations of nucleotides. After washing out and quenching of the remaining – not phagocytized – particles, only the intracellular particles were measured either with a plate reader or by flow cytometry.

In this experiment we were expecting – as the referred authors described – an increase of phagocytosis by the administration of extracellular nucleotides. A higher number of phagocytized bio particles per cell should be detectable as a higher fluorescence in the concerned well in a plate reader, and as higher mean fluorescence intensity and a different distribution pattern in a dot plot graph in flow cytometry.

Thereby we wanted to show an effect of extracellular nucleotides on both inflammatory/immunity effects of macrophages, i.e. cytokine release and phagocytosis.

2 A) Effect of extracellular nucleotides on cytokine release in primary murine Kupffer cells after stimulation with LPS

2.1 Introduction (idea & goal)

As I described above in more detail, the goal and hypothesis of this part was, to show an altered cytokine (i.e. TNFα) release by LPS-stimulated primary murine Kupffer cells upon stimulation with extracellular nucleotides (e.g. ATP, ATP γ S, ADP, and others), using an ELISA-assay.

2.2 Material & methods

To harvest murine Kupffer cells we used a terminal in vivo digestion model with subsequent serial centrifugation steps including a double percoll gradient, based on a previously described method (Aldeguer, 2002).

2.2.1 Animals

Animals were housed in accordance with the guidelines from the Swiss Veterinary Office in the animal facility of the "Departement für Klinische Forschung" (DKF) in Bern. Mice had free access to standard mouse chow. We studied BL/6 strain wild-type mice (DKF animal facility, Bern) and P2Y2-null mice (kindly provided by Wolfgang G. Junger [Beth Israel Deaconess Medical Center, HMS, Boston], originally provided for another project).

All the experiments were terminal during the cell harvesting procedure causing death by blood loss. Death at the end was confirmed by producing large bilateral pneumothoraxes.

Operative procedures were performed by – or under supervision of – Guido Beldi, Adrian Keogh, Markus Trochsler or René Fahrner.

2.2.2 Narcotics

As narcotics we used either a triple anesthesia for mice or ketamine anesthesia for mice. Triple anesthesia consists of Dormitor® (Medetomidine) 1mg/ml and 500µg/kg, Climasol® (Climazolam) 10mg/ml and 5mg/kg, Fentanyl 0.5mg/ml and 50 µg/kg. Ketamine was applied at 100-200 mg/kg.

2.2.3 Buffers and reagents

For the liver perfusions a Chelation buffer containing EGTA (ethylene glycol tetra acetic acid), and a collagenase Buffer was used. All the buffers were prepared in clean conditions and then filtered or autoclaved, any further handling was done in sterile conditions in a laboratory bench. All buffers were stored at 4°C.

2.2.3.1 Chelation Buffer

Chelation Buffer was composed of 1x Leffert's Buffer (which was made by a 10 dilution of 10x Leffert's Buffer) with 0.5 mM EGTA (ethylene glycol tetra acetic acid) (which was achieved my adding 0.625 ml of a 0.2 M EGTA stock into a final volume of 250 ml 1x Leffert's Buffer).

2.2.3.2 Leffert's Buffer

10x Leffert's Buffer is composed of 250 mM (59.7 g/l) HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1.15 M (69.27 g) NaCl (sodium chloride), 50 mM (3.73 g) KCl (potassium chloride), 10 mM (1.36 g) KH_2PO_4 (potassium dihydrogen phosphate), and was produced in 1 liter ion free water and calibrated to a pH of 7.4, and then filtered.

2.2.3.3 Collagenase Buffer

Collagenase Buffer was composed of 1x Leffert's Buffer (which was made by a 10 dilution of 10x Leffert's Buffer) with 2 mM $CaCL_2$ (Calcium chloride) (This was achieved by adding 0.5 ml of a 1 M $CaCL_2$ stock into a final volume of 500 ml 1x Leffert's Buffer.), and 100 mg/l Collagenase (Type IV, Sigma-Aldrich). Collagenase Buffer was produced in a bulk of 500 ml, filtered into 50 ml tubes, and stored at minus 20°C.

2.2.3.4 Culture medium

As culture medium we used RPMI-1640 (Roswell Park Memorial Institute 1640 medium) GlutaMAX™, (GIBCO/Invitrogen #61870) with 10% FBS (Fetal Bovine Serum, Heat Inactivated) (GIBCO/invitrogen #10500) and 1% Penicillin/Streptomycin (GIBCO/invitrogen # 15140122). This medium I call RPMI/FBS.

2.2.3.5 Percoll

Percoll (GE Healthcare # 17-0891-01) is used for gradient centrifugations. Percoll itself is hypotonic. Isotonic "stock-percoll" is produced by the addition of 9 parts percoll to 1 part 10x HBSS (Hanks Basic Salt Solution, 10x concentrated cell culture medium) (GIBCO/Invitrogen #14065-049). We used a double percoll Gradient: 50% (2.5ml) / 20% (3ml) in a standard 15 ml tube. It was produced by putting 3ml of 20% percoll in a 15 ml tube and gently layering 2.5ml of 50% percoll below. 20% percoll consists of 2 parts stock-percoll and 8 parts RPMI/FBS. 50% percoll consists of 5 parts stock-percoll and 5 parts RPMI/FBS.

2.2.3.6 Nucleotides

ATP (Adenosine 5'-triphosphate disodium salt hydrate): Sigma Aldrich #A26209; ADP (Adenosine 5'-diphosphate): Sigma Aldrich #A2754; ATPγS (Adenosin 5'-(gamma-thio)-triphospate): Jena Bio Science #NU-406; BzATP (2'(3')-*O*-(4-Benzoylbenzoyl)adenosine-5'-triphosphate): Tocris Bioscience #3312; UTP (Uridine 5′-triphosphate): Sigma Aldrich #U6750

2.2.3.7 LPS

Lipopolysaccharides from Salmonella enterica serotype abortus equi Sigma Aldrich #L6636

2.2.4 Isolation of murine liver Kupffer cells

Kupffer cells were isolated using an adapted previously described protocol (Aldeguer, 2002).

Bl/6 mice, usually 4 at the time, were put into narcosis with intraperitoneal injection of a Triple-Anesthesia or Ketamine (mixture and doses see above). Once the mice were in deep narcosis – confirmed by lack of muscle contraction after a potential painful

stimulus in the tail or lower extremities – a large transverse laparotomy was performed to get access to the liver and the inferior caval vein. The operations were performed using a binocular microscope.

The inferior caval vein was punctured with a 22G sized standard infusion cannula and the portal vein was cut. With this installation and a roller pump perfusing the cannula at a speed of 1.72 ml/min, a retrograde perfusion of the liver could be achieved, which was controlled visually. Outflow of clear buffer fluid out of the cut portal vein, and brightening of the liver within a few minutes demonstrated good perfusion of the liver.

The liver was perfused first with Chelation Buffer for 9 minutes, followed by the actual congestion with Collagenase Buffer for 13-18 minutes. The exact end point of liver congestion could be visually determined under the microscope, when the liver tissue within the capsule started to lose its structure. The congestion was performed under a warmth lamp, and with warmed buffers and pump to have an estimated in situ temperature of approximately 37°C.

After the perfusion, the livers were removed, collected and pooled together in a cell culture dish with some RPMI/FBS-Medium inside.

All the following steps were done in sterile conditions under the laboratory hood.

The livers were cut into small pieces. Gentle shaking released the cells from the capsule. Then we filtered the cells through a polyamide mesh with 60 µm mesh opening (Sefar AG MEDIFAB® #03-60/42) into 50 ml tubes. The tubes were filled up with RPMI/FBS-Medium.

In a first centrifugation step with 50g for 15 minutes the hepatocytes were pelleted while Kupffer cells remained in the supernatant. Secondly the supernatant containing Kupffer cells was centrifuged with 500g for minutes. This pellet was then resuspended in RPMI/FBS-Medium and layered on a double percoll gradient (50%/20%).

In this double percoll gradient, a density centrifugation was performed with 800g for 15 minutes. After the density centrifugation the interface between the 20% and 50% percoll layers– where the Kupffer cells are – was taken out with a sterile Pasteur pipette, and washed once with 500g for 7 minutes.

The newly pelleted Kupffer cells were resuspended and dispersed in about 36 wells of a 96 well cell culture plate and given 15 minutes to sink and adhere to the bottom. After another washing step in the plate Kupffer cells were incubated over night (Aldeguer, 2002).

2.2.5 Experimental design

To activate the Kupffer cells we stimulated for 6 hours with LPS in different concentrations (mostly 25ng/ml) and compared to culture medium as a control. Immediately before stimulation with LPS we administered agonists (ATP, ADP, ATP γ S, UTP, and Bz-ATP) and antagonists (suramin) in various concentrations.

All stimulating substances were added at the same time point. The substances were added in very small volumes with accordingly higher concentrations to achieve final concentrations as indicated in the detailed protocols. We then incubated the cells for 6 hours at 37°C with 5% CO2 in otherwise normal air before using the supernatant for

the measurements of cytokines. We measured TNFα (or IL-6) concentrations in the supernatants using an ELISA assay. Experiments were mostly performed in triplicates, some experiments (or parts) in duplicates only, because of a low amount of cells available at a time.

Cells were not counted or standardized between the experiments, concentration values therefore cannot allow a statement about the amount of cytokines released per cell. This also does not allow comparing absolute values between different experiments, and also statements about the amount of cytokine produced per cell cannot be taken. Also for this reason, experiments will be presented separately.

In the later sections I am going to present the setup and the results of the experiments separately. Afterwards I am summarizing the results of all experiments and taking general conclusion.

2.2.6 Measurement of cytokine release (TNFα and IL-6)

At the end of the stimulation we removed the supernatants. The according cytokine concentrations were measured using a commercial ELISA assay kit (PeproTech: TNFα No 900-K54, IL-6 No 900-K50).

2.2.7 Statistical analysis and graphs

All calculations and statistical analysis were made with Microsoft Excel. To estimate statistical significance of the results, a two-sided student's t-test, type 2, was performed without previously analyzing values for their statistically normal distribution. The result we regarded as a p-value. P-values below 0.05 were considered significant. P-values (simply named p) are noted compared to the positive control (i.e. condition with LPS but without nucleotides), or to the negative control (i.e. condition without any stimulation at all) to control the effect of LPS alone.

The error bars in the graphs represent the standard deviations from the mean of the triplicates/duplicates of every sample.

2.3 Results of the individual experiments

In this chapter I am presenting the experiments and hypotheses behind, following the line of argument. TNFα concentrations (in parenthesis standard deviations of triplicates/duplicates) are in pg/ml in the supernatant, but not absolutely comparable between experiments. TNFα concentrations in the supernatant are interpreted as TNFα release. The individual experiments are numbered for a better traceability and orientation of the writer.

For the sake of completeness all experiments are mentioned here, including experiments with technical problems at the end.

2.3.1 Is TNFα release by Kupffer cells in response to LPS dose dependent?

Hypothesis

Extracellular LPS is associated with TNFα release by primary murine Kupffer cells in a dose dependent manner.

Setup

In one experiment (ch08-003) Kupffer cells were stimulated with 0 and 25 ng/ml LPS. In another experiment (ch08-002) Kupffer cells were stimulated with 25, 100, and 400 ng/ml. Both experiments were performed with co-stimulations with 0, 25, 100, and 400 µM ATP.

Results

LPS at 25 ng/ml compared to 0 ng/ml is associated with an approximately five-fold increase of TNFα in the supernatant, $p < 0.001$ (figure 2). A further increase of LPS from 25 to 100 or 400 ng/ml did not alter the TNFα-concentrations significantly, independently of the stimulation with ATP (no figure shown).

Conclusion

We therefore concluded, that LPS is associated with TNFα release by primary murine Kupffer cells, but not in a dose dependent manner within the tested range.

2.3.2 Does ATP impact on TNFα release?

Hypothesis

Extracellular ATP impacts on TNFα release by LPS-stimulated primary Kupffer cells in a dose dependent manner.

Setup

In three experiments (ch08-002, 003, 004), we stimulated Kupffer cells with 0, 25, 100, and 400 µM ATP and 25 ng/ml LPS. In one of them (ch08-002) Kupffer cells were also stimulated with 100 and 400 ng/ml, in another (ch08-003), ATP was also added without stimulation with LPS.

Results

In all experiments ATP 25 µM was associated with a significant decrease of TNFα release compared to controls. In two of the experiments, the effect of ATP was throughout dose dependent, e.g. compared with 0 ng/ml LPS. Increasing concentrations of ATP during the stimulation were associated with lower concentrations of TNFα, down to basal levels at 100 and 400 µM ATP ($p < 0.001$ against positive control without ATP). Using 25 µM ATP TNFα decreased 50% ($p = 0.014$ against positive control) (figure 2).

Only in one experiment (ch0-002), at 400 µM ATP the TNFα release was higher again than at 25 and 100 µM ATP. This was the first experiment of the series.

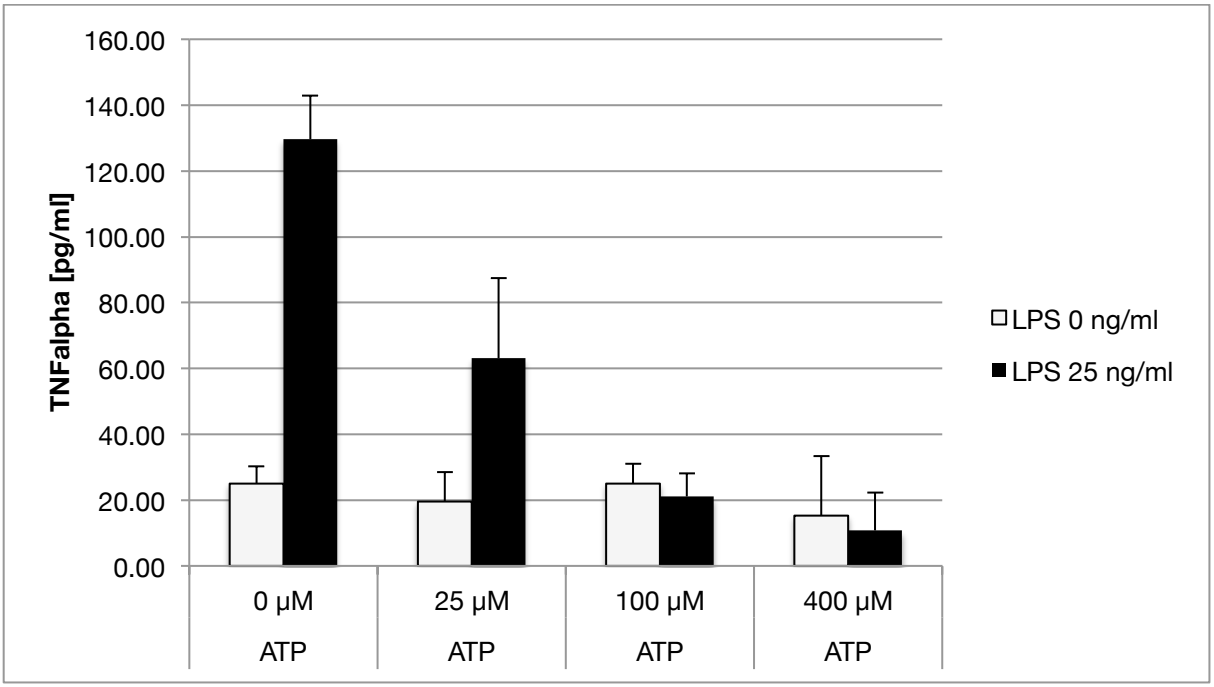

Figure 2: ch08-003_1: The figure shows the effect of different concentrations of extracellular ATP on the TNFα release of Kupffer cells with and without stimulation of LPS. (The error bars show standard deviations. P-value for 25µM ATP 0.014, for 100, and 400µM ATP <0.001)

Conclusion

Extracellular ATP decreases TNF secretion by primary KC in a dose dependent manner.

Non-physiological high concentrations of extracellular ATP (400uM) abrogate TNFα release of LPS stimulated Kupffer cells.

2.3.3 Which specific P2 receptor agonists and antagonists impact on TNFα release by Kupffer cells?

Next we wanted to identify if the observed ATP effect is mediated via specific P2 (ATP) receptors. We postulated P2 receptor signaling to be the key mediator of this effect.

Therefore, we used various specific agonists and antagonists to various P2 receptors to narrow down the probable set of responsible P2 receptors.

2.3.3.1 ATPγS

ATPyS is a non-hydrolysable ATP analog. Therefore, this agonist can be used to exclude relevant effects of AMP or Adenosine. ATPγS is the main agonist for P2X2, P2X5, P2Y11 (Burnstock, Purinergic signalling: Its unpopular beginning, its acceptance and its exciting future , 2012), and agonist for P2Y1, P2Y12, and P2Y13 (IUPHAR, 2012). A similar effect of ATPγS compared to ATP would therefore suggest that one of these receptors mediate inhibition of TNF secretion by KC.

Hypothesis

ATPγS decreases TNFα release by LPS-stimulated primary Kupffer cells in a dose dependent manner.

Setup

We stimulated Kupffer cells with 0, 25, 100 and 400 µM ATPγS with 25 ng/ml LPS (Figure 3; ch08-004). In the same experiment, Kupffer cells were also stimulated with 0, 25, 100, and 400 µM ATP and 25 ng/ml LPS. All conditions were in triplicate samples.

Results

Stimulation with LPS alone increased the TNFα concentration. A concentration of both 25 µM ATP and 25 µM ATPγS reduced TNFα by 50% ($p < 0.005$ and < 0.0002 respectively, compared to positive controls without nucleotides). A further increase of the nucleotide dose decreased TNFα additionally (figure 3).

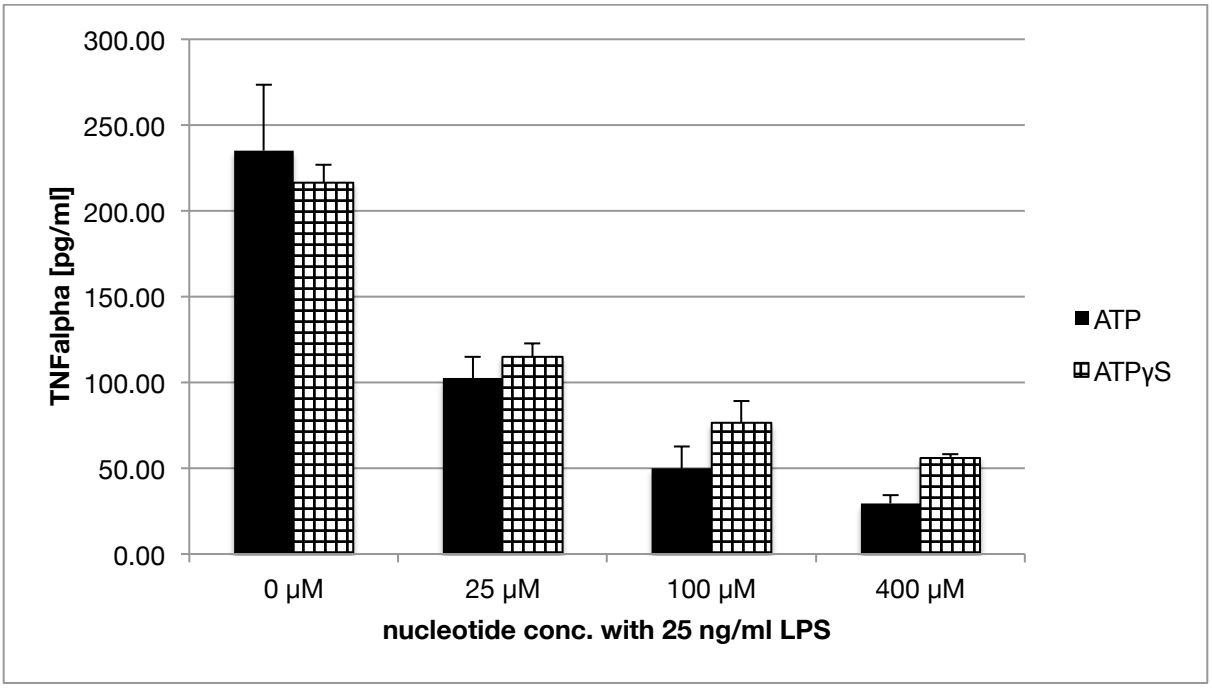

Figure 3: ch08-004_1 TNFα release by LPS-stimulated Kupffer cells, modulated by extracellular ATP and ATPγS.

Conclusion

ATPγS decreases TNFα release by LPS-stimulated primary Kupffer cells in a dose dependent manner. Based on these results we exclude major adenosine or AMP dependent effects.

2.3.3.2 ADP

ADP is an agonist for P2Y1, P2Y6, P2Y12, and P2Y13 (Burnstock, Purinergic signalling: Its unpopular beginning, its acceptance and its exciting future , 2012) (IUPHAR, 2012). A similar effect of ADP compared to ATP would therefore suggest an involvement of one of these receptors in the previously observed effects.

Hypothesis

ADP decreases TNFα release by LPS-stimulated primary Kupffer cells in a dose dependent manner.

Setup

In one experiment (ch08-005) we stimulated Kupffer cells with 0, 5, 25, 100, 400, and 1000 µM ADP with 25 ng/ml LPS. All conditions were in triplicate samples.

Results

ADP decreased TNFα-release with increasing nucleotide concentrations. Due to a large standard deviation in the control samples, a $p < 0.05$ was only achieved in the comparison of 0 µM ADP to 1000 µM ADP (figure 4).

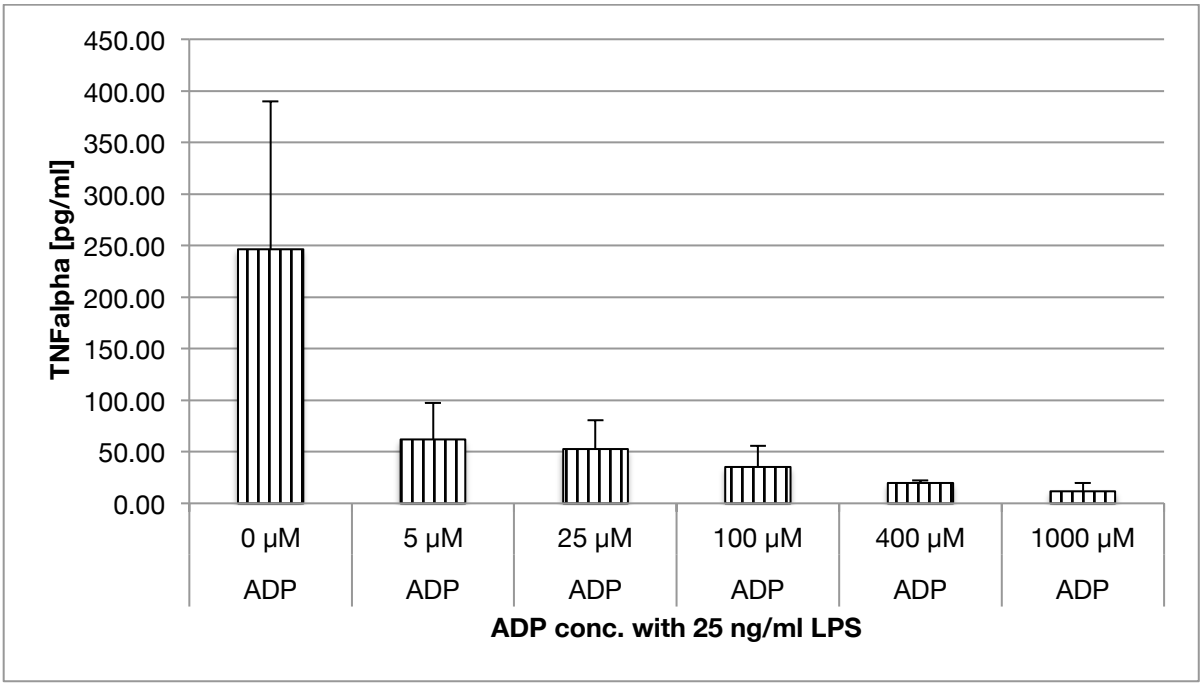

Figure 4 (ch05-008): TNFα release by LPS-stimulated Kupffer cells, modulated by extracellular ADP.

Conclusion

ADP may decrease TNF secretion by primary Kupffer cells in a dose dependent manner, but to achieve clear and consistent significance, further experiments are necessary. An involvement of ADP sensitive P2 receptors (i.e. P2Y12, P2Y13) seems possible, but may not be dominant in purinergic signaling in Kupffer cells.

The results in this experiment are not significant enough to be definitely interpreted. That's due to the wide standard deviations, which are probably because of technical problems. But the trend in this experiment shows a dose dependent decrease of TNFα release after stimulation with ADP. This makes further repetitive experiments promising.

2.3.3.3 UTP

UTP is an agonist for P2Y2, P2Y4, P2Y6, and P2Y11 (Burnstock, Purinergic signalling: Its unpopular beginning, its acceptance and its exciting future , 2012) (IUPHAR, 2012). A similar effect of UTP compared to ATP would therefore suggest an involvement of one of these receptors in the previously observed effects.

Hypothesis

UTP decreases TNFα release by LPS-stimulated primary Kupffer cells in a dose dependent manner.

Setup

Altogether we performed three experiments (ch08-003, 004, 005) with UTP. Within these experiments, taken together, all conditions were applied once; only 100µM UTP and the controls without UTP were performed twice and three times, respectively. We stimulated Kupffer cells with 0, 2.5, 5, 10, 25, 40, 100 (in two experiments), 400, and 1000 µM UTP and 25 ng/ml LPS.

Results

Extracellular UTP is associated with a modest decrease of TNFα release in LPS stimulated Kupffer cells.

In one experiment (ch08-003) a 50% decrease of TNFα was achieved with 100 µM UTP, $p = 0.028$. Lower UTP-concentrations showed dose dependent decreases, too, but were statistically not significant ($p > 0.1$) (figure 5).

In another experiment (ch08-004), UTP at 10 and 40 µM also reduced TNFα net of 12, respectively 18 %, $p < 0.05$, and 100 µM UTP leaded to a further decrease $p = 0.05$ (figure 6).

In the third experiment (ch08-005), UTP showed a trend to a dose dependent decrease of TNFα with increasing nucleotide concentrations (400, and 1000 µM UTP). Because of wide standard deviations this result was not significant (figure 7).

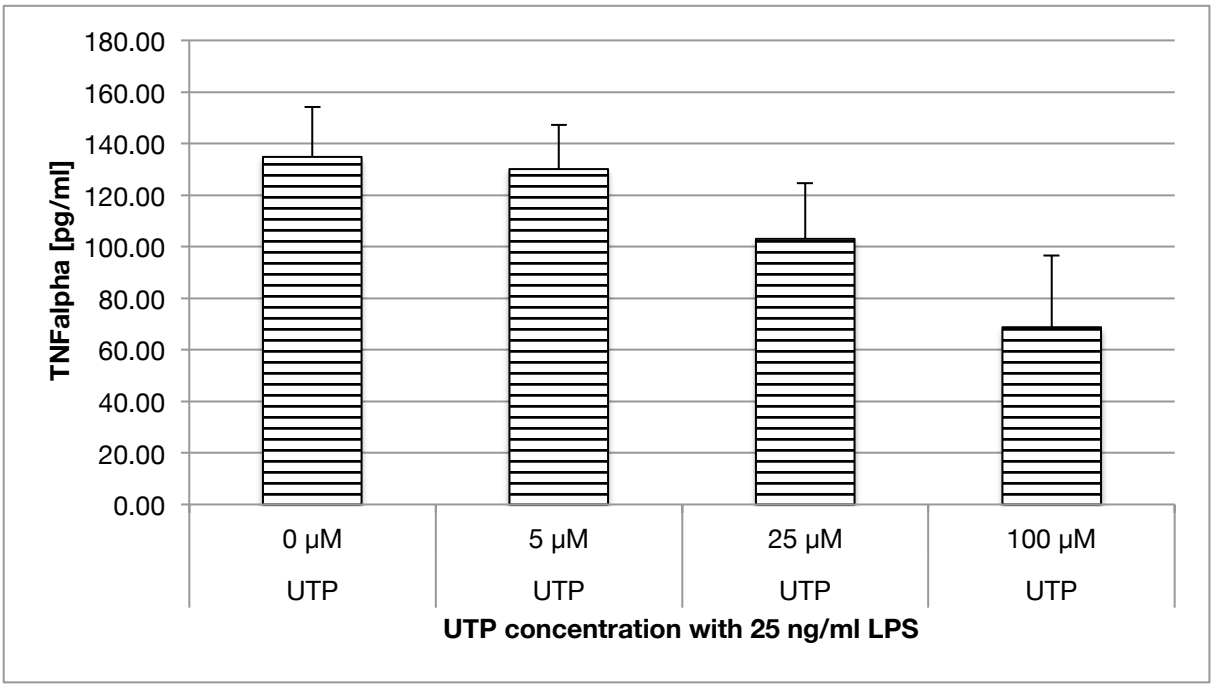

Figure 5: ch08-003_2 TNFα release by LPS-stimulated Kupffer cells, modulated by extracellular UTP.

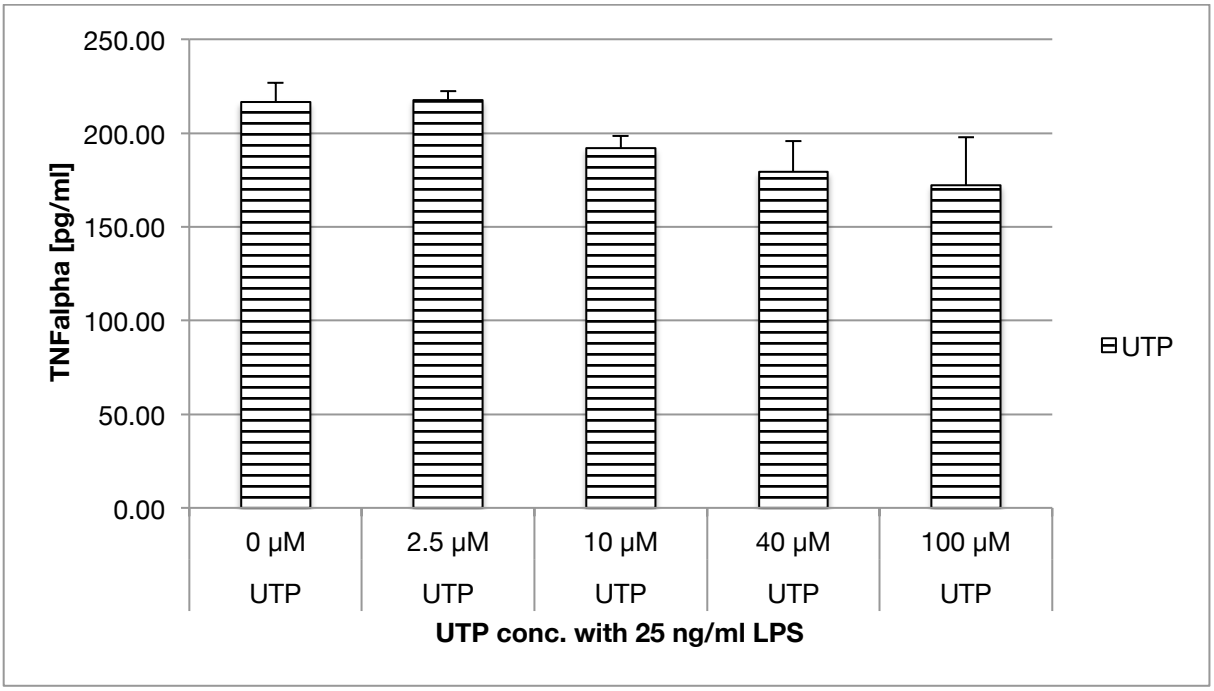

Figure 6: ch08-004 TNFα release by LPS-stimulated Kupffer cells, modulated by extracellular UTP.

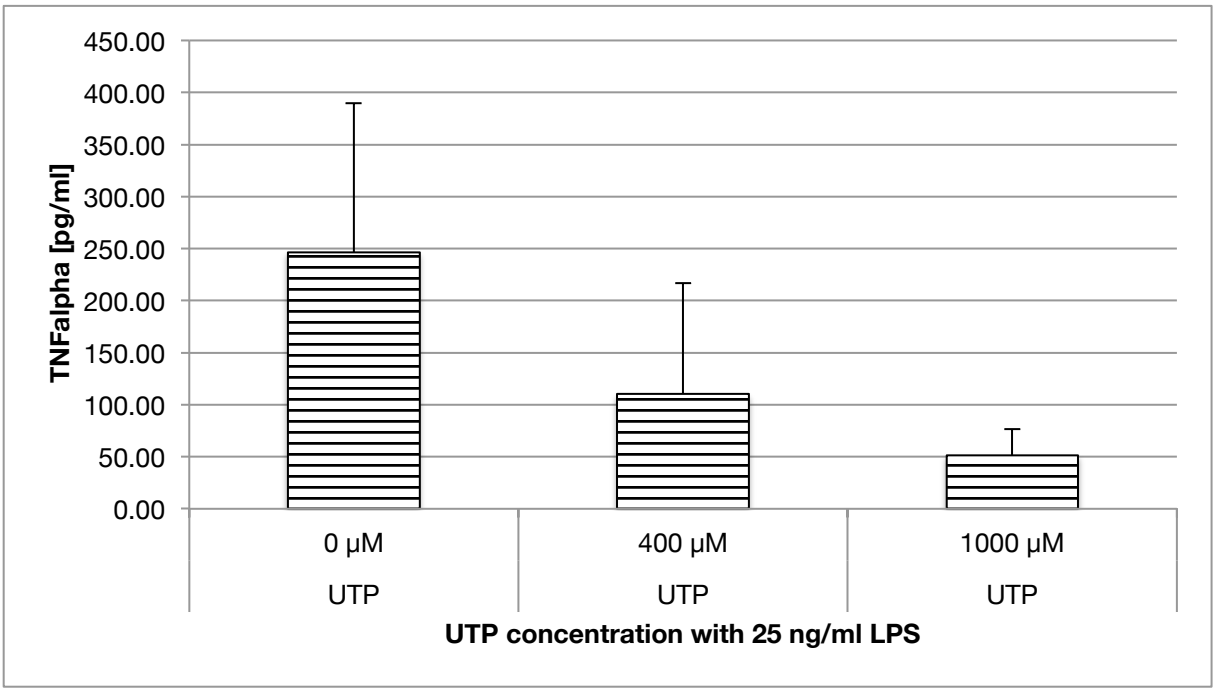

Figure 7: ch08-005_1 TNFα release by LPS-stimulated Kupffer cells, modulated by extracellular UTP.

Conclusion

UTP may decrease TNF secretion by primary Kupffer cells in a dose dependent manner, but to achieve clear and consistent significance, further experiments are necessary. In repetitive and controlled experiments, the UTP concentrations needed for a certain effect on TNF release should be determined with smaller technical variations. Based on the presented results it is possible, that UTP sensitive P2 receptors (i.e. P2Y2, P2Y4, P2Y6, P2Y11) are at least partly involved in purinergic signaling of Kupffer cells. UTP is only an agonist of P2Y receptors, but not of P2X

receptors. Therefore it is also probable, that G-protein coupled signaling is a key mechanism of how extracellular nucleotides impact on TNF release. But with these results alone, an effect of P2X receptors cannot yet be excluded.

2.3.3.4 BzATP

BzATP is an agonist for P2X1, P2X3, P2X7, and P2Y11 (Burnstock, Purinergic signalling: Its unpopular beginning, its acceptance and its exciting future , 2012). A similar effect of BzATP compared to ATP would therefore suggest an involvement of one of these receptors in the observed effects.

Hypothesis

BzATP decreases TNFα release by LPS-stimulated primary Kupffer cells in a dose dependent manner.

Setup

In one experiment (ch08-006) we stimulated Kupffer cells with 0, 1, 10, and 100 µM BzATP with 25 ng/ml LPS. All conditions were in triplicate samples.

Results

Extracellular BzATP decreased secretion of TNFα by LPS activated KC. TNFα secretion was decreased with 1 µM BzATP around 30% (p = 0.0157), with 10 µM around 50% (p = 0.0076), and with 100 µM Bz-ATP around 75%, compared to the positive control (p = 0.00046) (figure 8).

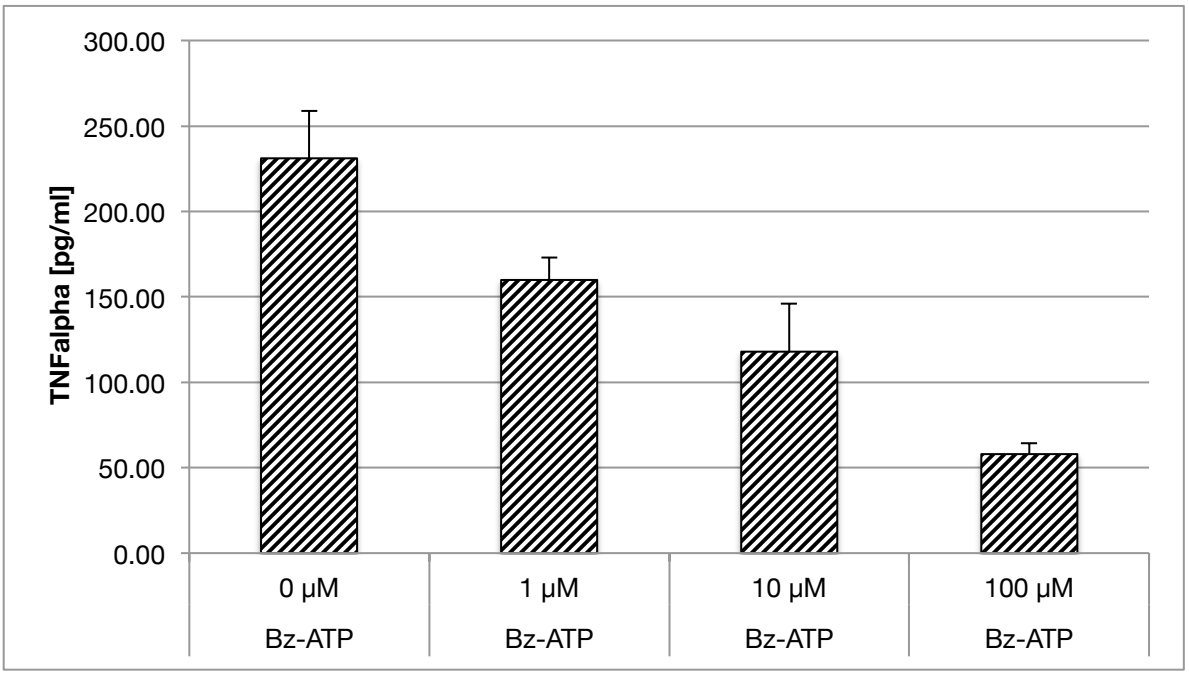

Figure 8: ch08-006_2 TNFα release by LPS-stimulated Kupffer cells, modulated by extracellular BzATP.

Conclusion

BzATP is associated with a strong decrease of TNFα release by LPS-stimulated primary Kupffer cells in a dose dependent manner. Based on these results we conclude that one of the BzATP sensitive receptors (i.e. P2X1, P2X3, P2X7, P2Y11) is probably involved in purinergic signaling in murine Kupffer cells.

2.3.3.5 Suramin

Suramin is an antagonist for P2X2, P2X5, P2Y2, P2Y4, and P2Y11 (Burnstock, Purinergic signalling: Its unpopular beginning, its acceptance and its exciting future , 2012). A partial or complete inhibition of the effect of extracellular ATP with Suramin would suggest, that the signaling of extracellular ATP is relevantly mediated by one of these receptors.

Hypothesis

Suramin – applied together with ATP – diminishes or even abolishes the effect of extracellular ATP and therefore leads to a dose-dependent increase of TNFα, compared to the ATP-suppressed TNFα-release.

Setup

Two experiments with Suramin were carried out (ch08-005, 006). In one experiment (ch08-005) Suramin was added at concentrations of 0, 10, 100, and 400 µM together with 25 ng/ml LPS. 30 minutes later a 100 µM ATP-concentration was administered. In the other experiment (ch08-006) Suramin was added at concentrations of 0, 1, and 10 µM together with 25 ng/ml LPS. 30 minutes later a 100 µM ATP-concentration was administered.

Additionally a control for Suramin in the same concentrations was made without administering ATP, to evaluate the effects of Suramin itself. Both experiments were carried out with triplicate samples in all conditions.

Results

In one experiment (ch08-005) with LPS and ATP stimulated Kupffer cells, 10 µM extracellular Suramin did not alter TNFα concentration. 100 µM Suramin increased TNFα threefold (p 0.01) (figure 9). With 400 µM Suramin we measured values of zero or close to zero in the ELISA-assay. They are not shown in the figure, because a technical problem is likely.

In the other experiment (ch08-006) Suramin 1µM and 10 µM – without ATP – TNFα decreased 35-40%, $p < 0.05$. When Suramin was added in the same concentrations together with 100 µM ATP, TNFα secretion, which has already shown a 78% decrease after stimulation with ATP, further decreased, $p < 0.001$ and < 0.00005 (Figure 10).

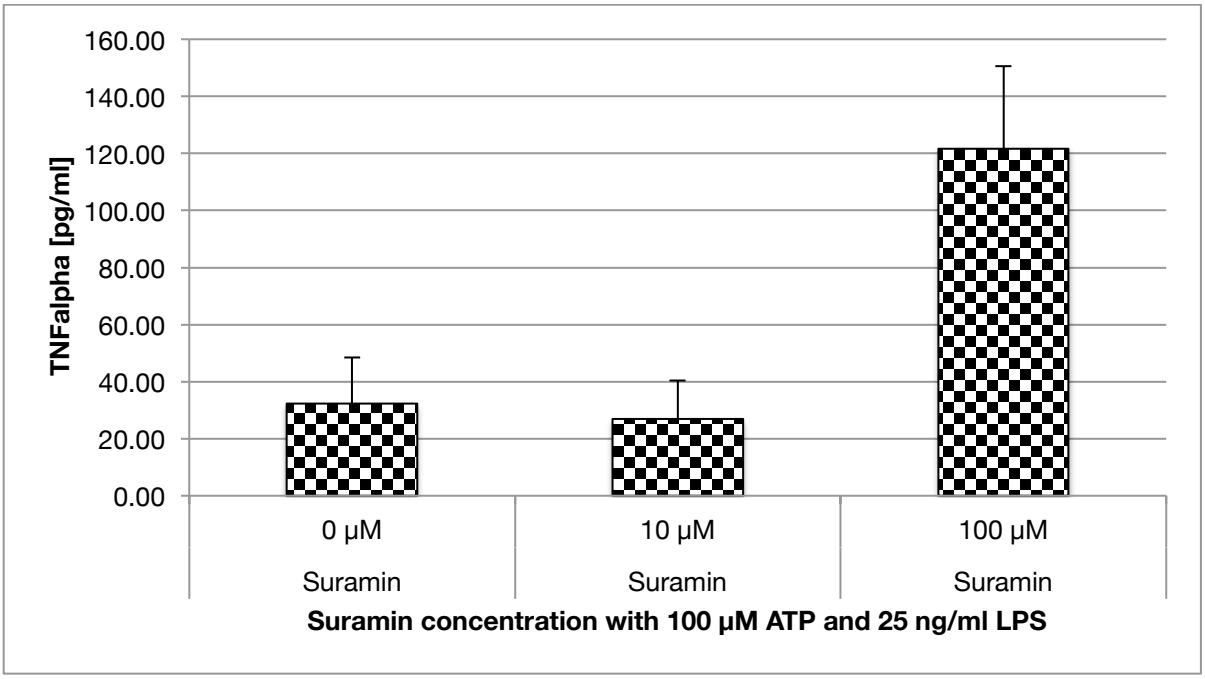

Figure 9: ch08-005 TNFα release by LPS-stimulated Kupffer cells, modulated by extracellular ATP and Suramin.

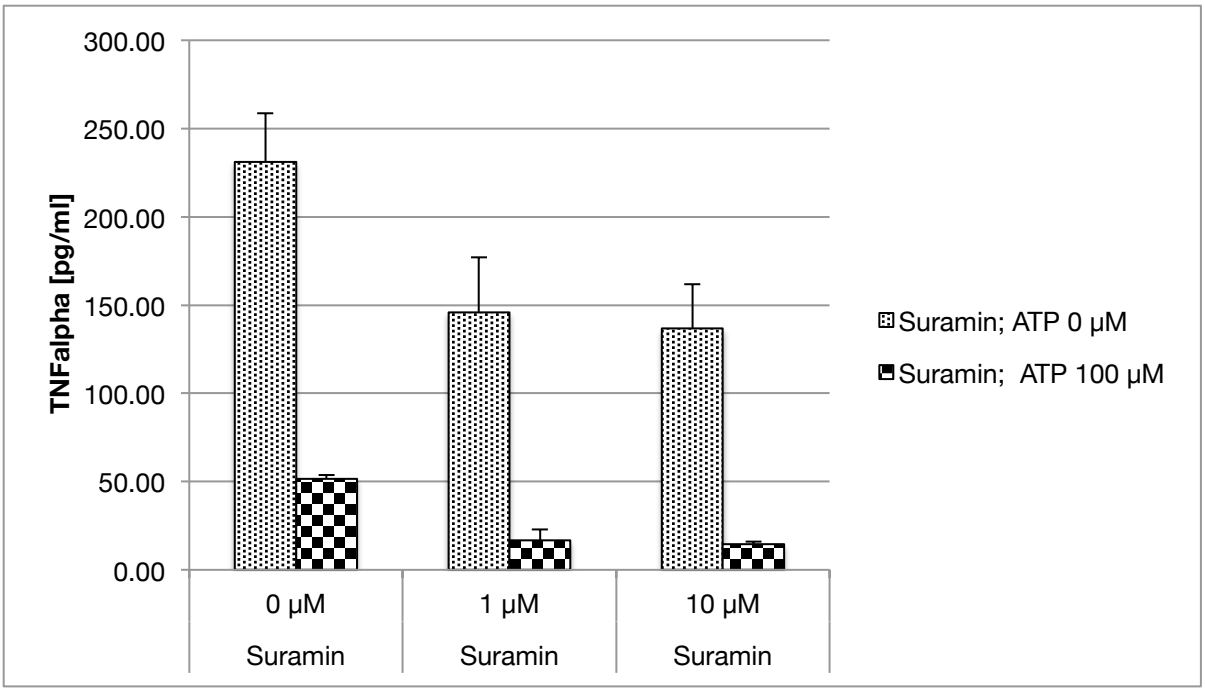

Figure 10: ch08-006_1 TNFα release by LPS-stimulated Kupffer cells, modulated by extracellular ATP and Suramin.

Conclusion

The results of these experiments were not conclusive. Suramin showed divergent effects at different concentrations. Suramin may therefore antagonize the purinergic signaling of Kupffer cells at higher concentrations, but further experiments are needed to verify these results and to determine the necessary concentration.

Other groups have showed effects of suramin in relation with purinergic signaling: Marques-da-Silva et al (Marques-da-Silva, 2011) could abolish a response of

macrophages to UTP with suramin, measuring phagocytosis of latex beads. Yebdri et al (Yebdri, 2009) could show that 100μM suramin diminishes P2Y2 and P2Y6 receptor mediated IL-8 release of monocytes, which is responsible for TLR-2 mediated neutrophil migration.

2.3.3.6 P2Y2

P2Y2 -/- knock out mice lack P2Y2 receptor. If P2Y2 is the mainly responsible receptor for purinergic signaling of extracellular ATP in Kupffer cells, we expected an effect of extracellular ATP to lack on P2Y2 -/- knock out mice.

Hypothesis

ATP does not decrease TNFα release in LPS stimulated Kupffer cells from P2Y2 -/- knock out mice, or its effect is significantly lesser than in wild type mice.

Setup

For this experiment (ch09-022) only two mice were used, instead of normally four. Conditions were only in duplicate samples. Kupffer cells from P2Y2 -/- mice were stimulated with 0, 5, 25, 100 μM of ATP, UTP, and ADP respectively, and with 25 ng/ml LPS.

Results

In P2Y2 -/- mice ATP TNFα decreased 20, 55, and 75% with 5, 25, and 100 μM ATP respectively (figure 11). P <0.05 only with 100 μM ATP. UTP and ADP didn't have a significant effect on TNFα-release in Kupffer cells of P2Y2-/- knock out mice (not shown in the figure).

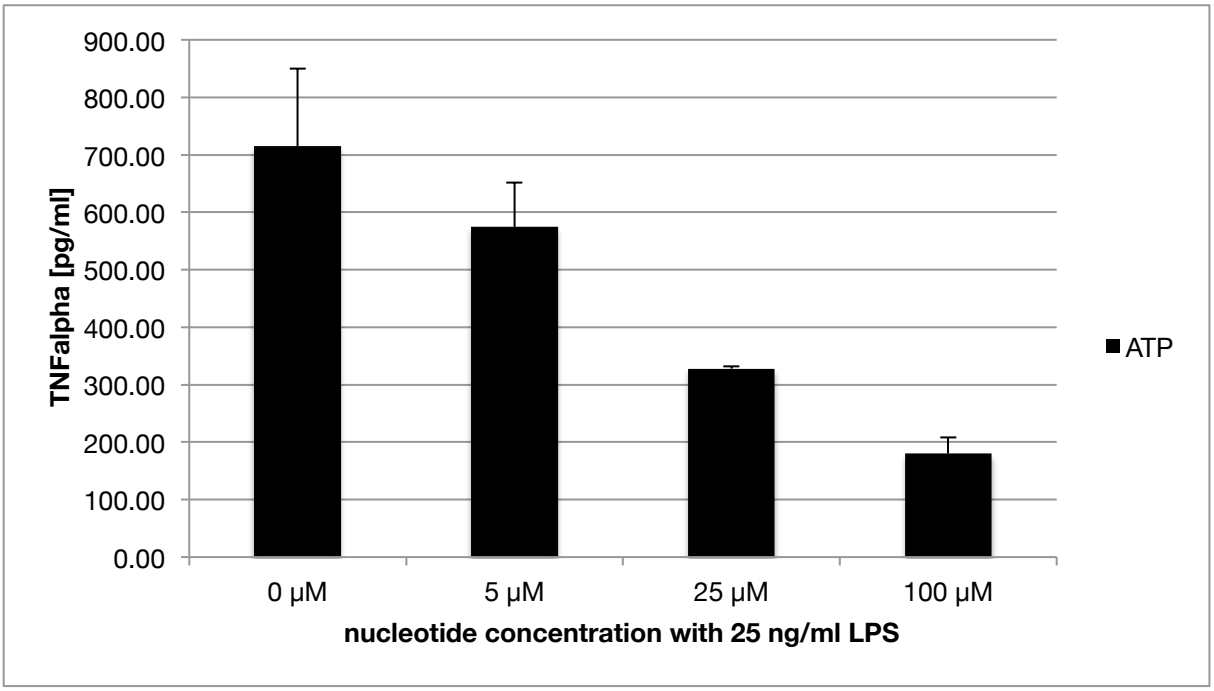

Figure 11: ch09-022_1 TNFα release by LPS-stimulated Kupffer cells from P2Y2-/- knock out mice, modulated by extracellular ATP.

Conclusion

P2Y2 is not a single key receptor for ATP in purinergic signaling of Kupffer cells.

A 50% decrease of TNFα with the administration of 25 μM extracellular ATP is the same effect as presented in previous experiments. Therefore the knocking out of P2Y2 in Kupffer cells does not alter puringergic signaling. Either this may be because P2Y2 is not the responsible receptor or because it can be sufficiently bypassed by redundant signaling pathways with other receptors. However, in order to exclude a minor effect, these experiments need to be performed in parallel with wild type cells.

2.3.3.7 CD39

CD39 -/- knock out mice lack CD39 ectonucleotidase. If CD39 is relevantly cleaving extracellular ATP we expected stronger effects of extracellular ATP in CD39 -/- knock out mice. It is hard to predict whether this effect is more relevant at higher or lower concentrations of extracellular ATP.

Hypothesis

ATP at lower concentration leads to a more distinct dose dependent decrease of TNFα release in LPS stimulated Kupffer cells from CD39 -/- knock out mice than in wild type mice.

Setup

For this experiment (ch09-022) only one mouse was used, instead of normally four. Conditions were only in duplicate samples. Kupffer cells from CD39 -/- mice were stimulated with 0, 5, 25, 100 μM ATP and with 25 ng/ml LPS.

Results

In CD39 -/- Kupffer cells, ATP decreased TNFα in a dose dependent manner up to 25 μM ATP. 100 μM ATP did not further decrease TNFα. From 810 (67) pg/ml without ATP, TNFα decreased 28, 50, and 49% with 5, 25, and 100 μM ATP respectively, $p < 0.05$ (figure 12).

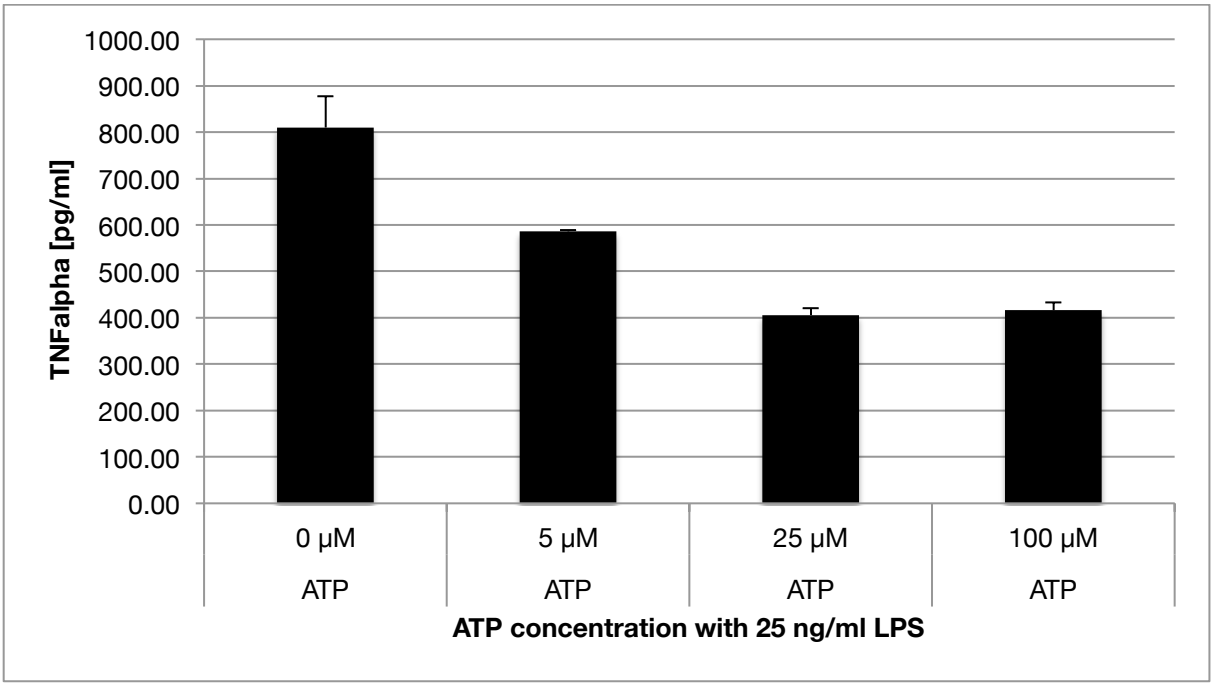

Figure 12: ch09-022_2 TNFα release by LPS-stimulated Kupffer cells from CD39-/- knock out mice, modulated by extracellular ATP.

Conclusion

CD39 ectonucleidase is probably not relevant in purinergic signaling of Kupffer cells.

A 50% decrease of TNFα after stimulation with 25 µM ATP is equal to wild type cells. Therefore CD39 probably does not relevantly diminish extracellular ATP concentration in vitro regarding the effect of ATP on TNFα release. However, in order to exclude a minor effect, these experiments need to be performed in parallel with wild type cells.

2.3.4 Interleukin-6

In one series of experiments (ch08-003) we carried out ELISA assays for Interleukin-6 (IL-6). In these experiments Kupffer cells were stimulated with 0, 25, 100, 400 µM ATP both without LPS and with 25 ng/ml LPS, and with 0, 5, 25, 100 µM UTP. All conditions were in triplicate samples. The ELISA assay for IL-6 showed no significant effect of extracellular ATP or UTP.

2.3.5 Experiments with technical problems

For the sake of completeness two experiments which were performed, but showed obvious technical problems are mentioned here.

In the very first experiment (ch08-001) Kupffer cells were stimulated with 0, 25, and 100 µM ATP, and with 25 µM UTP and all these conditions were stimulated with 25, 100, and 400 ng/ml LPS. Because of a mistake in the ELISA development (none of the necessary Hydrogen peroxide was added to the ABTS Liquid substrate) no results could be generated. All conditions were reproduced in later experiments described above.

In another experiment (ch09-022) Kupffer cells from two wild type mice were stimulated with 0, 5, 25, 100 µM of ATP, UTP, and ADP respectively, and with 25 ng/ml LPS. This experiment was performed together with the two knock out experiments described above. In this experiment no detectable TNFα concentrations

could be measured in the ELISA assay, probably because the amount of cells isolated from the two mice was not large enough.

2.4 Summarized results

Secretion of TNFα by LPS-stimulated Kupffer cells in response to different nucleotides, such as ATP (figure 2), ATPyS (figure 3), BzATP (figure 7), was measured in supernatant using ELISA. Our experiments show a decrease of TNFα in response to stimulation with nucleotides.

In summary, the figures show that extracellular LPS stimulate release of TNFα in murine Kupffer cells and that extracellular nucleotides inhibit this effect in a dose dependent matter.

In the tested range, i.e. from 25 to 400 ng/ml extracellular LPS, the effect of LPS was not dose-dependent; higher LPS concentrations did not lead to higher TNFα release (no figure shown). The effects of extracellular nucleotides were dose dependent; higher nucleotide concentrations were associated with a stronger decrease of TNFα release and even abolished the effect of LPS.

In figure 3 we have shown that the effects of ATP and ATPyS only differ at higher concentrations. In figure 8 we have shown that the effect of BzATP is very similar to the effect of ATP. The effects of UTP and ADP at the same concentrations are lower compared to ATP, as described and shown in figures 5 -7.

We could consistently and significantly reproduce both the effect of LPS resulting in higher, and the effect of nucleotides in lower TNFα concentrations in the supernatants of LPS-stimulated primary murine Kupffer cells. Especially ATP, ATPγS and BzATP showed very conclusive results. UTP (Figures 5-7) and ADP instead only showed tendencies towards a similar effect, but less effectively and less significantly.

Especially we could show the same effects with the non-hydrolysable ATPγS (figure 3) and the P2-receptor agonist Bz-ATP (figure 8), while an antagonizing effect of Suramin has not clearly been shown (figure 9 and 10).

3 B) ATP release of human platelets after stimulation and co-stimulation with LPS

3.1 Introduction (idea & goal)

As I described in the Background section, the goal of this series of experiments was, to reproduce a nucleotide release by platelets/thrombocytes upon (co-) stimulation with extracellular LPS. The idea then was, to combine this effect, with the first series of experiments: We planned to use the ATP released by stimulated platelets, to modulate TNFα release of stimulated Kupffer cells.

3.2 Material & methods

To isolate human platelets we tried two different methods, modified from various methods we found in the actual literature. The two main principles applied, starting from human full blood were the followings:

On one hand we used a two-step serial centrifugation with different speeds and times to first get platelet-rich blood plasma, of which the platelets are then pelleted (Weissmüller, 2008). This concept was applied in variations using different buffers.

On the other hand we used a Sepharose based approach in which we filtered platelet-rich plasma using a Sepharose gel (Alberio, 2000).

We measured ATP/ADP release with an ATP Luminescence assay kit (Perkin Elmer, ATPlite, No. 6016943), which we used in a modified manner.

3.3 Experiments with technical problems – results

Even if we were using the same or only slightly varied protocols as used and published by other groups before, we were not able to reproduce consistent results regarding the nucleotide release of platelets. Neither was it possible to reproduce their results, nor to generate new effects.

Generally we observed both very large variations within duplicates/triplicates of the same conditions in a single experiment (i.e. huge standard deviations), and different tendencies between multiple versions of the same experiments, too.

Unfortunately we were not able to resolve our technical problems.

The most (even if not very) consistent result was the unsurprising effect of collagen and thrombin on platelets. In these conditions we normally got an increased signal in our ATP assay. We could not show a consistent effect of LPS in a co-stimulation together with collagen or thrombin as others have showed it. This means we had neither a consistent positive nor negative effect nor a consistent missing effect of LPS on the ATP release of platelets.

The expected increase of ATP-release by platelets after stimulation with LPS could not be produced. Other unexpected different effects of LPS could not be observed as well.

Therefore these experiments are considered as unsuccessful and no data or graphs are shown and materials and methods are not described in more detail. Even though, multiple data exist showing ATP release by platelets. We therefore think it was a technical problem that leaded to the unsuccessfulness of the planned experiments.

3.4 Conclusion/discussion

It is difficult to tell why the experiments didn't work. Obviously platelets seem to be very fragile cells to isolate, with different isolation protocols in the literature for almost every publication. Even minor changes in the conditions, such as the temperature, or shear forces (maybe the ones when aspirated through the needle already enough) may activate the platelets and thereby falsify the results of the following experiments.

The kinetics of platelet activation and nucleotide release seems to be very fast. Therefore it may be quite difficult to measure precisely and reliably, and only possible with adequate equipment used by other groups. Aggrego-luminometers for example can measure clot formation and nucleotide release of platelets simultaneously and in real-time, but we did not dispose of one.

A more intensive search for a solution of the difficulties noted above with optimized technical infrastructure and protocols was unfortunately not within the range of our temporal and financial capacities. Further research in the future seems reasonable and promising, anyway, because others could already generate promising results.

4 C) Effect of extracellular Nucleotides on phagocytosis of fluorescein labeled inactivated E. coli bio particles by RAW 264.7 and U-937 cell culture macrophages.

4.1 Introduction (idea & goal)

As noted in the background-section the goal of these experiments was, to reproduce experiments performed by other groups showing that extracellular nucleotides increase the phagocytosis of macrophages, with our cell types. Various authors (Marques-da-Silva, 2011) (Elliott, 2009) could demonstrate an effect of extracellular macrophages on different aspects of the behavior of macrophages, such as phagocytosis and migration.

After having consistently reproduced this effect, the next step would be to go for the search of the responsible receptor(s) and signaling pathway(s).

This series of experiments consists of two experimental setups with different measurements. One was based on using a plate reader, which measures the fluorescence of cell culture wells. In these experiments, we used RAW 264.7 cell (described below). The other setup was based on flow cytometry measurement. For these experiments we mostly used U-937 cells (described below).

4.2 Material & methods

The following protocols are adapted from Anita Born and René Fahrner who did similar experiments in our laboratory, and from other authors cited above (Marques-da-Silva, 2011).

Because the different cell lines used needed different handling, I am separating this section in two blocks, one for each cell line.

4.2.1 RAW 264.7

The cell line RAW 264.7 (ATCC® Number TIB-71™) (Raschke, 1978) is derived from ascites monocytes/macrophages of mice with Abelson murine leukemia virus-induced tumor (LGC Standards - ATCC). The cells were handled as described by ATCC (LGC Standards - ATCC).

RAW 264.7 cells we used mainly for the phagocytosis assay with the plate reader (experiments No. ch11-001-006; 008-010), and once in an experiment using flow cytometry (ch11-007).

4.2.1.1 Buffers, materials and reagents

Bio Particles

For phagocytosis we used killed fluorescently labeled Escherichia coli (K-12 strain) BioParticles® (bio particles, BP) with approximate Absorption/Emission maxima of 494/518 nm (Molecular Probes/invitrogen # E-2861) that were prepared as described by the manufacturer.

Media

DMEM: Dulbecco's Modified Eagle Medium (DMEM), high glucose, GlutaMAX-I, pyruvate (GIBCO/Invitrogen # 31966); FBS: Fetal Bovine Serum (Heat Inactivated)

Origin: EU Approved (South American) (GIBCO/invitrogen #10500); PBS: Dulbecco's Phosphate-Buffered Saline; DPBS, no calcium, no magnesium; (GIBCO/invitrogen #14190); (P/S) Penicillin/Streptomycin (GIBCO/invitrogen #15140122).

The culture media was used with or without – depending on the specific protocol – 10% FBS, and with 1% P/S.

Nucleotides

ATP (Adenosine 5'-triphosphate disodium salt hydrate): Sigma Aldrich #A26209 for the experiments with the plate reader and #A2383 for experiments with flow cytometry; ADP (Adenosine 5'-diphosphate): Sigma Aldrich #A2754; ATPγS (Adenosin 5'-(gamma-thio)-triphospate): Jena Bio Science #NU-406; BzATP (2'(3')-*O*-(4-Benzoylbenzoyl)adenosine-5'-triphosphate): Tocris Bioscience #3312; UTP (Uridine 5′-triphosphate): Sigma Aldrich #U6750

Other materials and instruments

In addition we used the following products: Trypan Blue: 80mg in 40ml PBS for 0.2% w/v (Sigma #T6146); Trypsin-EDTA (GIBCO/invitrogen #15400-054), tissue cell culture plates of 96 wells (Sarstedt Cell+ # 83.1835.300) and tissue culture plates of 24 wells (Sarstedt # 1 83.1836) and 12 wells (nunc #150628); cell scraper (Sarstedt #83.1830); FACS-tube: 5ml round bottom Polystyrene tube, (Sarstedt #55.476.013 PS).

Standard plate reading measurements were done with Infinite® M200 (Tecan).

4.2.1.2 Preparation of the cells

The entire handling of the cells was done in sterile conditions under a laboratory hood. All incubation and treatment steps were at 37°C and 5% CO2.

The RAW 264.7 cells were plated in a sterile 96-well plate in a concentration of 100'000 cells/50µL in each well and incubated over night. According to the specific protocol the incubation was with or without FBS.

For the experiment with flow cytometry, larger wells and amounts of cells were used.

4.2.1.3 Basic stimulation protocol

The next day we pre-treated the cells with either nucleotides (dissolved in DMEM media) or control media (DMEM) in an additional 50µL/well. After the pre-treatment we added E. coli bio particles dissolved in 100µL DMEM in a concentration of approximately $10x10^6$ particles/well (for 100 BP/cell, and adapted for specific protocols) and incubated again for different times.

To end the stimulation/phagocytosis, and to quench the fluorescein on the remaining extracellular bio particles, the supernatant was removed and 100µL Trypan Blue 0.2% (in distilled water) was added for 2 minutes. The wells were then washed three times with Phosphate buffered saline (PBS), or until the wells were clear.

As a visual control the cells with phagocytized bio particles could be viewed in a fluorescence microscope where it was clearly visible, that the fluorescent bio particles were not homogenously distributed in the wells, but only visible in small, roundish-shaped areas, resembling the forms of cells (no photo documentation).

For the experiment with flow cytometry, to the RAW 264.7 cells 500µL Trypsin/EDTA were added for 5 minutes at room temperature to release the cells from the wells. The

cells were then scraped and washed off the plate with PBS, transferred in a FACS tube, washed once and resuspended in PBS for flow cytometry.

4.2.1.4 Measurement of phagocytosis by fluorescence, using a plate reader

For a quantitative measurement of the engulfed particles we used the plate reader Tecan infinite M200 at 488/522nm (Excitation=Absorbance/Emission).

All the results are in arbitrary light units.

4.2.1.5 Statistical analysis and graphs

All calculations and statistical analysis were made with Microsoft Excel. To estimate statistical significance of the results, a two-sided student's t-test, type 2, was performed without previously analyzing values for their statistically normal distribution. The result we regarded as a p-value. P-values below 0.05 were considered significant.

The error bars in the graphs represent the standard deviations from the tetraplicates/triplicates/duplicates of every sample.

4.2.2 U-937

The cell line U-937 (ATCC® Number CRL-1593.2™) derives from human histiocytic lymphoma monocytes (Sundström, 1976). It was handled as described by ATCC (LGC Standards - ATCC) and used for experiments with flow cytometry (experiments No. ch11-007; 011-018).

4.2.2.1 Buffers, materials and reagents

Bio particles, Nucleotides, and other reagents for U-937 cells were identical with the ones for RAW 264.7 cells.

The media used was RPMI: Roswell Park Memorial Institute 1640 medium; RPMI-1640, GlutaMAX™, (GIBCO/Invitrogen #61870).

The culture media was used with or without – depending on the specific protocol – 10% FBS, and with 1% P/S.

SORP LSRII™ (BD Bioscience) was used for flow cytometry measurements.

4.2.2.2 Preparation of the cells

The entire handling of the cells was done in sterile conditions under a laboratory hood. All incubation and treatment steps were at 37°C and 5% CO2.

The U-937 cells were plated in a sterile 96-well plate in a concentration of 100'000 cells/100µL and incubated over night. According to the specific protocol the incubation was with or without FBS.

4.2.2.3 Basic stimulation protocol

The next day we pre-treated the cells with either nucleotides (dissolved in RPMI) or control media for 30 min. Then we stimulated them with bio particles in concentrations according to the specific protocol for 2 hours (other times according to specific protocols).

To end the stimulation/phagocytosis, cells were put on ice (Marques-da-Silva, 2011).

The U-937 cells were transferred into FACS tubes and centrifuged (2000rpm ≅ 800g for 7 minutes at 4°C).

To quench the fluorescein on the remaining extracellular bio particles, 500µL Trypan blue 0.2% were added for 2 minutes. The tubes respectively, were then washed three times, or until the wells were clear with Phosphate buffered saline (PBS). The non-adherent U-937 cells needed to be centrifuged (800g for 7 min) in every washing step.

The U-937 cells were directly resuspended in PBS for flow cytometry after the last washing step.

4.2.2.4 Measurement of phagocytosis by fluorescence, using flow cytometry

Flow cytometry was performed in the SORP LSRII machine with the Absorption/Emission properties set on the ones for Fluorescein isothiocyanate (FITC). As a positive control in the first experiments we used highly diluted bio particles (1 to 1000 times diluted compared to the concentration during the stimulation of the cells).

4.2.2.5 Statistical analysis and graphs

We determined mean fluorescence of FITC positive cells. FITC positive cells were defined as cells with higher fluorescence activity than approximately 95% of the cells in the negative control.

The mean values from the experiments and dotplot graphs were generated with FlowJo software and prepared for Microsoft Excel. Statistical analyses were performed with Microsoft Excel equally to the experiments with the plate reader. Dotplot graphs did not show any visual differences and are therefore not added to this work.

4.3 Experimental design

Macrophages were pre-stimulated with different concentrations of various nucleotides (ATP, ADP, ATPγS) for 30 minutes or the nucleotides were added simultaneously with the bio particles. Then bio particles were added in different concentrations for different durations. After the stimulation period, the cells were put on ice to stop the phagocytosis, and the fluorescence of the remained extracellular bio particles was quenched with trypan blue. The cells were then washed and prepared for the particular measuring method used, as previously described.

The amount of phagocytosis by the macrophages was then determined by the fluorescence in the well in the plate reader, or by the mean fluorescence of the cells within the population of viable macrophages in flow cytometry.

4.4 RAW 264.7 – results of individual experiments

4.4.1 RAW 264.7 macrophages phagocyte fluorescence marked bio particles independently from serum starvation

Hypothesis

Macrophages phagocyte bio particles, which can be detected by an increase of fluorescence in the plate reader. We expected serum-starved cells to phagocyte more bio particles, because they may be more "hungry" after serum starving.

Setup

In one experiment (ch11-001) cells were incubated over night (16 hours) as described above either with or without FBS in the Medium. The next day they were stimulated with or without 100 bio particles/cell for two hours. After quenching, washing, and fluorescence measuring was performed as previously described. Conditions were in triplicates.

Results

We could observe an increase of fluorescence of more than 100% in both conditions, $p < 0.0001$. No difference was observed between serum-starved and not-serum-starved cells for both, absolute values and the relative change of fluorescence (no figure shown).

Conclusion

We could confirm that RAW 264.7 macrophages phagocytize bio particles. Serum starving, however, had no effect on the phagocytic properties of the cells in this setup.

Additionally, we observed relatively high fluorescence from the plate itself – approximately 50% of the maximum signal. Taking this into account there was no baseline fluorescence from the cells themselves.

This plated dependent fluorescence could not be eliminated completely, despite using different cell culture plates.

4.4.2 RAW 264.7 phagocyte bio particles in a dose and time dependent manner, and may be saturated with 1000 bio particles per cell

Initially we planned to do further experiments with saturated macrophages. Therefore we searched for the saturating concentration of bio particles. We expected, that the phagocytic properties of macrophages have certain limits, i.e. we expected that the phagocytosis of macrophages could be saturated by a sufficiently high concentration of bio particles, and by a sufficiently long stimulation time.

Hypothesis

The phagocytosis of macrophages with bio particles increases with concentration of bio particles and stimulation time, but reaches a plateau of fluorescence at a certain bio particle concentration and stimulation time. This plateau shows the phagocytic saturation of macrophages.

Setup

Three experiments were performed to examine this question.

Once (ch11-002) macrophages were stimulated with 100 BP/cell for 30, 60, 90, 120, 150, and 180 min (triplicate samples, figure 14), and with 10 and 1000 BP/cell for 30 and 120 min (not shown in the figure).

Once (ch11-004) macrophages were stimulated for 2.5 hours with 0, 100, 200, 400, 600, 800, and 1000 BP/cell (duplicate samples, figure 13).

Once (ch11-003) macrophages were stimulated with 100 and with 1000 BP/cell for 30, 60, 90, 120, 150, and 180 min (triplicate samples, but duplicate samples for 150 and 180 min).

Results

Experiments showed significant ($p < 0.05$) and clearly relevant increase of fluorescence (i.e. phagocytosis) after stimulation with increasing bio particle concentration. The increase was almost linear and did not show any plateau (figure 13).

After stimulation with 100 BP/cell, a time dependent increase of fluorescence could be detected, which was for all conditions significant ($p < 0.05$) compared to the samples with 30 min stimulation time, and significant for most conditions, compared to their respective neighbors (figures 14 and 15).

After stimulation with 1000 BP/cell, no significant time dependence of phagocytosis could be shown, as the standard deviations became larger (figure 15).

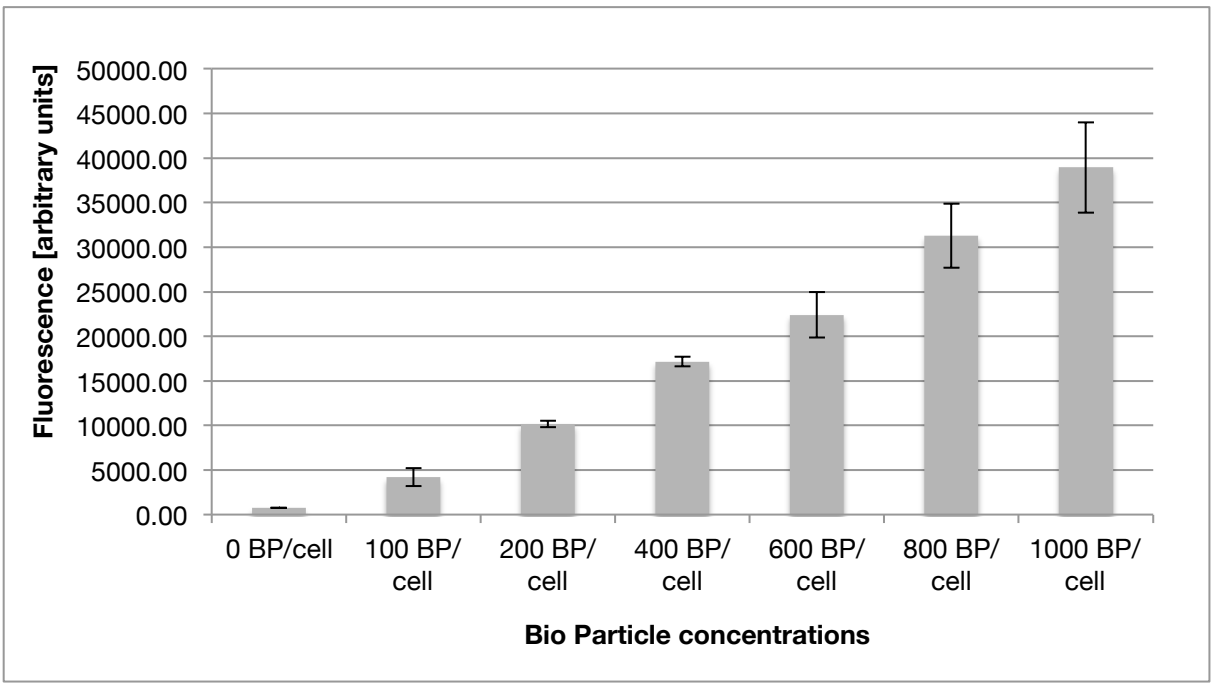

Figure 13: ch11-004_2 Detected fluorescence by plate reader after stimulation for 2.5 hours with different BP concentrations.

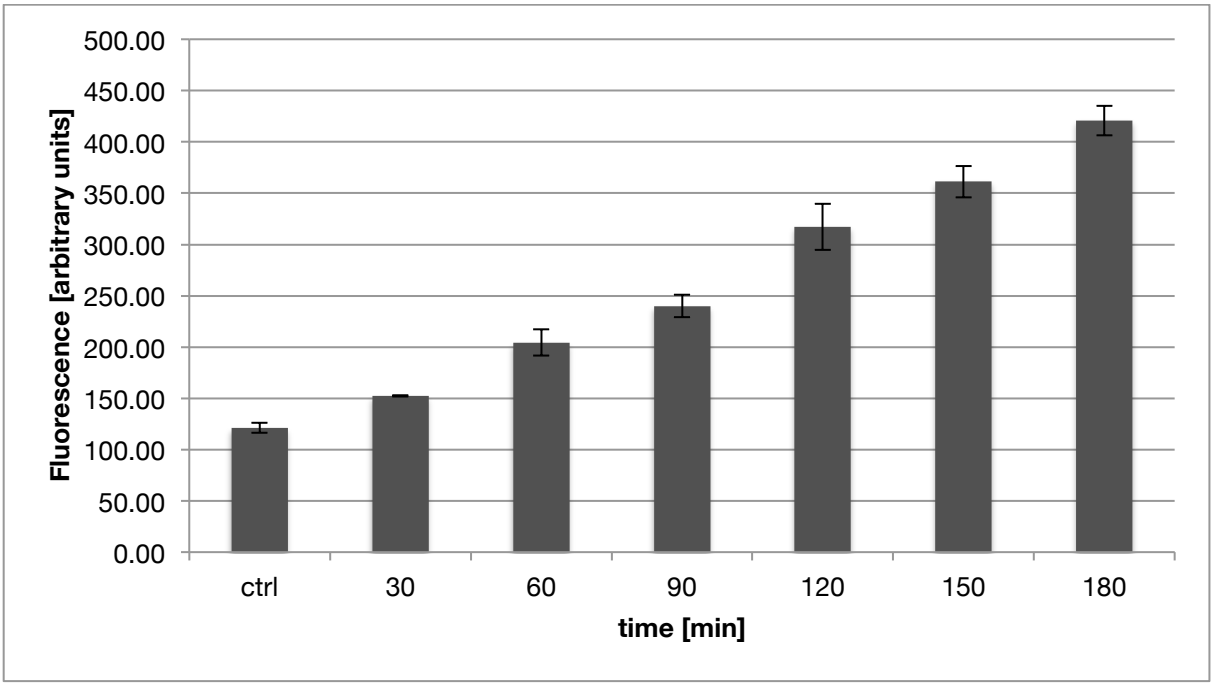

Figure 14: ch11-002_1 Detected fluorescence by plate reader after stimulation with 100 BP/cell for 30 to 180 min.

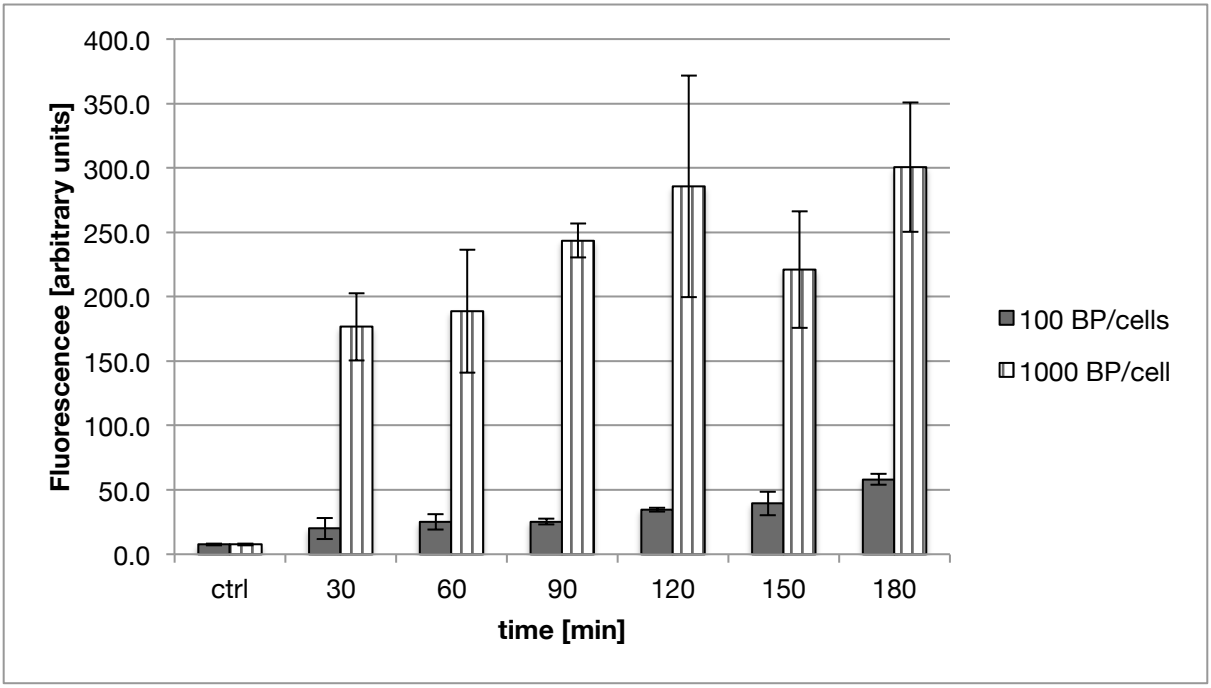

Figure 15: ch11-003_1 Detected fluorescence by plate reader after different BP concentrations and stimulation times.

Conclusion

A saturation concentration of extracellular bio particles was searched with the intention to perform further experiments using saturated macrophages. The goal was to examine whether saturated macrophages can be stimulated to increase their phagocytosis, or to lower their saturation dose, respectively. A saturation concentration would be visible as a plateau effect of the fluorescence level beginning at the saturation dose.

When cells were stimulated with 100 BP/cell, these experiments show no evidence of a time dependent plateau, i.e. of a saturation of the phagocytosis after 3 hours of stimulation with bio particles.

When cells were stimulated for 2.5 hours with increasing bio particle concentration, the results show no evidence of a dose dependent plateau yet.

There may be a sign for a plateau effect after 2 hours of phagocytosis with 1000 BP/cell (figure 15). But because of the very wide standard deviations, this observation needs to be interpreted carefully. For a certain conclusion of this effect, the same experiment, additionally with even longer stimulation times should be repetitively performed to achieve smaller standard deviations.

After these experiments, we decided, also for economic reasons, not to search further for a phagocytic saturation of macrophages, and therefore not to use saturated cells for the future experiments.

4.4.3 ATP has no effect on phagocytosis of RAW 264.7 macrophages

In the first series of preliminary experiments with primary Kupffer cells we could show, that extracellular ATP and other nucleotides diminish cytokine secretion. As explicated in the background chapter, Elliott (Elliott, 2009) could demonstrate a more efficient recruitment of macrophages after pre-treatment with ATP and other nucleotides. Marques-da-Silva (Marques-da-Silva, 2011) could increase phagocytosis of latex beads by macrophages with ATP and other nucleotides. We aimed to reproduce these results in our experimental setting, using RAW 264.7 cell culture macrophages.

Hypothesis

Extracellular ATP increases phagocytosis of fluorescence labeled bio particles by RAW 264.7 cell cultured macrophages. The increased phagocytosis can be measured using a fluorescence plate reader.

Setup

The above hypothesis was tested in three experiments (ch11-004/005/008). Cells were treated or pre-treated with different concentrations of ATP and stimulated with different concentrations of bio particles, before fluorescence was measured. In detail:

Once (ch11-004), cells were pre-treated for 30 min with ATP 0.125 µM, 0.25 µM, 1.25 µM, 2.5 µM, 3.75 µM, 5 µM, 6.25 µM, 12.5 µM, before the stimulation with 100 and 200 BP/cell for 2.5 hours (conditions in triplicates, figure 16).

Once (ch11-005), cells were pre-treated for 30 min with ATP 0 µM, 0.125 µM, 0.25 µM, 1.25 µM, 2.5 µM, 3.75 µM, 5 µM, 6.25 µM, 12.5 µM, 25 µM, 50 µM, and 250 µM before the stimulation with 100 BP/cell for 2.5 hours. Additionally cells were treated with ATP 0.25 µM, 2.5 µM, and 25 µM ATP without stimulating with bio particles afterwards, to exclude a fluorescing effect of ATP itself (samples in quadruplicates, no figure shown).

Once (ch11-008), ATP 0, 100, 500 and 1000 µM was added immediately prior to the 2 hours stimulation with 100 BP/cell (samples in quadruplicates, figure 17).

Results

None of the experiments showed consistent and/or significant effect of extracellular ATP on phagocytosis of bio particles, see figures 16 and 17.

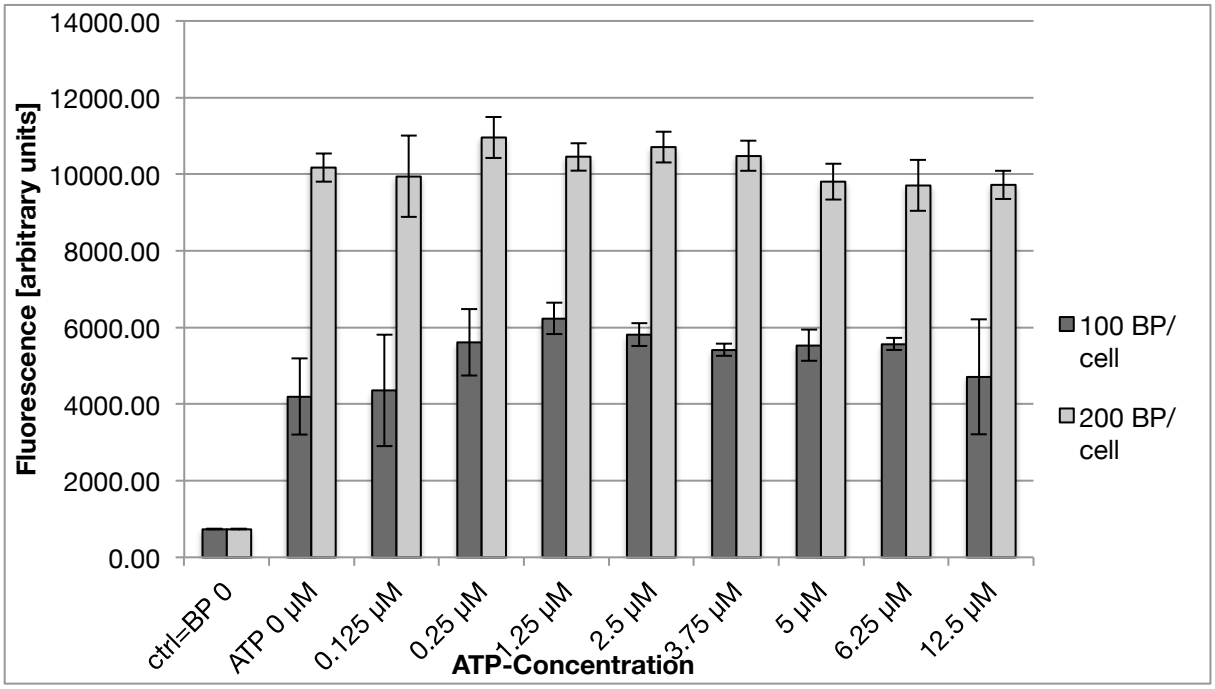

Figure 16: ch11-004_1 Detected fluorescence by plate reader after pre-stimulation with different extracellular ATP concentrations and 100 and 200 BP/cell.

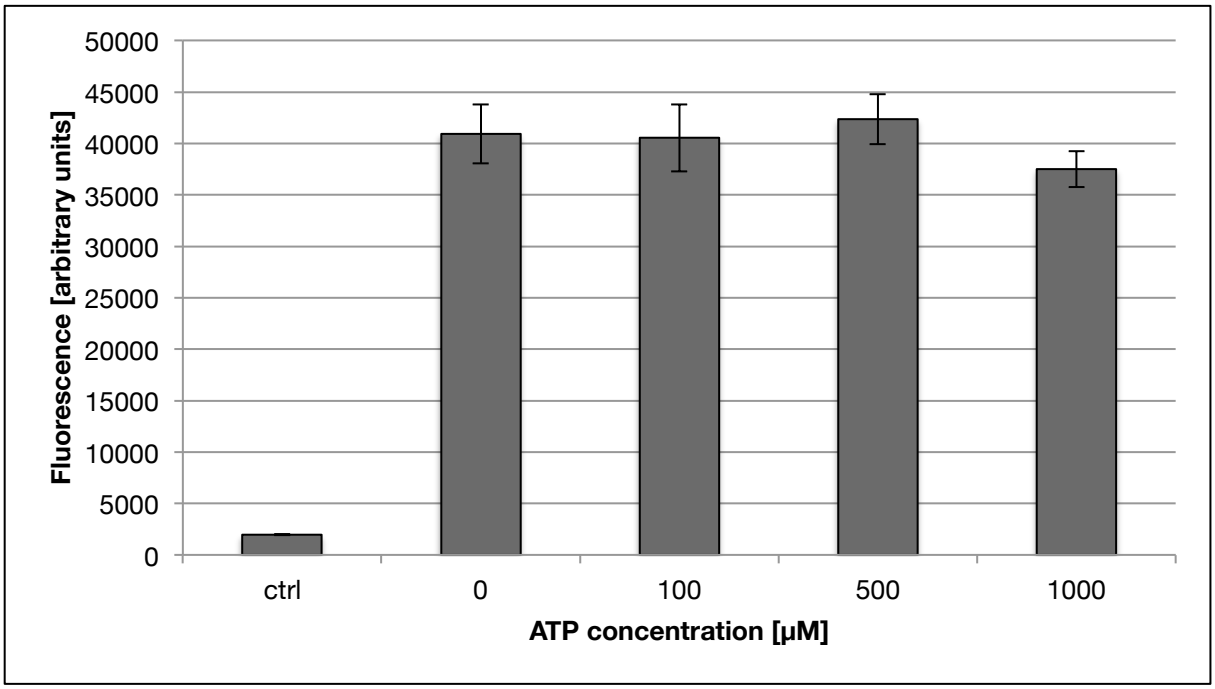

Figure 17: ch11-008 Fluorescence after stimulation with different concentrations of extracellular ATP and 100 BP/cell.

Conclusion

We could exclude ATP itself as a possible biasing factor on our assay by showing that ATP itself had no effect on the fluorescence signals (no figure shown).

Again we could not reproduce the effect of ATP on phagocytosis, which others (Marques-da-Silva, 2011), (Elliott, 2009) published before. Extracellular ATP had no effect even at higher concentrations on phagocytosis in our settings.

Either the used RAW 264.7 cells are not sensible to extracellular ATP concerning their phagocytic properties, or small variations in phagocytosis that are generated cannot be detected in our settings.

In one experiment reveal some increased phagocytosis in response to ATP-treatment at even lower concentrations may be of interest for further investigations.

Instead of performing the same experiment again and again with different slight variations, searching for an effect, we decided to change the detecting methods in favor of flow cytometry, a more precise instrument to detect smaller variations.

4.5 U-937 – results of individual experiments

4.5.1 Flow cytometry

Using flow cytometry we can measure the same fluorescence labeled bio particles used for the previous experiments. In contrast to a plate reader, which measures the fluorescence of all the cells in a cell culture well together, flow cytometry will measure the fluorescence of thousands of single cells individually. Additionally it is possible to measure other properties of the cells.

This technique gives the possibility to select only the viable populations of the cells from the experiment and to calculate a mean value of the fluorescence of many single cells. Additionally the graphs generated by flow cytometry can show more detailed information on the distribution of phagocytosis within a population of cells.

Briefly, flow cytometry can potentially generate more detailed and precise information about the behavior of a population of phagocytizing cells concerning their “nutritional behavior”. We hoped to discover an effect of extracellular nucleotides on phagocytosis of macrophages by this more sophisticated method.

4.5.2 Preliminary experiment – RAW 264.7 and U-937 cells phagocytize bio particles, detectable by flow cytometry

We used a first preliminary experiment (ch11-007) to establish the method for our cells. Materials and methods are described above.

Setup

For both cell lines RAW 264.7 and U-937, we ran single conditions with and without bio particles, with and without serum starving over night, with and without different concentration of extracellular ATP as pre-stimulation.

Results/conclusion

We could show, that both cell lines, RAW 264.7 and U-937, phagocytize the used bio particles. An effect of the pre-stimulations with ATP could not be shown. The not-serum-starved cells, which were incubated over night with FBS in the culture media, showed lesser standard deviations (no figures shown).

Because the soluble U-937 cells, compared to RAW 264.7 cells which attach to cell culture plates and flow cytometry tubes, are much simpler and faster in handling for flow cytometry and phagocytize bio particles as well, we decided to use this cell line for the future experiments. Also we were not serum starving the cells any more.

4.5.3 U-937 cells phagocytize bio particles in a dose dependent manner, detectable by flow cytometry

Hypothesis

The results from the phagocytosis assay with RAW 264.7 cells can be verified with U-937 cells by flow cytometry. Therefore, U-937 cells will phagocytize bio particles in a dose dependent manner.

Setup

U-937 cells were stimulated for two hours with different number of bio particles reaching from 0 to 100 bio particles per cell (0, 5, 10, 20, 50, 75, 100 BP/cell).

Phagocytosis was measured with flow cytometry according to the previously described protocol.

Results

Analysis of the mean fluorescence values of the fluorescein isothiocyanate positive cells (abbreviated with FITC, which is frequently used in flow cytometry) showed an increasing value of fluorescence with increasing concentrations of BioParticles.

The mean (FITC) values increased in a dose dependent manner (figure 18).

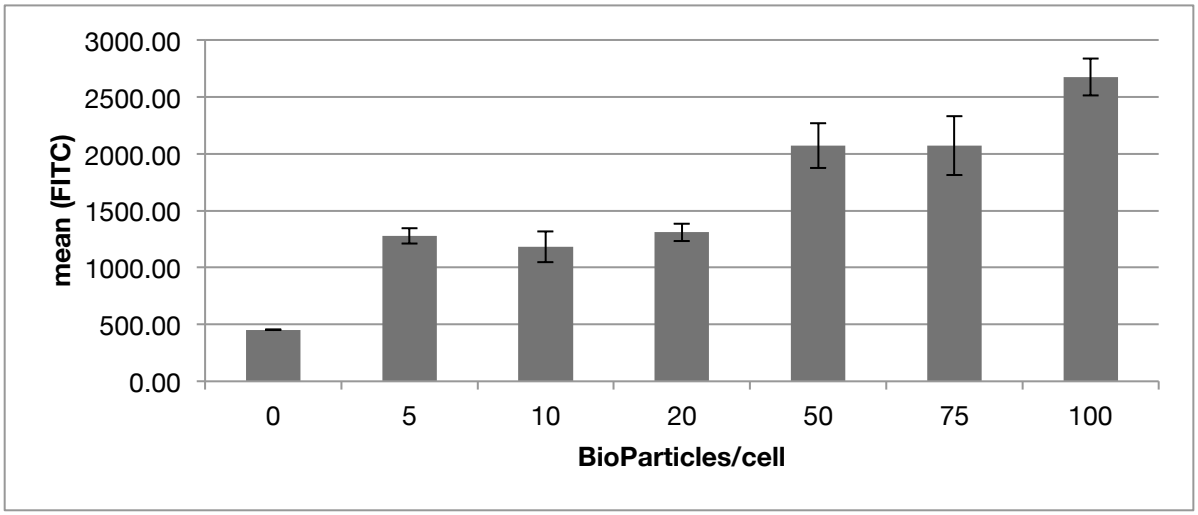

Figure 18: ch11-011_1 Mean Fluorescence [arbitrary units] after 2 hours stimulation with different amounts of BP/cell

Conclusions

Dose dependent increase phagocytosis of bio particles can be shown in flow cytometry. The result confirms our findings in the phagocytosis assay.

For the further experiments we decided to use lower amounts of bio particles. Thereby we thought to facilitate the detection of minor changes in phagocytosis due to extracellular nucleotides. With higher bio particle concentrations they may be overridden by the high amount of bio particles.

4.5.4 U-937 cells do not phagocytize bio particles in a time dependent manner

To reproduce another effect shown in the previous experiments with flow cytometry, we tested the time dependency of phagocytosis by U-937 cells.

Hypothesis

U-937 cells phagocytize bio particles in a time dependent manner.

Setup

Cells were stimulated with 10 BP/cell for 15, 30, 60, 120 min. (ch11-014, conditions in duplicates)

Results

Mean fluorescence was not affected by different stimulation times, even though standard deviations were only 1-2% of the fluorescence values (no figure shown).

Conclusion

Unlike in the previous results we gained with RAW 264.7 cells, the phagocytosis of U-937 cells within the tested settings was not time dependent.

Instead of further searching of a time dependency with other conditions, we decided to continue with experiments to examine the effect of extracellular ATP on phagocytosis.

4.5.5 Extracellular ATP does not alter phagocytosis of U-937 cells

Hypothesis

Extracellular ATP increases phagocytosis of U-937 cells.

Setup

Altogether we performed five experiments with extracellular ATP.

Cells were pre-treated for 30 min with 0, 0.01, 0.1, 1, 10, 100 µM ATP, then stimulated for two hours with 50 BP/cell (ch11-011, triplicate samples), or with 2.5, 5, and 10 BP/cell (ch11-012, duplicate samples). In another experiments cells were pre-treated for 30 min with 10, 100, 400 µM ATP, then stimulated with 10 BP/cell for two hours (ch11-013, triplicate samples).

In two other experiments, cells were pre-treated for 30 min with ATP 50 µM (only ch11-016), 100, and 300 µM (ch11-016 and 017) before a 30 min stimulation with both 5 and 10 BP/cell (ch11-016 in triplicate samples, ch11-017 in duplicate samples).

Results

A significant effect of ATP on phagocytosis was not detected (as example: figure 19, from the experiment ch11-012). Neither did the distribution of fluorescence within the population of cells change, as determined in the graphs from flow cytometry (no figures shown).

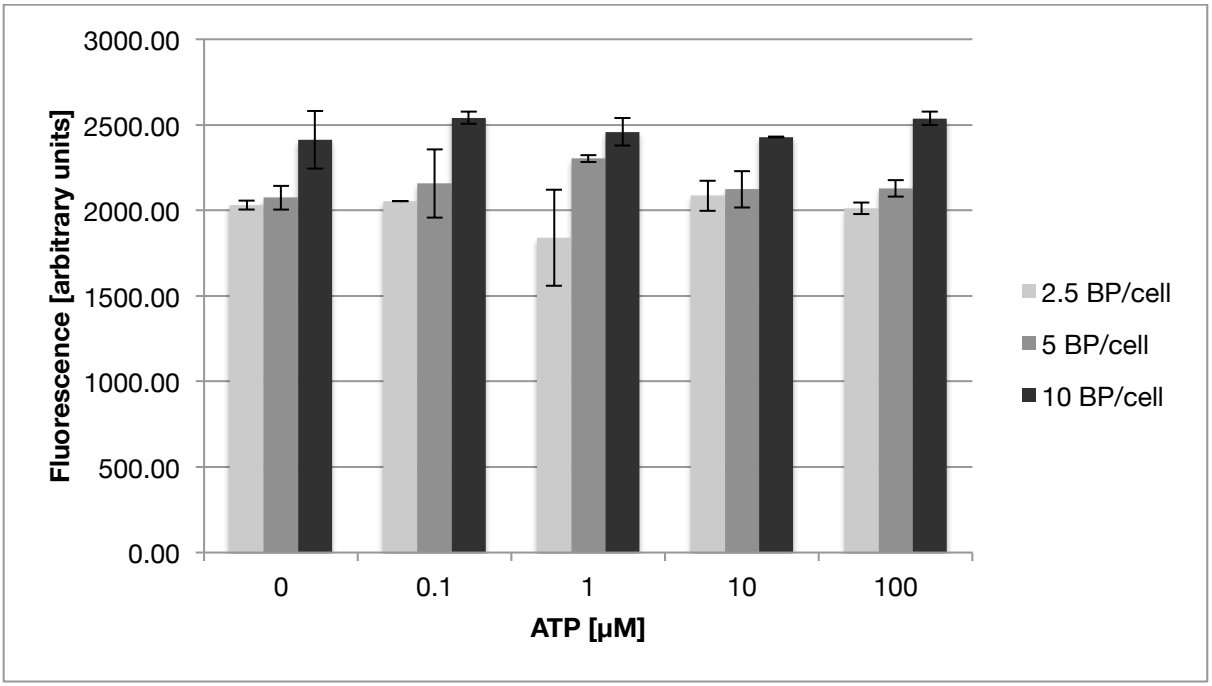

Figure 19: ch11-012_2 Mean Fluorescence of FITC positive cells after pre-stimulation for 30 min with different concentrations of extracellular ATP and stimulation for 2 hours with different concentrations of bio particles.

Conclusion

ATP has no effect on phagocytosis of bio particles by U-937 cells in the examined conditions. These results with human cell culture monocytes/macrophages are contradictory to the published results by Marques-da-Silva with murine primary macrophages (Marques-da-Silva, 2011).

The differences between the different cells used are a possible explanation of the different results.

4.5.6 Extracellular ATPγS does not alter phagocytosis of U-937 cells

Another reason why ATP has no effect on the used cells is, that ATP is hydrolyzed by ectonucleidases of U-937 cells. To exclude, that the hydrolysable properties of ATP is not responsible for false negative results, we performed the same experiments with ATPγS, too.

Hypothesis

Extracellular ATPγS increases phagocytosis of U-937 cells.

Setup

Altogether four experiments were performed answering this question.

In one experiment cells were pre-treated for 30 min with 10, 100, 400 µM ATPγS, then stimulated with 10 BP/cell for two hours (ch11-013, triplicate samples). In one experiment cells were pre-treated for 30 min with 0, 1, 10, 100 µM ATPyS and then stimulated with 10 BP/cell for 15, 30, 60, 120 min (ch11-014, conditions in duplicates).

In two other experiments, cells were pre-treated for 30 min with ATPγS 50 µM (only ch11-016), 100, and 300 µM (ch11-016 and 017) before 30 min stimulation with both 5 and 10 BP/cell. (ch11-016 in triplicate samples, ch11-017 in duplicate samples)

Results

No significant effect of ATPγS on phagocytosis could be shown (no figure shown).

Conclusion

Also with the non-hydrolysable ATPγS, no purinergic effect on phagocytosis could be demonstrated in our setting.

4.5.7 Apyrase, ADP, and UTP do not alter phagocytosis of U-937 cells

To further search for purinergic effects on phagocytosis of U-937 cells, we used two other nucleotides, i.e. ADP and UTP. Additionally we used Apyrase, which should degrade possible extracellular base level ATP and therefore lower its effect.

Hypothesis

Extracellular ADP and UTP increase phagocytosis of U-937 cells. Apyrase decreases phagocytosis of U-937 cells.

Setup

In two experiments (ch11-016 in triplicates, ch11-017 in duplicates) cells were pre-treated for 30 min with 100, 200, and 300 µM ADP and with 100 and 300 µM UTP and then stimulated for 30 min with 5 and 10 BP/cell.

In one experiment (ch11-013, in triplicates) cells were pre-treated with 0.01, 0.1, 1, 10 U/l Apyrase for 30 minutes and then stimulated with 10 BP/cell for 2 hours.

Results

Neither ADP, nor UTP, nor Apyrase could significantly and consistently alter phagocytosis of U-937 cells (no figure shown).

Conclusion

A purinergic effect with other nucleotides than ATP (i.e. ADP and UTP) also could not be shown. Additionally an effect of possible base level ATP (e.g. released by the cell culture itself) could not be abolished by hydrolysis using Apyrase.

4.6 Summarized results

Phagocytosis of bio particles by RAW 264.7 murine macrophages with different exposure concentrations of bio particles (figure 13), different exposure times (figures 14, 15), and in response to ATP (figures 16, 17) has been measured by a plate reader fluorescence assay.

Phagocytosis of bio particles by U-937 human monocytes/macrophages with different exposure concentrations of bio particles (figure 18) and in response to ATP (figure 19) and other nucleotides and apyrase (no figures) has been measured by flow cytometry.

4.6.1 Results from the experiments with RAW 264.7 cells using the plate reader phagocytosis assay.

Summarized, from the first series of experiments performed as described above with RAW 264.7 cells and a phagocytosis assay, measured in a fluorescence plate reader, we gained the following findings:

- Macrophages from the RAW 264.7 cell line phagocyte fluorescence labeled bio particles. The phagocytosis is not influenced by serum starvation.
- The amount of phagocytized bio particles is dependent on both exposure time and concentration of the exposed bio particles. A saturating effect (a plateau) could not be demonstrated with low concentration of bio particles.
- The phagocytosis of bio particles by RAW 264.7 macrophages cannot be influenced by extracellular ATP in an extend that could be measured by the fluorescence of entire wells.

4.6.2 Results from the experiments with U-937 cells using flow cytometry

In summary, from the second series of experiments performed as described above with U-937 cells phagocytizing FITC labeled bio particles, we gained the following findings, measured with flow cytometry:

- Monocytes/Macrophages from the U-937 cell line phagocyte fluorescence labeled bio particles.
- The amount of phagocytized bio particles is dependent on the bio particle concentration. An effect of exposure time could not be demonstrated, but would need further investigation for more precise statements.
- Extracellular ATP cannot significantly influence the phagocytosis of bio particles by U-937 monocytes/macrophages.

5 Discussion

5.1 Kupffer cell release of TNFα upon stimulation with LPS can be inhibited by extracellular nucleotides

We could reproduce, that LPS has a stimulating effect on Kupffer cells, leading to a cytokine (i.e. TNFα) release and other effects (Decker, 1990) (Lin, 2005).

Especially we could demonstrate, that extracellular nucleotides, in particular ATP, can lower and even abolish cytokine – i.e. TNFα – release of murine Kupffer cells, in response to bacterial LPS. Furthermore we could show, that the effect of ATP on TNFα-release is dependent on a stimulation of Kupffer cells, e.g. with LPS.

All nucleotides used, i.e. ATP, ATPγS, ADP, UTP, and BzATP, showed similar qualitative effects but were effective in different concentrations.

By the use of ATPγS we could show that the response to extracellular ATP is mediated via activation of P2 type receptors and not in response to dephosphorylated nucleosides such as AMP or Adenosine. We conclude, that the effect is, at least partly, mediated by one of the ATPγS dependent receptors P2X2, P2X5, P2Y1, P2Y11, P2Y12, or P2Y13 receptors.

Because of the shown effect of BzATP, we conclude, that the effects of ATP are, at least partly, mediated by one of the BzATP dependent receptors P2X1, P2X3, P2X7 or P2Y11 receptor. P2Y11 is therefore the known common receptor of both agonists.

Additionally, based on the results we achieved with UTP, effects of ATP are, at least partly, mediated by one of the UTP dependent receptors P2Y2, P2Y4, P2Y6, P2Y11. UTP is only an agonist for P2Y receptors, but not for P2X receptors. Therefore we propose that G-protein coupled signaling is a key mechanism of how extracellular nucleotides impact on TNF release.

Experiments with the P2 receptor antagonist Suramin, however, were inconclusive.

A potential common intersection of the receptors of ATPγS, BzATP, UTP, and Suramin is P2Y11 receptor. I therefore postulate, that P2Y11 is a relevant or even a key receptor for purinergic signaling in Kupffer cells.

All of the above experimental data and conclusions and the fact that three of the used nucleotides – ATP, ATPγS, and BzATP – showed very clear effects on TNFα-release by Kupffer cells motivates to keep searching for the receptors mediating this effect. P2Y11 would be my favorite next target.

However, by the performed experiments, involvement of other P2Y G-protein coupled receptors, or even of P2X ion-channel receptors, cannot definitely be excluded.

5.1.1 Comparison to other publications

Searching the literature, a clear and consistent effect of extracellular ATP on TNFα-release of macrophages is not yet established. Both increased and decreased TNFα-release (with and without stimulation with LPS) has been shown (Rayah, 2012) (Gabel, 2007). Haskò et al showed similar results as we do, i.e. a dose dependent decrease of TNFα in response to ATP and other nucleotides by murine peritoneal macrophages (Haskò, 2000). Lambert et al demonstrated a decrease in LPS-induced TNFα release in

human microglia and macrophages in response to ATP (Lambert, 2010). Additionally, in the same paper, they describe increased migration of microglia in response to extracellular ATP. On the other hand Hide et al describe an increase of TNFα release in response to ATP in rat microglia cell cultures (Hide, 2000).

Although, in contrast to our results, the results of Sakaki et al show an increase of cytokine release in response to ATP, Sakaki et al identify P2Y11 receptor to be relevant in the autocrine regulation of macrophage activation (Sakaki, 2013). In vitro, they demonstrated a P2Y11 dependent increase of IL-6 release by LPS-stimulated macrophages in response to ATP. Sakaki et al suppressed blood serum levels of TNFα, IL-6 and other cytokines using an antagonist of P2Y11 receptor in mice stimulated with intraperitoneal administration of LPS (Sakaki, 2013). These results are supporting our results with which we propose that P2Y11 mediated anti-inflammatory effects of extracellular ATP.

5.1.2 Methodical weaknesses of the study

Further discussion of this work shows also methodical weaknesses, which are to be addressed, when further experiments are performed:

Main deficits are on the one hand, that Kupffer cells were not counted and/or incubated at a standardized concentration of cells per well. Therefore no statements can be made neither about the concentration of nucleotides per cell, nor about the amount of TNFα released per cell with specific stimulations. Additionally, different experiments with same settings cannot be taken together to achieve higher significance in the statistical evaluation. On the other hand, only few (sometimes just one) experiments have been performed for each setting. Therefore this work has to be viewed as a series of interesting preliminary experiments, and no final conclusions can be drawn before every single experiment has been reproduced.

Furthermore, viability of the cells after the assay has not been tested consequently. Theoretically, the changes in TNFα release could be due to ATP and LPS induced cell death by necrosis or apoptosis via P2X7 receptor, as it has been described (Le Feuvre, 2002) (Di Virgilio, Cytolytic P2X purinoceptors, 1998). If this would be the case, we would have shown a dose dependent cell toxicity of ATP in co-stimulation with LPS. To exclude that, experiments needed to be reproduced and completed with a reliable cell viability assay or with pharmacologically or genetically antagonizing of P2X7. Since on rat Kupffer cells no P2X7 receptor has been shown (Xiang, 2006), this theory seems rather improbable.

5.1.3 Conclusion

However, we could show a decrease of TNFα in the supernatants of primary murine LPS-stimulated Kupffer cells after co-stimulation with nucleotides. As described above, we postulate a P2-receptor modulated pathway is responsible for this effect, possibly P2Y11. But further research needs to be done to explore the responsible receptors and intracellular pathways generating the observed effect.

Taking together our results and the mentioned publications, we conclude that extracellular nucleotides at least in the liver can possibly have an effect on the immune system, and that this effect – at least partly – is mediated by cytokine release of Kupffer cells.

A next step could be a systematic use of other more specific P2-receptor agonists and antagonists in different combinations to restrict the possible receptors for closer investigation. More sophisticated techniques, such as genetically knocked out cells for specific receptors, or others, could follow.

But as introduced in the first chapter, in this work we further searched for other effects of extracellular nucleotides, which modulate the immune system and are presented below.

5.2 Effect of extracellular Nucleotides on phagocytosis of fluorescein labeled inactivated E. coli bio particles by RAW 264.7 and U-937 cell culture macrophages.

We could demonstrate phagocytosis of fluorescence labeled bio particles by both macrophage cell lines RAW 264.7 and U-937. We also showed a dose dependency of phagocytosis. The amount of phagocytized bio particles is depending primarily on the concentration of bio particles available to the macrophages. A saturating concentration could not be determined.

Depending on the cell type, phagocytosis of bio particles could also be increased by longer exposure times of the cells. While RAW 264.7 cells showed a continuous increase of phagocytosis with time, this effect was not reproducible within the same exposure times in U-937 cells.

However, in all our settings, both with RAW 264.7 and U-937 cell lines, we repetitively could not reproduce an effect of extracellular nucleotides on phagocytosis of macrophages, as published by other authors (Marques-da-Silva, 2011) (Elliott, 2009). Our results with murine and human cell culture macrophages and monocytes/macrophages are contradictory to the ones of Marques-da-Silva with murine primary macrophages (Marques-da-Silva, 2011).

We therefore conclude, that extracellular nucleotides, especially ATP, have no effect on phagocytosis by RAW 264.7 and U-937 cells. Since, others have showed an effect of extracellular nucleotides on phagocytosis in different conditions, it remains to be explored, which factors influence the effect of ATP on phagocytosis.

5.2.1 Comparison to other publications

Different explanations are imaginable for the noted discrepancy, either looking for differences within the cells or in the methods used in the different experiments.

A possible explanation of the different results are differences between the different cells used, such as e.g. the difference between primary and cell culture macrophages, or between murine and human macrophages. Different macrophages may have different receptor expressions. Marques-da-Silva et al propose P2X1, P2X3, P2X7, and/or P2Y1 receptors to be responsible for their described effects (Marques-da-Silva, 2011). The cells we used may lack these receptors. Further genetical, immunological, or other analyses needed to be done to compare the different cell types for these receptors to determine whether the existence or lack of one of these receptors may be relevant to explain differences in the obtained results.

Theoretically otherwise, the changes in phagocytosis could be due to ATP and LPS induced cell death by necrosis or apoptosis via P2X7 receptor, as it has been described (Le Feuvre, 2002) (Di Virgilio, Cytolytic P2X purinoceptors, 1998). If some of

the macrophages underwent phagocytosis in response to ATP, surviving macrophages would have more bio particles per cell available for phagocytosis. This would explain higher phagocytosis per cell. This effect may not be relevant in the cell culture cells we used, that are derived from tumor cells. Tumor cells are known to have lesser tendency to undergo apoptosis. But until proven, this theory remains hypothetic.

Possibly also, in our experiments macrophages may be not sufficiently saturated with bio particles. Insufficiently saturated cells would phagocyte all available bio particles, independent of any co-stimulation with nucleotides. If this was the case, even if nucleotides had an effect, it could not be demonstrated, because of the insufficient saturation. The fact, that a saturation of phagocytosis could not be demonstrated, even using 1000 bio particles per cell, indicates very high capacities of phagocytosis and supports the theory of an insufficient saturation. Possibly, the affinity of macrophages is higher for bio particles than for latex beads. This would explain, why in our settings cells were insufficiently saturation, compared to the settings with latex beads used by other groups. Otherwise, the fact, that phagocytosis is time dependent in RAW 264.7 cells, is contradictory to this theory.

Additionally, it has been shown, that phagocytosis by macrophages is depending on size of the particles and on the opsonizing density, i.e. on the amount of Fc ligands (Pacheco, 2013). On the other hand, the same work showed, that the phagocytosis of larger particles was lesser, and could not be influenced by Fc density on their surface. Bio particles we used may be larger and therefore less phagocytized than latex beads used by other. Additionally phagocytosis of larger particles seems to be less prone to modulation than smaller particles. This could explain, why other groups could modulate phagocytosis of latex beads while for us it was not possible to modulate phagocytosis of bio particles.

5.2.2 Conclusion

However, various factors probably have relevant influence on the characteristics of either nucleotide signaling or phagocytic properties of macrophages, such as type and derivation of macrophages. Additionally, speed or capacity of phagocytosis, or both, seem to depend on type and derivation of macrophages as well as size and other properties of the phagocytized particles.

5.3 General conclusion

Extracellular nucleotides act as signaling molecules on cells and can modulate cell functions (Eltzschig, Sitkovsky, & Robson, 2012). An effect of extracellular nucleotides is postulated for years and has been shown in many different studies (Eltzschig, Sitkovsky, & Robson, 2012) (Burnstock, Purinergic signalling: Its unpopular beginning, its acceptance and its exciting future , 2012) (Bours, 2006).

Our experiments with primary Kupffer cells showed, that various extracellular nucleotides, especially ATP and ATPγS, and other P2 receptor agonists (i.e. BzATP) have an inhibiting effect on TNFα release of LPS-stimulated Kupffer cells.

On the other hand, Sakaki et al describe suppressed blood serum levels of TNFα, IL-6 and other cytokines using an antagonist of P2Y11 receptor in mice stimulated with intraperitoneal administration of LPS (Sakaki, 2013). In the same paper, they describe a pro-inflammatory polarization of peritoneal and spleen macrophages in LPS-

induced sepsis in mice in response to ATP, that could be suppressed using an antagonist of P2Y11 receptor. Their data also pointed out the importance of purinergic signaling in sepsis by changes in cytokine release. But in contrast to us, they conclude ATP to have a pro-inflammatory effect via autocrine regulation of macrophages.

The discrepancies between the different studies shows the complexity of the topic and further research is required to better understand the effects of purinergic signaling in sepsis. However, we conclude, that one important mechanism how extracellular nucleotides modulate the immune system is via the cytokine release of Kupffer cells and possibly other macrophages in the body, too.

The relevance of various in vitro effects in an entire organism in vivo will need further research, to determine, whether nucleotides in vivo have pro- or anti-inflammatory effects. Also, the effect of extracellular nucleotides on other macrophages in the body, the knowledge about responsible receptors, detailed signaling pathways, and possibly other nucleotides, needs further investigation.

Our other experiments with cell culture macrophages – in contrast to published data from other authors – showed no sign of an effect of extracellular nucleotides on the phagocytic properties of macrophages. If further research seems required the appropriate conditions have to be explored in detail. However, an effect of extracellular nucleotides on phagocytosis still seems probable. Possibly this effect is not very distinct or not intrinsic to all species of macrophages, so that our cells didn't show an effect.

Finally, we show some evidence that extracellular nucleotides are a – possibly important – factor in the immune system and especially inflammatory response to gram-negative bacterial infection and modulate inflammatory response from macrophages. We could demonstrate an inhibition of TNFα release in response to extracellular ATP but no effect on phagocytosis. Further effects of extracellular nucleotides on the immune system, their signaling mechanisms, and their relevance in vivo need further experimental research both in vitro and in vivo before being able to discuss and evaluate possible clinical implementations.

5.3.1 Therapeutic targets of purinergic signaling

The clinical relevance of purinergic signaling can theoretically be investigated in every context of inflammation. Especially in the context of sepsis due to bacteremia, which stands for a generalized inflammation. It may be possible to reduce clinical severity of sepsis by altering the release of inflammatory cytokines in response to bacteremia. Whether ATP, or other mediators in purinergic signaling are the key mediators still needs to be shown in future.

Lecut et al describe a higher susceptibility of P2X1 -/- knockout mice to LPS-induced sepsis, even if they could not demonstrate an altered cytokine release (Lecut, 2012). Together with their other results, they conclude that P2X1 ion channel receptors play a protective role in endotoxemia by negatively regulating systemic neutrophil activation.

Various other diseases are associated with deregulated inflammation and can therefore potentially be addressed by immunomodulation via purinergic signaling. In a perspective article Arulkumaran et al describe different renal diseases where purinergic signaling has pathological effects (Arulkumaran, 2013).

In allergic asthma for example, purinergic signaling has also been named as a potential therapeutic target by Idzko et al. In their study they describe an increased ATP level in the airways of asthmatic patients and mice (Idzko, 2007). In a murine model of asthma they could reduce various cardinal features of asthma by either degrading ATP in the airways using apyrase, or by treating mice with different P2 receptor antagonists.

Surgery is a stressful event for the human organism, and perioperative stress can lead to major changes of hemodynamic status of patients, and to ischemic stress of organs for different reasons. This makes perioperative organ protection to another relevant topic in the further research of the therapeutic use of purinergic signaling. Eltzschig (Eltzschig H. , Targeting Purinergic Signaling for Perioperative Organ Protection, 2013) summarizes different approaches to target purinergic signaling for perioperative organ protection.

Ischemia and reperfusion itself is known as a major stressful factor for organs in different settings such as cerebral or myocardial infarction, but also in the perioperative period, leading to different inflammatory changes (Eltzschig H. , Ischemia and reperfusion—from mechanism to translation , 2011). In his profound review, Eltschig names purinergic signaling as one of many mechanisms in the regulation of ischemia and reperfusion injuries and points out potential therapeutic approaches in this context.

5.3.2 Questions for the future research

As pointed out, modulation of inflammation and immune system by purinergic signaling is a possible target for future therapeutic approaches of various diseases. But until a broad and safe clinical use can be achieved, knowledge about purinergic signaling and its effects on inflammation and the immune system needs to become much more profound. Various questions yet need to be answered, for example:

- Which cells changes their inflammatory properties in response to extracellular nucleotides and in what is the effect?
- Which cytokines are up- or downregulated by which cells?
- Which cytokine patterns lead to an up- or downregulation of inflammatory responses in the tissue?
- What is the clinical consequence and benefit of a modulation of purinergic signaling in the human organism?
- Do extracellular nucleotides have effects only in autocrine/paracrine signaling or can their effects be used systemically.
- Do extracellular nucleotides only play relevant roles in acute situations, such as allergic asthma or ischemia and reperfusion, or can they be used also in chronic inflammatory states?
- What are the dangers of immunomodulation via purinergic signaling?

Obviously, many questions remain to be answered, but the enormous amount of promising research encourages to further exploring the possibilities opened by purinergic signaling.

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