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Role of extracellular ATP in immunity and intestinal defence: effects on intestinal permeability and enterocyte-driven inflammatory response

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The project presented in this thesis was performed within the Nutrition and Toxicology Research Institute Maastricht (NUTRIM), which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

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Voor mam

CONTENTS

CHAPTER 1

Introduction

In this chapter, I will introduce the concepts and background that are of importance for a clear understanding of the main line of thought of this thesis. First, general definitions of nucleotides and adenosine 5'-triphosphate (ATP) are given. Second, previous results are described, which lay the foundations of the present project, and the concept of purinergic signaling and its role in immunity and inflammation are introduced. Third, current knowledge on purine-based treatment modalities in chronic inflammatory diseases is summarized and a working hypothesis for immunoregulation by ATP is presented. Fourth, underlying ideas are presented from which the experiments described within this thesis ensued. Finally, the concept of mucosal defence, comprising both mucosal permeability and mucosal immunity, is introduced as background for two areas of investigation related to inflammatory bowel disease.

1. Nucleotides

A nucleotide is a phosphate ester of a nucleoside, which consists of a heterocyclic purine or pyrimidine base and a pentose sugar deoxyribose or ribose. The most common pyrimidine bases are uracil and cytosine, and the primary purine bases are guanine and adenine. Nucleotides can either be directly synthesized (*de novo synthesis*), or formed by recycling of preformed nucleobases (*salvage synthesis*). Nucleotides are involved in many critical cellular functions:

i. They form the monomeric units of DNA and RNA;
ii. They play a key role in energy metabolism;
iii. They are components of coenzymes;
iv. They serve as physiological mediators.

The principal purine and pyrimidine compounds found in cells are the 5'-nucleotide derivatives. ATP is a purine nucleotide consisting of the nucleoside adenosine and three phosphate groups (Fig. 1).

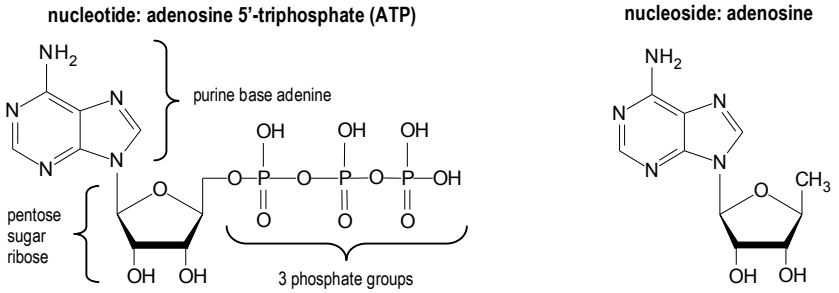

Figure 1. *Structural formulae of ATP and adenosine.*

It is well-known that intracellular ATP is the principal form of chemical energy that is directly available to cells. Since a few decades however, evidence is accumulating that ATP also is a physiological mediator

in the extracellular compartment. Extracellular ATP appears to be involved in the regulation of various biological processes as a ubiquitous signaling molecule. A wide variety of regulatory functions in the nervous, respiratory, gastrointestinal, cardiovascular and immune system have been described for ATP [1].

2. Extracellular functions of ATP

The project described within this thesis was based on the results of a previous randomized clinical trial, reported in 2000 by Agteresch et al. [2], in which effects of ATP infusions in patients with advanced non-small-cell lung cancer (NSCLC, tumor stage IIIB and IV) had been investigated. In this randomized clinical trial, 28 NSCLC patients randomized to the experimental group received supportive care plus intravenous 30-hour ATP infusions every 2 to 4 weeks over a period of 24 weeks (maximally 10 infusions); 30 NSCLC patients receiving supportive care alone served as a control group. The following beneficial effects of ATP treatment were observed relative to control (no ATP) [3-5]: (i) inhibition of progressive loss of weight, fat mass and fat free mass, (ii) inhibition of loss of muscle strength, (iii) maintenance of quality of life, (iv) inhibition of deterioration in nutritional status (appetite, energy intake), and (v) increase in survival of weight-losing stage IIIB NSCLC patients.

In addition to the above clinical effects of ATP infusions, analysis of blood parameters from NSCLC patients showed that ATP treatment completely prevented the decrease in plasma concentrations of albumin which was seen in the control group [2]. Since albumin levels are affected by both nutrition and an acute phase response [6], it was hypothesized that the observed stabilization of albumin levels by ATP may have been caused by inhibition of the acute phase response [2]. For this reason, it was decided also to evaluate levels of C-reactive protein (CRP), a positive acute phase protein that is an important indicator of inflammation [7]. Results showed that plasma CRP levels increased in the control group, but remained stable in the ATP-treated NSCLC patients [8].

The remarkable finding of combined stabilization of albumin and CRP levels following ATP treatment was highly suggestive of an inhibition of the acute phase response by ATP, indicating that the favourable clinical effects of ATP infusion in cancer cachexia could have been partly mediated through immunomodulatory properties of ATP. This fuelled the notion that, by inhibiting acute phase responses, ATP could also exert favourable effects in the treatment of chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease. The present project was initiated in order to further explore this notion, which was completely novel at the time, by a thorough literature review as well as several pilot experiments related to inflammatory conditions.

The majority of physiological functions of extracellular ATP are closely related to one of its breakdown products, adenosine. Both ATP and adenosine exert extracellular functions by signaling through a family of membrane-bound purinergic receptors, which are widely expressed throughout body tissues. Receptors activated by ATP are P2 receptors and those activated by adenosine are P1 receptors. At

present, fifteen subtypes of P2 receptors and four subtypes of P1 receptors are defined. Extracellular ATP is rapidly metabolized by the co-ordinated action of several ecto-enzymes, which are located on cell surfaces or are present in soluble form in the extracellular compartment. Ecto-enzymes control extracellular concentrations of ATP and adenosine via the so-called purinergic cascade (Fig. 2).

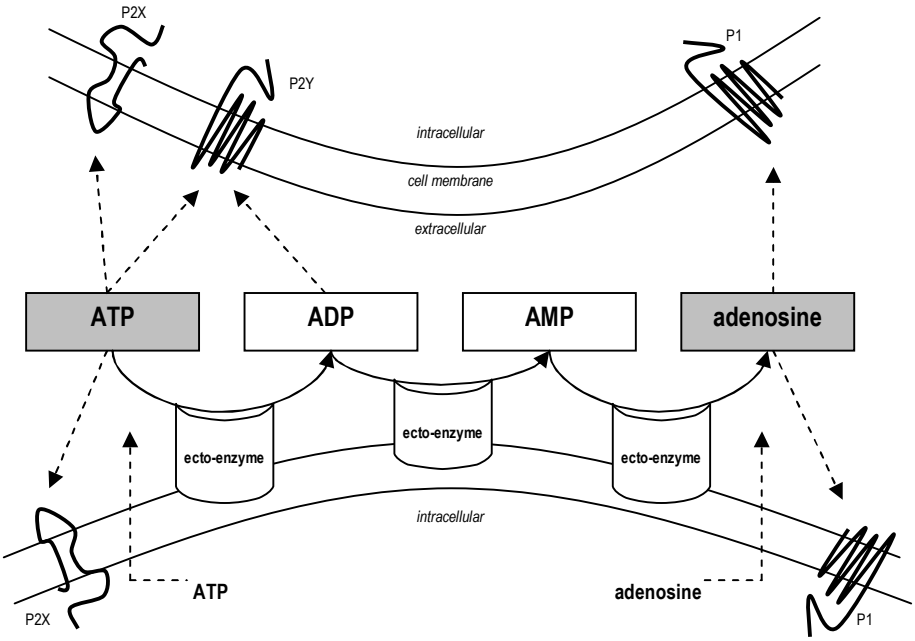

Figure 2. *ATP and adenosine are released from cells into the extracellular space during events of cellular stress. Ecto-enzymes catalyze the sequential degradation of ATP to adenosine. ATP and its metabolites mediate autocrine and paracrine effects by signaling through P2 and P1 receptors.* *(ADP: adenosine 5'-diphosphate, AMP: adenosine 5'-monophosphate)*

Since purinergic receptors and ecto-enzymes are often co-expressed on the same cell, receptor-mediated cellular effects of ATP and adenosine are partly modulated by the ecto-enzyme-driven purinergic cascade. Over the past decades, evidence has accumulated indicating that extracellular ATP, together with its breakdown products, is involved in the complex regulation of immunity and inflammation as an immunomodulatory molecule operating in the cellular microenvironment (Fig. 2).

The immune system is an extremely complex system, in which various immune and non-immune cells co-operate in order to preserve homeostasis and to protect the host from a wide variety of dangers, both self and nonself. Countless numbers of extracellular and intracellular messenger molecules, including cytokines and transcription factors, are crucial in efficacious immunity by constituting an intrinsic signaling network by which the various immune and non-immune cells communicate with each other [9, 10]. The constant flow of immunologic information is aimed at fine-tuning inflammatory and immune responses in such a way that dangers to the host are eliminated efficiently with minimal damage to healthy tissues [11]. For this purpose, there is a redundancy of both cellular and non-cellular effectors with diverse functions during the course of inflammation and immune reponses.

The notion that ATP appears to have an evident role in immunity and inflammation, which is extensively reviewed in this thesis *(chapter 2)*, justified further pilot studies focusing on the potential of ATP in the treatment of chronic inflammatory diseases.

3. Chronic inflammatory diseases

Chronic inflammatory diseases are immune-mediated diseases in which aberrant immune responses result in chronic inflammation and tissue damage. Two prominent diseases in the broad spectrum of chronic inflammatory diseases are rheumatoid arthritis (RA) and inflammatory bowel disease (IBD).

RA is a progressive autoimmune disease characterized by symmetrical and destructive inflammation of multiple joints, which leads to pain and joint failure, eventually resulting in joint disfiguration and disability [12]. Epidemiological studies, which have mostly been carried out in Northern European and North American areas, report an estimated prevalence for RA of 0.5-1.1% of the general population and annual incidence rates of 20 to 50 newly diagnosed RA cases per 100,000 inhabitants [13]. In the Netherlands, there were between 120,000 and 156,000 people with RA in 2000 (10.0-12.6 per 1000 inhabitants) and incidence rates that year were between 2,400 and 4,200 new cases of RA (0.2-0.4 per 1000 inhabitants) [14].

IBD encompasses Crohn's disease and ulcerative colitis, two conditions that probably represent opposite ends of a disease continuum. The hallmark of IBD is chronic uncontrolled inflammation of the intestinal mucosa, which can affect any part of the gastrointestinal tract [15]. Crohn's disease and ulcerative colitis share many clinical symptoms, such as diarrhea, bloody stools, weight loss, abdominal pain and fatigue, but each condition also has unique features [16, 17]. Crohn's disease and ulcerative colitis are traditionally considered to be common in the Western world, with as many as 1.4 million people in North America and as many as 2.2 million people in Europe suffering from these diseases [18]. Incidence rates in Northern and Western Europe, which have mostly been reported in the late-1980s and mid-1990s, range from 3.6 to 9.8 cases per 100,000 person-years for Crohn's disease and from 3.2 to 20.3 cases per 100,000 person-years for ulcerative colitis [18]. High incidence rates have also been observed in the southeastern region of the Netherlands. A four-year prospective study between 1991 and 1994 found incidence rates of 6.9 ± 1.0 (mean ± 95% confidence interval) cases per 100,000 inhabitants per year for Crohn's disease and 10 ± 1.2 cases per 100,000 inhabitants for ulcerative colitis [19]. More recently, high incidence rates of pediatric IBD (< 18 years) were reported in the Netherlands. Yearly incidence over a three-year period (1999-2001) was 2.1 newly diagnosed cases per 100,000 children for Crohn's disease and 1.6 cases per 100,000 children for ulcerative colitis [20].

RA and IBD are now considered to have some commonality in pathogenesis. Both are multifactorial diseases that are believed to result from the interaction of both genetic and environmental factors. They are characterized by dysregulated immunity and an increased acute phase reaction, which is associated with disease activity and clinical symptoms. It has been demonstrated that increased CRP levels

correlate with disease activity, functional outcome and joint damage in RA [21, 22]. CRP has also been proposed as an important biomarker for IBD, reflecting ongoing inflammation in the gut and correlating with disease activity [23].

Immunoregulatory defects in innate and adaptive immune responses are believed to underlie the etiology of RA and IBD in which chronic destructive inflammatory processes are perpetuated by a Th_1–Th_2 imbalance. Nowadays, a diversity of therapeutic modalities are available as a treatment regime for patients with RA and IBD. One of these is methotrexate. Originally known as an effective antiproliferative drug in the treatment of cancer at high doses, it is now well-established that low-dose methotrexate has profound anti-inflammatory effects. Despite recent development of targeted biological therapies, methotrexate is still one of the most commonly prescribed disease-modifying antirheumatic drugs (DMARDs) in the first-line treatment of RA today [24]. Methotrexate is also frequently applied as an immunomodulatory drug in IBD, particularly in Crohn's disease [25]. Although the mechanisms of action by which methotrexate at a low dose modulates inflammation have not been fully elucidated, Cronstein and co-workers proposed that the anti-inflammatory effects of methotrexate are exerted in part by promoting extracellular release of adenine nucleotides and adenosine with subsequent suppression of inflammation by adenosine acting at P1 receptors [26-28].

Methotrexate is a folic acid antagonist, which is believed to elevate extracellular adenosine concentrations by interfering with folate-dependent reactions involved in *de novo* synthesis of purines and pyrimidines. Following cellular uptake, methotrexate is polyglutamated inside cells. The polyglutamated form of methotrexate then inhibits an important enzyme involved in purine biosynthesis: 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase. Inhibition of AICAR transformylase leads to intracellular accumulation of AICAR and its metabolite 5-aminoimidazole-4-carboxamide ribonucleoside (AICAr or acadesine), which are intermediates in the purine synthesis pathway. These intermediates eventually promote the accumulation of intracellular adenosine by inhibition of adenosine deaminase (ADA, the enzyme catalyzing the conversion of adenosine to inosine) and AMP deaminase (AMPDA, the enzyme catalyzing the conversion of AMP to IMP). Extracellular adenosine concentrations eventually rise by its release into the extracellular space and also by extracellular conversion of released adenine nucleotides to adenosine (Fig. 3).

Strong *in vivo* evidence supporting the 'adenosine hypothesis' derives from animal models of acute and chronic inflammation, in which anti-inflammatory effects of methotrexate were shown to be abrogated by administration of either ADA or an adenosine receptor antagonist [29-32], by an inhibitor of ecto-5'-nucleotidase (CD73) [33], and more recently by knock-out of adenosine receptors [34-36] and of CD73 [37]. Clinical significance has been demonstrated also in human studies. Increased adenosine levels have been detected in methotrexate-treated patients [36, 38, 39], which indicates that methotrexate induces adenosine release *in vivo*. Also, in RA patients treated with methotrexate, significant treatment failure was observed in heavy coffee drinkers [40]. This was confirmed by another study which showed that methotrexate-treated RA patients with high intake of caffeine experienced less improvement in clinical disease symptoms compared to patients with low caffeine intake [41]. These data suggest that

caffeine interfered with the efficacy of methotrexate, and, since caffeine is a nonselective adenosine receptor antagonist, provide evidence for involvement of adenosine in the treatment efficacy of methotrexate. In addition, it has also been proposed that the adenosine-mediated anti-inflammatory action of methotrexate is shared by sulphasalazine, another common drug used in the treatment of IBD [33, 42, 43].

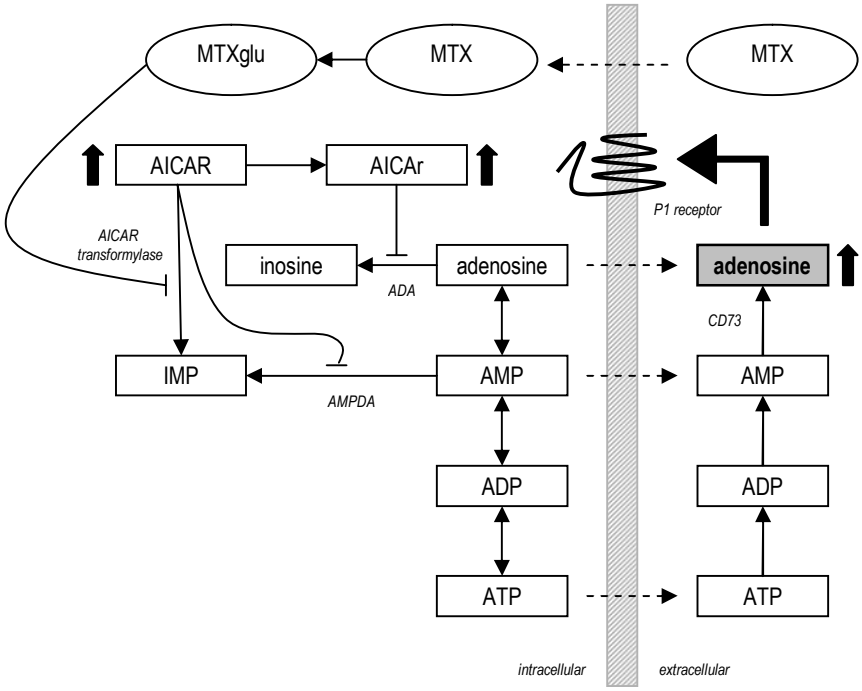

Figure 3. *The 'adenosine hypothesis' as an explanatory mechanism for the anti-inflammatory action of methotrexate (see text for detailed explanation).* *(ADA: adenosine deaminase, AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide, AICAr: 5-aminoimidazole-4-carboxamide ribonucleoside, AMPDA: AMP deaminase, CD73: ecto-5'-nucleotidase, IMP: inosine 5'-monophosphate, MTX: methotrexate, MTXglu: glutamated methotrexate)*

Further indications underlining the potential benefit of purinergic signaling in chronic inflammatory diseases derive from a number of studies using several animal models of RA and IBD, which unequivocally demonstrate that applying purinergic pathways *in vivo* leads to substantial regulatory effects on markers of chronic inflammation and clinical signs of disease activity [44-66].

Taken together, the growing body of evidence on the immunoregulatory role of ATP and its metabolites warranted investigation of the potential of purine-based treatment of RA and IBD. Although it would appear that adenosine is responsible for most of the proposed purinergic anti-inflammatory effects, our main attention remained focused on the clinical application of ATP, which in the study by Agteresh et al. (2000) appeared to exhibit immunomodulatory properties by inhibition of the acute phase response. Furthermore, recent *ex vivo* experiments by our group indicate that ATP at relatively low extracellular concentrations may exhibit marked anti-inflammatory properties, even under conditions of severe

oxidative stress that accompany uncontrolled inflammatory processes [67-69]. In lipopolysaccharide/ phytohemagglutinin (LPS/PHA)-stimulated whole blood, which resembles the *in vivo* situation more closely than isolated cell culture models, ATP was shown to inhibit TNFα production via $P2Y_{11}$ receptor activation and to stimulate IL-10 production via $P2Y_{12}$ receptor activation. ATP thus simultaneously affected both pro- and anti-inflammatory cytokine responses through activation of different P2 receptor subtypes in the *ex vivo* stimulated blood model, suggesting that ATP has strong immunomodulatory properties.

As it might be suggested that chronic inflammatory diseases that are characterized by dysregulated immunity would benefit more from efficient immunoregulatory treatment than sole immunosuppresive treatment, we hypothesized that by administering ATP at a chronic low level (*e.g.* by low-dose infusion therapy), combined effects of different P2 and P1 receptor subtypes could be induced on three levels, leading to effective immunomodulation with down-regulation of chronic inflammation (Fig. 4).

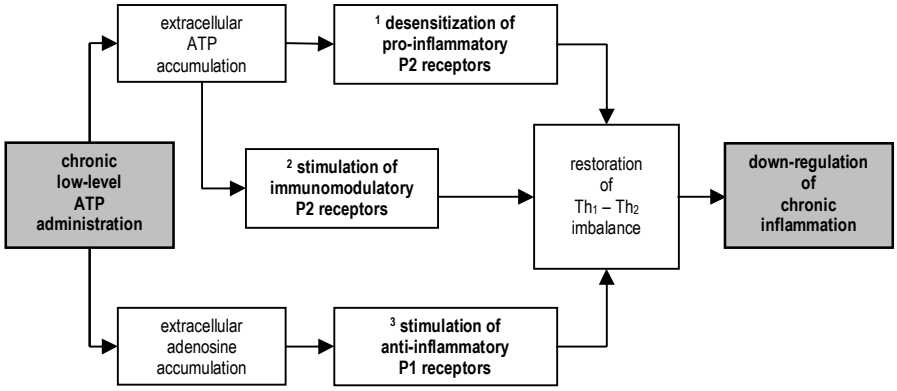

Figure 4. *Working hypothesis of immunoregulation by ATP. Chronic low-level ATP down-regulates chronic inflammation through combined effects at three levels: (1) desensitization of pro-inflammatory P2 receptors, (2) stimulation of immunomodulatory P2 receptors, and (3) stimulation of anti-inflammatory P1 receptors.*

In view of this hypothesis, two routes of investigation in chronic inflammatory diseases were initiated, one related to IBD and the other related to RA. Several experiments were initiated to explore the IBD-related route, which is the main subject of this thesis. To delineate the role of ATP (and adenosine) in intestinal defence, we focused on two areas of investigation related to two proposed pathways of disease pathogenesis in IBD [70, 71]. First, disrupted barrier function with hyperpermeability of the mucosal epithelium, the so-called leaky gut, is thought to facilitate increased antigen penetration from which exaggerated inflammatory reactions in the intestine ensue. Second, dysregulated mucosal immunity may lead to uncontrolled immune responses to luminal antigens and chronic inflammation. These factors are considered to be involved in a self-amplifying cycle wherein an initial event at the mucosal barrier may trigger immune activation with excessive cytokine responses, which further compromise barrier function. Both disrupted barrier function and dysregulated mucosal immunity may therefore be of considerable importance to defective intestinal defence in IBD.

In addition to IBD, the RA-related route, not further discussed in this thesis, was also explored. We initiated a 'proof of concept' study to investigate the potential of ATP infusion therapy in RA. In this study, which is designed as a double-blind randomized clinical trial (currently ongoing), patients with active RA receive either regular ATP or placebo infusions. Effects on outcome parameters of chronic inflammation and disease activity (and functional status) are assessed.

4. Intestinal defence

Epithelial cells lining mucosal surfaces in the intestine form a physical barrier, which separates the host's internal milieu from the external environment, thereby constituting a first line of defence against the aggressive gut milieu. This selective permeable barrier permits passive entry of luminal nutrients, ions and water while restricting access of pathogenic substances to underlying tissue compartments. It has been proposed that there are two major pathways for epithelial permeation in the intestine (Fig. 5).

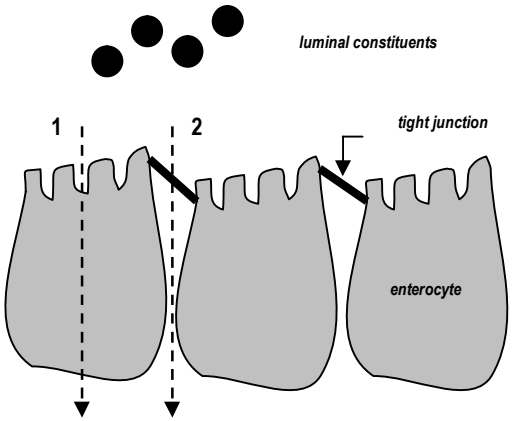

Figure 5. *Permeability pathways of the mucosal barrier: (1) transcellular pathway, (2) paracellular pathway.*

The first is a transcellular pathway, which is constituted by lipophobic and lipophilic pores located in the enterocyte brush border membrane. The second pathway is a paracellular pathway, which is formed by an apical intercellular junctional protein complex that allows selective passage across the epithelium through the tight junctions between adjacent enterocytes. Although initially thought to have static permeability properties, the tight junctional protein complex is now believed to be a dynamic structure that is able of altering its permeability status in response to a variety of extracellular stimuli. Inflammatory stimuli, including pro-inflammatory cytokines, cause disassembly of the junctional proteins, which are thought to contribute to the permeability defects seen in IBD.

Permeability defects are also seen in chronic users of nonsteroidal anti-inflammatory drugs (NSAIDs). It has become clear over the past decades that long-term NSAID use is commonly accompanied by mucosal lesions in the gastrointestinal tract, which can eventually result in serious pathological

conditions such as perforations, ulcers and strictures [72]. Although NSAID effects in the upper gastrointestinal tract (stomach) are well-characterized, adverse effects of NSAIDs in the lower gastrointestinal tract (small intestine) are now also generally recognized. Specific detrimental NSAID effects in the small intestine, which encompass intestinal inflammation associated with blood and protein loss, are collectively called NSAID enteropathy.

Besides comprising a mucosal barrier, the intestinal epithelial cells also participate in mucosal immunity by generating a variety of immunoactive molecules in response to various extracellular stimuli, including bacteria and their products. In this way, intestinal epithelial cells are an important part of mucosal defence mechanisms by contributing to inflammatory processes in the intestine through interaction with local immune cells [73].

As described above, we focused on two areas of investigation to explore the role of ATP and adenosine in intestinal defence. In the first part of our experiments, the concept of intestinal permeability was utilized. Permeability defects are nowadays considered to be critical in several gastrointestinal disorders, and have recently even been suggested to be involved in the initiation of extraintestinal autoimmune responses, such as in RA [74, 75]. Therefore, the concept of intestinal permeability forms an attractive candidate for investigating therepautic interventions with relevance for several pathologic conditions [76]. We used a human model of NSAID-induced permeability changes of the mucosal barrier to evaluate *in vivo* effects of ATP and adenosine on epithelial permeability as part of intestinal defence *(chapters 3 and 4)*. In the second part of our experiments, which was aimed specifically at mucosal immunity as part of intestinal defence, we examined effects of ATP and adenosine on an inflammatory response mediated by human enterocytes *in vitro (chapter 5)*.

5. Outline of the thesis

In **chapter 2**, existing literature on the role of extracellular ATP and adenosine in immunity and inflammation is extensively reviewed, with special emphasis on their interplay. Overwhelming evidence indicates that ATP and adenosine are versatile extracellular messengers contributing to immune regulation by signaling through widely expressed purinergic receptors. A conceptual framework is presented, which positions ATP and adenosine within the complex web of immunoactive signaling molecules regulating immunity and inflammation.

In **chapters 3 and 4**, two experiments on the effect of ATP and adenosine on NSAID-induced permeability changes in the human small intestine are described. Using a human model of early-stage small intestinal enteropathy, the efficacy of two modes of administration is evaluated: topical administration of ATP into the upper small intestine via a naso-intestinal tube (*chapter 3*) and administration of both ATP and adenosine via enteric-coated capsules as a more pratically feasible mode of administration (*chapter 4*).

In **chapter 5**, a cell experiment on the effects of ATP and adenosine on adhesion molecule expression and cytokine production by epithelial cells of the gut mucosa is described. Using human enterocyte-like colon adenocarcinoma Caco-2 cells as an *in vitro* cell culture model of small intestinal epithelial cells, findings are presented on: (i) ATP and adenosine metabolism, (ii) expression of purinergic receptor mRNA, (iii) effects of ATP and adenosine on adhesion molecule expression, and (iv) effects of ATP and adenosine on cytokine production.

In **chapter 6**, main findings and implications are discussed, and where relevant, directions for future research are suggested.

CHAPTER 2

Review

Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation

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Abstract

Human health is under constant threat of a wide variety of dangers, both self and nonself. The immune system is occupied with protecting the host against such dangers in order to preserve human health. For that purpose, the immune system is equipped with a diverse array of both cellular and non-cellular effectors that are in continuous communication with each other. The naturally occurring nucleotide adenosine 5'-triphosphate (ATP) and its metabolite adenosine (Ado) probably constitute an intrinsic part of this extensive immunological network through purinergic signaling by their cognate receptors, which are widely expressed throughout the body. This review provides a thorough overview of the effects of ATP and Ado on major immune cell types. The overwhelming evidence indicates that ATP and Ado are important endogenous signaling molecules in immunity and inflammation. Although the role of ATP and Ado during the course of inflammatory and immune responses *in vivo* appears to be extremely complex, we propose that their immunological role is both interdependent and multifaceted, meaning that the nature of their effects may shift from immunostimulatory to immunoregulatory or vice versa depending on extracellular concentrations as well as on expression patterns of purinergic receptors and ecto-enzymes. Purinergic signaling thus contributes to the fine-tuning of inflammatory and immune responses in such a way that the danger to the host is eliminated efficiently with minimal damage to healthy tissues.

1. Introduction

The naturally occurring nucleotide adenosine 5'-triphosphate (ATP) is normally present in every living cell of the human body and is well-known for its role in intracellular energy metabolism. In addition to this intracellular role, ATP in the extracellular compartment is thought to contribute to the regulation of a variety of other biological processes, including cardiac function, neurotransmission, muscle contraction, vasodilatation, bone metabolism, liver glycogen metabolism and inflammation [1, 77, 78].

The human immune system comprises an interactive network of lymphoid organs and immune cells, and is essential to host defence [79, 80]. Interaction between the various components of the immune system during activation is realized by multiple signaling molecules. These molecules, which can be released in response to tissue injury or exogenous pathogens, signal danger to the host and are necessary for initiating primary immune responses as well as for controlling the course and resolution of the concomitant inflammatory processes [81-85]. Extracellular nucleotides such as ATP may function as endogenous signaling molecules that control inflammation and immune responses [86-88]. Modulation of inflammatory processes and immune responses by extracellular ATP is complex and results from specific effects on a wide variety of both immune and non-immune cells.

ATP's role in immunity is closely related to one of its breakdown products, the nucleoside adenosine (Ado). Ado has an already established role in immunity [89-97], in which it may contribute to the engineering of inflammation and immune reponses by providing a suppressive tissue-protecting signal in a delayed, negative feedback manner [98-100]. The notion of an interrelation between ATP and Ado is firmly based on the presence of a large family of purinergic receptors (P1 and P2 receptors for Ado and ATP, respectively) that are mostly co-expressed by immune and non-immune cells. Several enzymes, which are also expressed by various immune and non-immune cells, are involved in a purinergic cascade by which extracellular purine levels and the ensuing purinergic signaling can be dynamically controlled during inflammatory and immune responses.

This review discusses the role of extracellular ATP and Ado in immunity and inflammation with special focus on their interplay.

2. Extracellular metabolism

Whereas intracellular concentrations of ATP are very high (3-10 mM), its extracellular concentrations are considerably lower. Physiological ATP concentrations in plasma are normally submicromolar (400-700 nM) [101-103]. Compared to ATP, plasma concentrations of Ado are usually about tenfold lower (40-80 nM) [104-106]. However, extracellular concentrations of both ATP and Ado can rise markedly under several conditions, including inflammation, hypoxia and ischemia [107-110]. Concentrations of ATP and Ado in the extracellular compartment are controlled by enzymes catalyzing their conversion (Fig. 1) [111-120]. These so-called ecto-enzymes are located on cell surfaces or may be found in soluble form in the

interstitial medium or in body fluids. The currently known ecto-enzymes, which are involved mainly in the breakdown of extracellular ATP, include four families that partially share tissue distribution and substrate specificity: (i) the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, (ii) the ectonucleotide pyrophosphatase/ phosphodiesterase (E-NPP) family, (iii) alkaline phosphatases, and (iv) ecto-5'-nucleotidase (CD73). The first family catalyzes the sequential degradation of extracellular nucleotide tri- and diphosphates. It includes seven members (NTPDase1 to -6 and NTPDase8) of which NTPDase1, -2, -3 and -8 are involved in the breakdown of ATP and adenosine 5'-diphosphate (ADP) to adenosine 5'-monophosphate (AMP). The second family consists of three members (NPP1, -2 and -3), which catalyze the hydrolysis of cyclic AMP (cAMP) to AMP, ATP to AMP and ADP to AMP. A splice variant of NPP2 (autotaxin) is involved in the conversion of AMP to Ado. The third family comprises a protein family of non-specific ectophosphomonoesterases catalyzing the degradation of nucleotide tri-, di- and monophosphates. Finally, the fourth family is represented by CD73 which catalyzes the hydrolysis of AMP to Ado. According to cellular location and kinetic properties, this enzyme can be grouped into four forms, of which a membrane-bound form and a soluble form are involved in extracellular metabolism of AMP. Besides the enzymes that degrade extracellular nucleotides, enzymes catalyzing the generation and interconversion of extracellular adenine and uridine nucleotides have also been described (Fig. 1) [121-127].

In addition to CD73, which constitutes the final enzymatic link of the purinergic cascade that leads to the formation of extracellular Ado, two other enzymes are important to the regulation of extracellular Ado levels, *i.e.*, adenosine deaminase (ADA) and adenosine kinase (Fig. 1). ADA is thought to be mainly a cytosolic enzyme, but it can also appear on the exterior plasma membrane of several immune and non-immune cells (ectoADA). EctoADA is considered to be a key enzyme in purine metabolism, catalyzing the irreversible deamination of Ado and deoxyadenosine to inosine and deoxyinosine, respectively. EctoADA therefore contributes to the removal of Ado from the extracellular compartment [115, 128, 129]. The observation that ADA deficiency leads to the severe combined immunodeficiency (SCID) syndrome points to the physiological importance of controlling extracellular Ado levels. Humans suffering from the ADA-SCID syndrome have a hypoplastic thymus with dramatically reduced numbers of peripheral T and B cells, which increases the risk of infections because of generalized immune suppression. Elevated levels of the toxic metabolite deoxyadenosine are believed to partly mediate lymphotoxicity in ADA-SCID. In addition, excessive purinergic receptor activation by increased levels of extracellular Ado may also contribute to the immune suppression by impairing the development and function of lymphocytes [130-137].

Levels of extracellular Ado are also regulated by adenosine kinase, an intracellular enzyme catalyzing the rapid phosphorylation of Ado to AMP [138-141]. Since cellular uptake of Ado from the extracellular compartment is driven by its concentration gradient, adenosine kinase indirectly regulates Ado uptake by controlling intracellular Ado concentrations. Administration of various adenosine kinase inhibitors has been shown to increase extracellular Ado levels *in vivo* with concomitant down-regulation of inflammation in various animal models of acute and chronic inflammation [50, 59, 66, 142-146].

Figure 1. *Overview of conversion pathways of ATP and adenosine (see text for detailed explanation).*

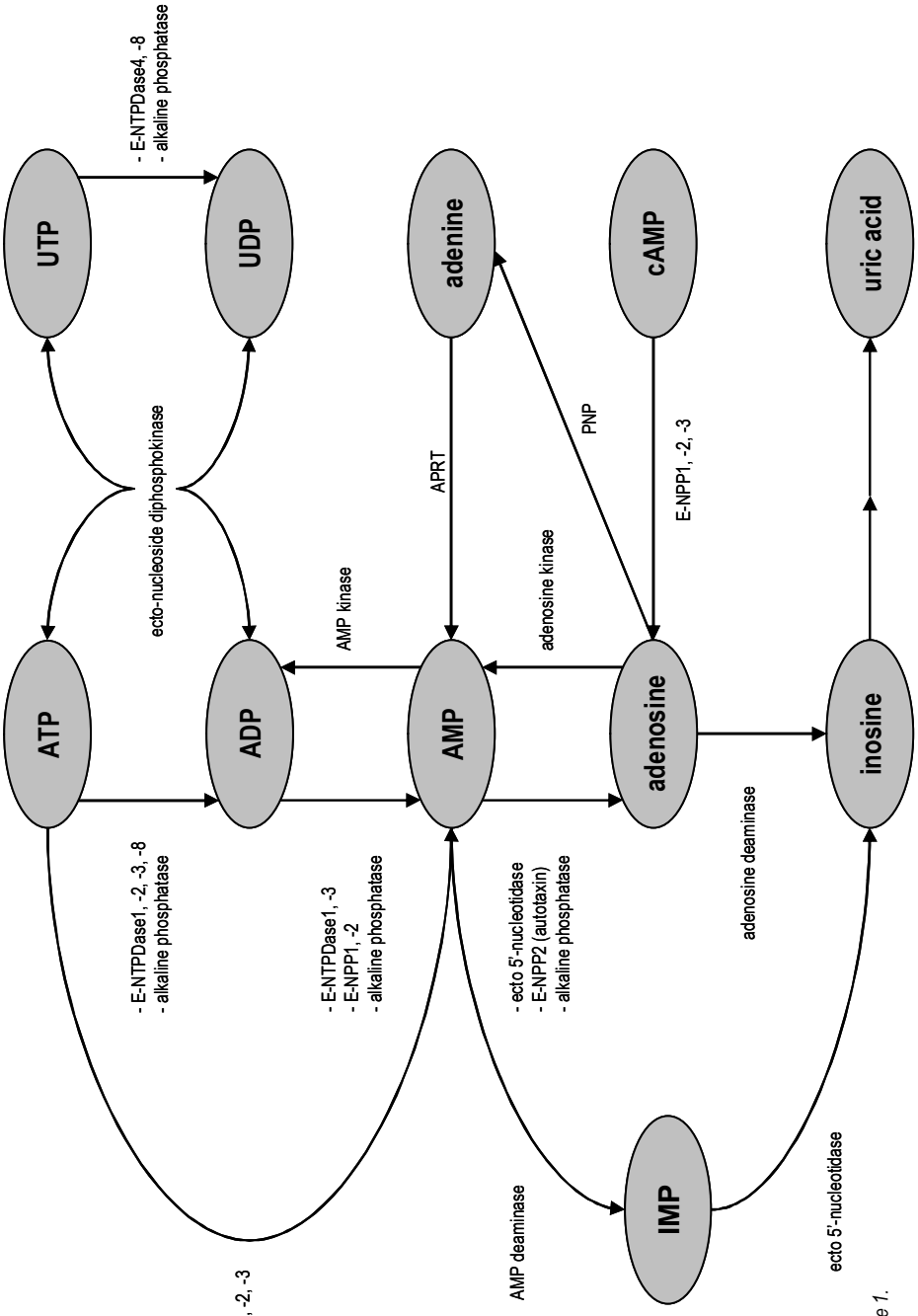
UTP
UDP
adenine
cAMP
uric acid
ATP
ADP
AMP
adenosine
inosine
IMP
- E-NTPDase4, -8
- alkaline phosphatase
ecto-nucleoside diphosphokinase
AMP kinase
APRT
PNP
adenosine kinase
E-NPP1, -2, -3
- E-NTPDase1, -2, -3, -8
- alkaline phosphatase
- E-NTPDase1, -3
- E-NPP1, -2
- alkaline phosphatase
- ecto 5'-nucleotidase
- E-NPP2 (autotaxin)
- alkaline phosphatase
adenosine deaminase
E-NPP1, -2, -3
AMP deaminase
ecto 5'-nucleotidase

Figure 1.

Taken together, a wide variety of enzymes are involved in the control of extracellular nucleotide and nucleoside levels. These enzymes are essential to the regulation of purinergic signaling by ATP and Ado. The co-existence of nucleotide-consuming, -generating and -interconverting pathways on the surface of immune and non-immune cells, which also co-express ATP and Ado receptors, is crucial in the duration, magnitude and nature of purinergic signaling.

3. Purinergic receptors

A large family of membrane-bound receptors mediates cell signaling by ATP and Ado. A large family of membrane-bound receptors mediates cell signaling by ATP and Ado. These so-called purinergic receptors ultimately determine the variety of effects induced by extracellular ATP and Ado. Two families of purinergic receptors have been defined to date, namely P1 and P2 receptors (Table 1) [147].

Table 1. *P1 and P2 receptor subtypes with estimated affinity for physiologic ligands and immune cell distribution (see text for further details and references).*

Subtype	Physiologic ligands	Immune cell distribution
P1 receptors		
A_1	Adenosine (EC_{50}: 0.18-0.53 μM) Inosine (EC_{50}: 290 μM)	Neutrophils; monocytes; macrophages; dendritic cellls
A_{2A}	Adenosine (EC_{50}: 0.56-0.95 μM) Inosine (EC_{50}: 50 μM)	Neutrophils; monocytes; macrophages; dendritic cells; T lymphocytes; B lymphocytes
A_{2B}	Adenosine (EC_{50}: 16.2-64.1 μM)	Neutrophils; monocytes; macrophages; dendritic cells; T lymphocytes
A_3	Adenosine (EC_{50}: 0.18-0.53 μM) Inosine (EC_{50}: 0.03-2.5 μM)	Neutrophils; monocytes; macrophages; dendritic cells; T lymphocytes
P2 receptors		
P2X subfamily		
$P2X_1$	ATP (EC_{50}: 0.05-1 μM)	Neutrophils; monocytes; macrophages; dendritic cells; T lymphocytes; NK cells
$P2X_2$	ATP (EC_{50}: 1-30 μM)	
$P2X_3$	ATP (EC_{50}: 0.3-1 μM)	
$P2X_4$	ATP (EC_{50}: 1-10 μM)	Neutrophils; monocytes; macrophages; dendritic cells; T lymphocytes; NK cells
$P2X_5$	ATP (EC_{50}: 1-10 μM)	Neutrophils; monocytes; macrophages; dendritic cells; T lymphocytes
$P2X_6$	ATP (EC_{50}: 1-12 μM)	
$P2X_7$	ATP (EC_{50}: 100-780 μM)	Neutrophils; monocytes; macrophages; dendritic cells; T lymphocytes; B lymphocytes; NK cells
P2Y subfamily		
$P2Y_1$	ADP (EC_{50}: 8 μM)	Neutrophils; monocytes; macrophages; dendritic cells; T lymphocytes
$P2Y_2$	UTP (EC_{50}: 0.14 μM) = ATP (EC_{50}: 0.23 μM)	Neutrophils; monocytes; macrophages; dendritic cells; T lymphocytes
$P2Y_4$	UTP (EC_{50}: 2.6 μM) >> ATP, UDP	Neutrophils; monocytes; macrophages; dendritic cells; T lymphocytes
$P2Y_6$	UDP (EC_{50}: 0.3 μM) >> UTP (EC_{50}: 6 μM)	Neutrophils; monocytes; macrophages; dendritic cells; T lymphocytes
$P2Y_{11}$	ATP (EC_{50}: 17 μM)	Neutrophils; monocytes; macrophages; dendritic cells; T lymphocytes; B lymphocytes
$P2Y_{12}$	ADP (EC_{50}: 0.07 μM)	Monocytes; macrophages; T lymphocytes
$P2Y_{13}$	ADP (EC_{50}: 0.06 μM) > ATP (EC_{50}: 0.26 μM)	Monocytes; dendritic cells; T lymphocytes
$P2Y_{14}$	UDP-glucose (EC_{50}: 0.3 μM)	Neutrophils; dendritic cells; T lymphocytes

P1 receptors belong to the superfamily of seven-transmembrane-spanning receptors which are subdivided into A_1, A_{2A}, A_{2B} and A_3 receptor subtypes [147, 148]. These receptor subtypes bind extracellular Ado with different affinities (Table 1). It must be noted that Ado's breakdown product inosine also exhibits immunomodulatory properties by agonistic action on A_1, A_{2A} and A_3 receptors at micromolar concentrations [149-152]. The P2 receptor family is subdivided in two subfamilies, *i.e.*, P2X and P2Y [78, 153, 154]. P2X receptors are ligand-gated ion channels of which seven subtypes have been characterized ($P2X_{1\text{-}7}$) [155, 156], and P2Y receptors are seven-transmembrane-spanning receptors of which eight subtypes have been identified to date ($P2Y_{1,\ 2,\ 4,\ 6,\ 11\text{-}14}$) [154, 157, 158]. In contrast to P2X receptors, which respond primarily to extracellular ATP, P2Y receptors show subtype-specific responsiveness to their physiologic ligands and can be subdivided into two groups based on sequence homology (Table 1) [158, 159]. Group 1 encompasses specific purinergic receptors ($P2Y_1$, $P2Y_{11}$), specific pyrimidinergic receptors ($P2Y_4$, $P2Y_6$) and a receptor of mixed specificity ($P2Y_2$). Group 2 encompasses two specific ADP receptors ($P2Y_{12}$, $P2Y_{13}$) and a receptor for UDP-glucose ($P2Y_{14}$).

Purinergic receptors are broadly distributed throughout body tissues, being expressed on a wide variety of both immune and non-immune cells. The role of extracellular ATP and Ado in immunity and inflammation particularly depends on purinergic receptor expression by cell types that are essential to efficacious inflammatory and immune responses (Table 1). Most immune cells co-express both P1 and P2 receptor subtypes, which suggests dual regulation of cell function through purinergic signaling. The outcome of purinergic receptor-mediated signaling is partly determined by the extent of receptor expression, *i.e.*, receptor density. Receptor density may actually change during the course of inflammatory and immune responses depending on the nature of those responses, as various immunomediators have been shown to modulate purinergic receptor expression in several cell types. For example, functional $P2X_7$ receptors in monocytes and macrophages are down-regulated by the anti-inflammatory cytokines interleukin (IL)-4 and IL-10, whereas they are up-regulated by the pro-inflammatory mediators tumor necrosis factor (TNF)-α, interferon (IFN)-γ and lipopolysaccharide (LPS) [160-164]. Concurrently with the up-regulation of $P2X_7$ receptors, inflammatory activation of monocytes and macrophages by LPS and IFNγ induces down-regulation of functional $P2Y_2$ receptors [161, 165]. P1 receptor expression can also be modulated. A_{2B} receptors are up-regulated by LPS, TNFα, IL-1 and IFNγ [166-169]. On the other hand, A_{2A} receptors are down-regulated by IFNγ, but up-regulated by TNFα and IL-1 [167, 170, 171]. Thus, functional expression of both P1 and P2 receptors appears to undergo constant modulation in inflamed tissues, which might contribute to the fine-tuning of inflammatory and immune responses.

In summary, the above indicates that signaling by purinergic receptors depends on a wide variety of factors, including receptor expression and receptor sensitivity, as well as extracellular levels of nucleotides and nucleosides. Purinergic signaling seems to be even more complex in inflammatory conditions during which signaling is subject not only to changing levels of extracellular nucleotides/nucleosides, but also to additional modulating factors, such as dynamic changes in the expression of purinergic receptors and ecto-enzymes in response to various immunomediators that are synthesized during the course of inflammatory and immune responses.

4. Effects of ATP and adenosine on immune cell function

For insight in the role of ATP and Ado in immunity and inflammation, an overview of their effects on major cell types involved in innate and adaptive immunity is outlined in the following sections.

4.1 Neutrophils

Neutrophils are the body's first line of defence against pathogens and are critical effectors in both innate and humoral immunity [172, 173]. Neutrophil-mediated destruction of pathogens plays a crucial role in the early stages of inflammation and the immune response. However, since this killing capacity also implies an implicit capability to destruct host tissues, tight regulation of neutrophil function is essential. ATP and Ado may contribute to the regulation of neutrophil function during inflammatory and immune responses [89, 97, 174-176]. Neutrophils have been shown to express CD39 (E-NTPDase) [177-179] and CD73 [180-183]. Furthermore, neutrophils are capable of releasing both ATP and Ado following inflammatory activation [179, 181-192]. The effector functions of neutrophils may thus be subject to autocrine and paracrine control by endogenous ATP and Ado.

4.1.1 ATP

Receptors. Neutrophils have been shown to express $P2Y_{1,2,4,6,11,14}$ and $P2X_{1,4,5,7}$ receptor subtypes [183, 193-199]. P2 receptor density in neutrophils may be subtype-specific. As a model of promyelocytic neutrophil progenitors, human leukemic HL-60 cells have been shown to weakly express $P2X_{1,5}$, moderately express $P2X_7$ and $P2Y_{1,11}$ and strongly express $P2Y_{2,4,6}$ receptor subtypes [165, 177, 178, 194, 200, 201]. Granulocytic differentiation of HL-60 cells was shown to induce up-regulation of $P2X_5$ and $P2Y_{11}$ receptor subtypes, but down-regulation of $P2X_7$ receptors [177, 200, 201]. ATP at high micromolar concentrations may contribute to the differentiation of HL-60 cells into neutrophil-like cells via stimulation of $P2Y_{11}$ receptors [200-202]. Although mRNA for the $P2X_7$ receptor has been detected in human polymorphonuclear neutrophils (PMN) [183, 194], functional expression of this receptor subtype by neutrophils remains controversial. Using a mouse anti-human $P2X_7$ receptor monoclonal antibody, Gu et al. (2000) detected little expression of $P2X_7$ receptor protein on the surface of PMN. However, these authors reported the presence of large intracellular amounts of $P2X_7$ protein in PMN, and suggested that these might constitute an intracellular receptor reserve from which $P2X_7$ receptors may be recruited to the surface following cellular activation [203]. In homology with other immune cells showing up-regulation of the $P2X_7$ receptor upon inflammatory activation [161, 163, 164, 196], such an up-regulation of this receptor subtype by neutrophils might be conceivable during inflammation.

Adhesion. Adhesion of neutrophils to the vascular endothelium with subsequent transendothelial extravasation is an important step in the recruitment of circulating neutrophils to extravascular inflammatory sites during the early stages of inflammation. Extracellular ATP has been shown to stimulate neutrophil adhesion to endothelial cells [204-210]. Up-regulation of endothelial adhesion molecules such as E-selectin near inflammatory sites allows circulating neutrophils to tether to the endothelium, which results in rolling of neutrophils. ATP at low millimolar concentrations has been shown to induce up-regulation of E-selectin through $P2X_7$ receptor-mediated activation of nuclear factor (NF)-κB

[211, 212]. During rolling along the endothelium, neutrophils are primed by various chemoattractants and chemokines secreted by endothelial cells. This priming leads to activation of the β_2-integrin macrophage antigen (Mac)-1 (CD11b/CD18) in neutrophils, which is a prerequisite for firm adhesion of neutrophils to the endothelium. Micromolar concentrations of ATP have been shown to induce a rapid up-regulation of Mac-1 in neutrophils [204, 207, 208]. Following firm adhesion, neutrophils extravasate by transmigrating through the vascular endothelium. Extracellular ATP may facilitate transmigration by increasing endothelial permeability via activation of P2Y receptors [213].

Migration. Once extravasated, neutrophils migrate to sites of inflammation or tissue damage; a process which is mediated by a variety of chemokines and chemoattractants. Effects of extracellular ATP on neutrophil migration are equivocal. At micromolar ATP concentrations, neutrophil motility (*i.e.* chemotaxis and chemokinesis) has been shown either to be: (i) unaffected [214-216], (ii) inhibited [217], or (iii) promoted via stimulation of $P2Y_2$ receptors [195, 218]. ATP may also indirectly affect neutrophil migration by modulating formation of the potent neutrophil chemoattractant leukotriene (LT)-B_4, which is formed from arachidonic acid (AA) through the 5-lipoxygenase pathway. High micromolar ATP concentrations were shown to inhibit the release of AA, whereas low micromolar concentrations stimulated AA release by activating neutrophil $P2Y_2$ receptors [219, 220]. These findings suggest that ATP may exert a dual modulatory role on neutrophil migration during inflammation. At low micromolar concentrations, ATP may promote neutrophil accumulation via $P2Y_2$ receptor activation, either directly by acting as a chemoattractant or indirectly by facilitating LTB_4 production. Moreover, since ATP has been shown to stimulate production of the CXC chemokine CXCL8 (also known as IL-8) by both eosinophils and astrocytes [221, 222], ATP-mediated stimulation of chemokine release by cells near sites of tissue damage may contribute to neutrophil recruitment towards these sites. Upon arriving at inflamed sites where ATP levels are the highest, neutrophil migration may be no longer affected, even inhibited by ATP, allowing the neutrophils to exert their bactericidal functions.

Bactericidal mechanisms. The first step in the bactericidal function of neutrophils is phagocytosis of pathogens [223], which has been shown to be stimulated by low micromolar concentrations of both ATP and ADP via activation of Mac-1 [224, 225]. Next, efficient pathogen destruction requires the mobilization of microbicidal molecules (*i.e.* degranulation and oxidative burst) either into the phagolysosome or into the extracellular space [226, 227]. Putatively through activation of $P2Y_2$ receptors [180, 197], micromolar concentrations of extracellular ATP stimulate the degranulation of both primary (azurophilic) [180, 197, 220, 228-236] and secondary (specific) granules [216, 220, 231, 233, 237]. $P2Y_2$ receptor-mediated LTB_4 generation, which may subsequently enhance granule secretion in an autocrine manner, has been proposed as a mechanism for nucleotide-induced neutrophil degranulation during inflammation [238-240]. In addition to stimulating degranulation, extracellular ATP has been shown to contribute to the initiation of the oxidative burst. ATP appears to prime neutrophils for functional responses to various inflammatory mediators, as indicated by increased production of reactive oxygen species (ROS, *e.g.* O_2^- and H_2O_2) [180, 183, 201, 204, 216, 218, 224, 228, 232, 234, 235, 241-255]. The $P2Y_2$ receptor has been suggested to be the P2 receptor subtype involved in neutrophil priming [180, 232, 244, 252, 255]. Extracellular ATP at millimolar concentrations may induce ROS production even in quiescent neutrophils via stimulation of $P2X_7$ receptors [194].

Apoptosis. Neutrophil apoptosis and subsequent ingestion by macrophages is considered as the main mechanism for clearing neutrophils from inflamed areas and thereby for promoting the resolution of acute inflammatory responses [256]. Extracellular ATP at micromolar concentrations has been shown to delay neutrophil apoptosis in synergy with the neutrophil survival factor granulocyte macrophage colony-stimulating factor (GM-CSF), thus extending the functional life span of neutrophils [257].

4.1.2 Adenosine

Receptors. Neutrophils express all four P1 receptor subtypes [183, 258-269]. P1 receptor expression on neutrophils may be affected by the presence of an inflammatory reaction, as changes in A_2 receptor expression by human neutrophils were found in some rheumatic diseases [265]. Fortin et al. (2006) have recently shown that A_{2A} receptors were up-regulated in human PMN in response to stimulation with LPS and TNFα [269].

Adhesion. The effects of Ado on neutrophil recruitment from the circulation appear to be bi-directional. At submicromolar concentrations, Ado has been shown to enhance neutrophil adhesion to the vascular endothelium by stimulation of A_1 receptors on both neutrophils [270, 271] and endothelial cells [272, 273]. A_1 receptor-mediated up-regulation of endothelial P-selectin expression as well as of the expression of Mac-1 by neutrophils may be responsible for the enhanced adhesion [273]. In contrast, extracellular Ado at micromolar levels inhibits adhesion of neutrophils to vascular endothelial cells [192, 264, 270-272, 274-290], which is thought to be mediated by A_{2A} and A_{2B} receptors expressed by neutrophils [192, 264, 282-284, 291]. Endothelial A_3 receptors may also contribute to the Ado-mediated inhibition of adhesion [279]. Extracellular Ado has been suggested to inhibit neutrophil rolling by interfering with binding of selectins [277]. Indeed, micromolar Ado concentrations were shown to inhibit E-selectin expression on vascular endothelial cells [292]. Firm adhesive processes may also be affected by extracellular Ado. Firm adhesion is thought to be mediated by binding of neutrophil integrins to cellular adhesion molecules expressed by endothelial cells. Although some data suggest that Ado-mediated inhibition of adhesion is β_2-integrin-independent [277, 287, 293], several studies have shown that Ado may inhibit up-regulation of Mac-1 expression by activated neutrophils [284, 294, 295]. Based on the finding that an Ado analogue down-regulated intercellular adhesion molecule (ICAM)-1 expression on endothelial cells [296], Ado-mediated inhibition of firm adhesion may thus involve attenuated binding of Mac-1 to ICAM-1 [285]. However, effects of Ado on ICAM-1 remain controversial [292, 293]. Recently, Sullivan et al. (2004) demonstrated that stimulation of A_{2A} receptors attenuated up-regulation of very late antigen (VLA)-4 (α_4/β_1-integrin) expression in immunostimulated neutrophils and inhibited vascular cell adhesion molecule (VCAM)-1-dependent adhesion to the vascular endothelium [291]. VCAM-1 expression has previously been shown to be down-regulated by micromolar Ado concentrations [292]. A role for endogenous Ado in neutrophil adhesion has also been implicated under inflammatory conditions *in vivo*. Li et al. (2000) showed that infusion of Ado (40 µg/kg/min for 4 hours) in humans inhibited endotoxin-induced leukocyte adhesion [297]. Moreover, the A_{2A} agonist ATL-146e (4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester) has been shown to reduce increased expression of VCAM-1, ICAM-1, platelet endothelial cell adhesion molecule (PECAM)-1 and P-selectin *in vivo* [298-300].

Both transmigration and extravasation of neutrophils following their adherence to the endothelium also appear to be affected by extracellular Ado. Local application of the P1 receptor antagonist 8-phenyltheophylline was shown to augment LTB_4-induced macromolecular permeability changes as well as granulocyte diapedesis in a hamster cheek pouch model of microcirculation, possibly as a result of antagonizing the inhibitory effects of endogenous Ado [301]. In accordance with this, Ado has been shown to enhance the barrier function of the vascular endothelium via A_{2B} receptor activation on endothelial cells, thereby decreasing paracellular permeability to neutrophils [184, 191, 302]. Both basal permeability and oxidant-induced increased permeability may be reduced by micromolar concentrations of extracellular Ado [303]. The reduction in permeability may result from A_{2B} receptor-mediated stimulation of endothelial CD73 activity, which leads to increased endogenous Ado concentrations by promoting AMP breakdown [304]. Endogenous Ado may thus be part of a positive feedback loop regulating endothelial barrier function, since CD73-generated Ado acting on A_{2B} receptors was recently identified as a key control point in preserving endothelial barrier function during hypoxia [305]. In addition to endothelial A_{2B} receptors, neutrophil A_{2B} receptors may also be involved since high micromolar Ado concentrations were shown to inhibit the release of vascular endothelial growth factor (VEGF) by neutrophils, thereby promoting endothelial barrier function and inhibiting transmigration of neutrophils [293]. Extracellular Ado has also been shown to attenuate neutrophil-induced damage to the endothelium [275, 285, 306].

Overall, although the above information would suggest that extracellular Ado may bi-directionally regulate neutrophil recruitment depending upon its extracellular concentration, Ado's inhibitory effects probably prevail under inflammatory and hypoxic conditions during which extracellular Ado levels rise markedly. Recently, Eltzschig et al. (2004) provided evidence that extracellular Ado participates in an endogenous pathway regulating leukocyte recruitment to inflamed sites [192]. By conducting experiments in wild-type as well as in CD39- and CD73-null mice, they demonstrated that activated neutrophils release ATP during hypoxia. Hypoxia-induced increase in CD73 and CD39 expression on the vascular endothelium resulted in the rapid formation of Ado from breakdown of the released ATP, which then inhibited neutrophil adhesion via A_2 receptor activation on neutrophils [192]. Extracellular Ado levels may remain elevated under hypoxic conditions by repressed equilibrative nucleoside transporter (ENT) expression on endothelial cells [307]. Endogenous Ado-mediated signaling may thus limit excessive accumulation of neutrophils within tissues.

Migration. After diapedesis, neutrophils migrate away from the vascular endothelium up a gradient of chemoattractants produced in inflamed tissues. By navigating through complex chemoattractant fields in a multistep process responding to one agonist source after the other, neutrophils migrate towards their final target within a tissue. Directed migration (*i.e.* chemotaxis) of neutrophils is promoted by nanomolar concentrations of Ado through activation of neutrophil A_1 receptors [308, 309]. Ado appears to have no effect on chemoattractant-induced neutrophil chemotaxis at higher concentrations (*i.e.* micromolar) [215]. However, Ado at these concentrations has been shown to counteract TNFα-mediated inhibition of neutrophil chemotaxis by restoring directed migration of neutrophils to the chemoattractant formyl-methionyl-leucyl-phenylalanine (fMLP) [310]. Micromolar concentrations of Ado may also indirectly affect neutrophil migration away from the endothelium via its inhibitory effect on the release of the chemokine

CXCL8 by activated endothelial cells [292]. Taken together, endogenous Ado might thus be involved in directing neutrophils and promoting their arrival at infected or damaged tissues. Ado may thereby minimize neutrophil-induced collateral damage to healthy tissues.

Bactericidal mechanisms. The protection of host tissues from potentially damaging neutrophils is also accomplished by extracellular Ado through modulation of the bactericidal functions of neutrophils (*i.e.* phagocytosis, oxidative burst, degranulation). Ado has been shown to dually regulate phagocytosis in that it enhanced phagocytosis by activating A_1 receptors, whereas Ado inhibited phagocytosis through activation of A_2 receptors [294, 311, 312]. Accordingly, Ado may also dually regulate the oxidative burst of neutrophils. Whereas submicromolar Ado concentrations were shown to enhance the generation of ROS via A_1 receptor activation [311], micromolar concentrations of Ado inhibit ROS generation by immunostimulated neutrophils through activation of A_{2A} receptors [180, 183, 185-188, 215, 216, 241, 242, 245-247, 249, 257, 259, 260, 263, 264, 269, 274, 279, 280, 283, 285, 286, 288, 300, 306, 309, 311, 313-340]. Ado-mediated inhibition of the oxidative burst has also been confirmed *in vivo.* In a porcine model of hyperdynamic endotoxemia, Thiel et al. (1997) showed that intravenous infusion of Ado (150 µg/kg/min for 6 hours) strongly inhibited the extracellular release of ROS, without affecting intracellular ROS production [341]. Extracellular Ado may thus protect healthy tissues from ROS-mediated injury without compromising the intracellular destruction of phagocytized pathogens. Additionally, neutrophils eliminate pathogens by degranulation of microbicidal molecules [226]. Ado at micromolar concentrations inhibits the degranulation of both primary [185, 231, 258, 279, 326, 328, 333, 335, 336, 340, 342, 343] and secondary granules [215, 231, 264, 316, 326, 328, 344] by activated neutrophils. Neutrophil A_{2A} [264, 333, 335, 336] and A_3 receptors [258, 336] may both be involved in the inhibitory effect of Ado on neutrophil degranulation. Ado at lower concentrations (*i.e.* submicromolar) appears to have no effect on degranulation of primary and secondary granules [286, 332], nor on the degranulation of secretory granules [332].

Inflammatory mediators. Besides playing an important role in eliciting and sustaining inflammation, neutrophils contribute to the regulation of immune responses by releasing a variety of inflammatory mediators [345]. Cadieux et al. (2005) recently demonstrated that A_{2A} receptor activation up-regulated the expression of cyclooxygenase (COX)-2 in neutrophils. It was shown that neutrophils from A_{2A} knock-out mice had less COX-2 induction than wild-type mice following LPS-induced inflammation in a murine air pouch model [346]. In immunostimulated human neutrophils, A_{2A} receptor activation also enhanced COX-2 expression and increased generation of prostaglandin (PG)-E_2, a prostanoid with anti-inflammatory properties [346, 347]. Ado-mediated inhibition of the generation of several inflammatory mediators by neutrophils has also been demonstrated. First, Ado was shown to inhibit the formation of LTB_4 by activated neutrophils due to a decreased A_{2A} receptor-mediated arachidonic acid release by neutrophils [348-352]. Second, Ado at micromolar concentrations was shown to inhibit the production of platelet activating factor (PAF) by fMLP-activated neutrophils [320]. Third, production of the pro-inflammatory cytokine TNFα, and of the chemokines CCL3, CCL4, CCL20 and CXCL2 (also known as macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-3α and MIP-2α, respectively) by LPS-stimulated neutrophils was shown to be profoundly inhibited by Ado via activation of A_{2A} receptors [353, 354]. Thus, by up-regulating COX-2 expression with resulting generation of anti-inflammatory PGE_2,

together with inhibiting the production of several pro-inflammatory mediators, purinergic signaling by endogenous Ado might counteract exuberant neutrophil activation.

Apoptosis. As mentioned before, the role of neutrophils during inflammatory processes is terminated by their apoptosis. High micromolar concentrations of Ado have been shown to induce apoptosis of HL-60 cells through stimulation of A_3 receptors [261]. Oppositely, P1 receptor agonists consistent with a pharmacological profile of the A_{2A} receptor subtype have been shown to delay the onset of neutrophil apoptosis [355, 356]. This has been confirmed *ex vivo* by the finding that low micromolar Ado levels in synovial fluids of patients with rheumatoid arthritis (RA) correlated with the anti-apoptotic activity of these synovial fluid samples on neutrophils [357, 358]. However, this is likely to be of minor relevance *in vivo*, since ADA activity is elevated in the synovial fluids of RA patients, which could lead to a decrease in Ado concentrations below its anti-apoptotic threshold in these fluids [359]. Although this increased ADA activity might counteract Ado-mediated effects on neutrophil apoptosis, it does probably not diminish the *in vivo* anti-inflammatory and anti-rheumatic effects of Ado [45, 46, 53, 54], which are thought to be mediated via multiple P1 receptor subtypes [29, 34, 46, 281].

4.1.3 Modulation of neutrophil function by ATP and adenosine

Neutrophil function appears to be reciprocally modulated by endogenous ATP and Ado, depending on their extracellular concentrations. This would suggest that changing levels of extracellular ATP and Ado in the microenvironment of neutrophils may contribute to the fine-tuning of neutrophil action during inflammatory processes. Neutrophil effector functions appear to be enhanced by co-ordinated effects of high ATP and low Ado levels, while increasing Ado levels mostly suppress effector functions of inflammatory neutrophils. Accordingly, neutrophil function has been shown to be affected in a biphasical manner, that is, initial stimulation by ATP via P2 receptor activation followed by P1 receptor-mediated inhibition by Ado, which is formed from ATP upon prolonged incubation of neutrophils [180, 183, 207, 216]. Increased extracellular levels of ATP, ensuing upon tissue damage and concomitant early inflammatory processes, constitute a danger signal, which triggers several pro-inflammatory effector functions of neutrophils. ATP breakdown may be delayed during early inflammatory processes by the action of several inflammatory mediators, which thereby amplify pro-inflammatory signaling by high levels of extracellular ATP. Activity of endothelial CD39 and CD73 has been shown to be inhibited by TNFα [360, 361], whereas the activity of ADA and adenosine kinase appears to be preserved [361]. Oxidative stress may also be involved in inhibiting the activity of endothelial CD39 and CD73 during acute inflammation [181, 360, 362, 363]. Rising of extracellular Ado concentrations may be prohibited in this way, so that several A_1 receptor-mediated pro-inflammatory effects may predominate during acute inflammatory responses and operate in synergy with the high ATP levels.

However, extracellular Ado concentrations will eventually rise upon continuation of the inflammatory response by breakdown of excessive extracellular ATP. Increased Ado levels provide a negative feedback signal that will mostly counteract the ATP-mediated neutrophil activation. High levels of extracellular Ado may be conserved by several mechanisms. Tissue hypoxia arising during ongoing inflammatory responses decreases nucleoside transporter expression and increases the activity of both CD73 and CD39 in endothelial and epithelial cells, thereby promoting and maintaining high Ado levels

[191, 192, 307, 364, 365]. Hypoxic stress also modulates endothelial Ado receptor expression towards a predominant A_{2B} receptor phenotype, which preserves endothelial barrier function and may contribute to angiogenesis and tissue healing [366]. Endothelial activity of both CD39 and CD73 may also be enhanced by shear stress due to increased blood flow [367]. Ado itself may even contribute to maintaining its increased extracellular levels by enhancing the expression and activity of endothelial CD73 [304]. Moreover, the activity of alkaline phosphatase is enhanced upon neutrophil activation [332], whereas the activity of ADA may be inhibited upon sustained inflammation [182]. Thus, upon progression of inflammatory responses, rising levels of extracellular Ado may provide negative feedback signals to prevent over-recruitment and over-activation of potentially harmful neutrophils. Up-regulation of neutrophil A_{2A} receptor expression in response to pro-inflammatory mediators, which are present in inflammatory exudates, further sensitizes inflammatory neutrophils to Ado-mediated negative feedback signaling [269, 368]. In this way, Ado may contribute to the resolution of acute inflammatory processes as well as the minimization of collateral tissue damage.

4.2 Monocytes and macrophages

Mononuclear phagocytes are innate immune cells that reside in the bloodstream as monocytes or in various tissues as macrophages. In contrast to neutrophils, which principally contribute to acute inflammatory responses, monocytes/macrophages (Mo/Mø) are a major component of chronic inflammatory responses. Monocyte-like precursors colonize extravascular sites early during embryogenesis to become resident macrophages, acquiring morphological and functional properties that are characteristic for the tissue in which they reside (*e.g.* Kupffer cells in the liver, microglial cells in the brain). During postnatal life, circulating monocytes are capable of migrating into various tissues in response to damage or infection, where they transform into macrophages. Macrophages are a major source of inflammatory mediators and thereby modulate the course of inflammatory and immune responses [369, 370]. Naive macrophages entering inflamed tissues have the unique ability to display distinct functional phenotypes during a progressive inflammatory response by continuously adapting their effector functions to the huge array of inflammatory factors that are present in the tissue microenvironment [371]. Depending on the profile of these environmental stimuli, inflammatory macrophages generally are either classically or alternatively activated. Classically activated macrophages, induced by IFNγ plus TNFα or Toll-like receptor (TLR) ligands, are typical effectors of cell-mediated immunity. Enhanced secretion of both pro-inflammatory cytokines and microbicidal molecules renders them immunostimulatory and cytotoxic as well as potentially injurious. Effector functions of classically activated macrophages must be tightly regulated to prevent uncontrolled excessive inflammation that would result in destruction of healthy tissues. Alternative activation of macrophages by IL-4 and IL-13 or by phagocytosis of apoptotic cells antagonizes classical activation. Alternatively activated macrophages secrete the anti-inflammatory cytokines IL-1ra, IL-10 and transforming growth factor (TGF)-β, thereby providing signals to deactivate macrophages as well as to mediate immunosuppressive and healing processes. Both macrophage phenotypes are present in inflamed tissues, and it is believed that coordinated switching between these two phenotypes determines the outcome of inflammatory processes [372-374]. Mo/Mø exhibit CD39 as well as CD73 activity [375-

378], and are capable of releasing both ATP and Ado upon activation [379-386], rendering them susceptible to autocrine and paracrine purinergic regulation by extracellular ATP and Ado.

4.2.1 ATP

Receptors. Mo/Mø express multiple P2 receptor subtypes, *i.e.*, monocytes express $P2Y_{1,2,4,6,11,12,13}$ and $P2X_{1,4,5,7}$ receptors [160, 162, 163, 165, 177, 178, 193, 198, 203, 376, 382, 387-396], and macrophages express the same receptor subtypes except for $P2Y_{13}$ [160, 162, 165, 177, 198, 388, 397-404]. P2 receptor expression may depend on maturation stage, since up-regulated expression of $P2X_7$ as well as P2Y receptors was noted upon differentiation of monocytes into macrophages [160, 388, 405]. P2 receptor expression may also depend on the nature of cellular activation, since $P2X_7$ receptor expression and function is up-regulated following classical activation of Mo/Mø by IFNγ, TNFα or LPS [161-164, 389, 391]. Classical activation of Mo/Mø with IFNγ and LPS has been shown to induce down-regulation of $P2Y_2$ receptor expression [161, 165]. In contrast, activation of rat alveolar macrophages by the Th_2 cytokines IL-4 and IL-10 was shown to induce down-regulation of functional $P2X_7$ receptors [164]. A recent study showed that $P2X_7$, $P2Y_1$ and $P2Y_2$ receptor subtypes were up-regulated during a chronic inflammatory reaction induced in rats [406].

Recruitment. During inflammatory and immune responses, circulating monocytes are recruited to inflamed sites. Recruitment of inflammatory monocytes involves adhesive interactions with vascular endothelial cells as well as chemoattractant-mediated directed migration [407-409]. Extracellular ATP and ADP have been shown to increase adhesiveness of human promonocytic U-937 cells [410, 411], suggesting that endogenous nucleotides may contribute to the adhesion of inflammatory monocytes to the vascular endothelium. Interaction of selectins with their respective ligands slows down rapidly flowing monocytes (*i.e.* monocyte rolling), a process to which ATP may contribute by up-regulating E-selectin expression through $P2X_7$-mediated activation of NFκB [211, 212]. ATP-induced up-regulation of E-selectin is augmented upon inflammatory activation of endothelial cells with TNFα [211]. Binding of VLA-4 to VCAM-1 causes further slowing down of monocyte rolling, which results in loose adhesion. At low micromolar concentrations, ATP was shown to promote VCAM-1-dependent loose adherence by enhancing VCAM-1 expression through activation of endothelial $P2Y_2$ receptors [412, 413]. Activation of adhered monocytes increases the affinity of the monocyte β_2-integrins Mac-1 and lymphocyte function-associated antigen (LFA)-1 (CD11a/CD18) for binding to cellular adhesion molecules on endothelial cells, which leads to firm adhesion. Both ATP and ADP at micromolar concentrations have been shown to enhance expression and function of Mac-1 in monocytes, possibly due to P2Y receptor stimulation [208, 414-417]. Firmly adhered monocytes subsequently transmigrate through the endothelium. Stimulation of monocyte $P2X_7$ receptors has been shown to induce shedding of L-selectin from the surface of monocytes [52, 392]. This might indicate that ATP at high extracellular levels contributes to activation and transmigration of monocytes, as leukocytes shed L-selectin upon activation and during (trans-)migration. Indeed, ATP was shown to stimulate transmigration of murine monocytes through an endothelial cell monolayer [416]. Transmigration of inflammatory monocytes occurs mainly by passing between adjacent endothelial cells, a process for which endothelial paracellular junctions need to be breached. ATP may facilitate transmigration by either inducing apoptosis of endothelial cells, which disturbs the integrity of the endothelial barrier [211, 212], or by inducing shrinkage of endothelial cells

through activation of P2Y receptors, which may also be a mechanism for increasing endothelial macromolecule permeability [213].

Subsequent to transmigration, monocytes migrate to inflammatory foci in response to various chemoattractants. Both ATP and ADP at micromolar concentrations have been shown to stimulate chemotaxis of murine monocytes as well as rat microglial cells [416, 418, 419]. Similar levels of extracellular ATP were also shown to stimulate chemokinesis (*i.e.* undirected cell motility) of human monocytes [396, 420]. Although the mechanism by which extracellular nucleotides mediate these chemotactic effects have not been clarified to date, stimulation of monocyte migration could be P2 receptor-mediated. ADP-induced chemotaxis was shown to be G-protein dependent, which is suggestive of P2Y receptor involvement [418, 419]. Goepfert et al. (2001) demonstrated the importance of CD39 in ATP-mediated stimulation of monocyte migration. In contrast to wild-type cells, monocytes from CD39-null mice showed no migratory response to extracellular ATP, which was suggested to be due to P2Y receptor desensitization [416]. Extracellular nucleotides may contribute to recruitment of additional leukocytes to inflamed sites by increasing chemokine production, since production of CXCL8 by LPS-activated THP-1 monocytes as well production of CXCL8, CXCL10 (also known as IFNγ inducing protein (IP)-10) and the CC chemokine CCL2 (also known as monocyte chemoattractant protein (MCP)-1) by U-937 monocytic cells were increased by uridine 5'-diphosphate (UDP) through activation of monocyte $P2Y_6$ receptors [382, 421].

Inflammatory mediators. Production of soluble immunomediators such as cytokines, chemokines and eicosanoids is essential in immunity and involves the activation of several transcription factors, including NFκB and activator protein (AP)-1. NFκB is activated in Mo/Mø by stimulation of $P2X_7$ receptors [390, 422-425]. P2Y receptors may also be involved, as suggested by the observation that both ADP and uridine 5'-triphosphate (UTP) can induce NFκB activation in macrophages. Whereas ADP induced NFκB activation in quiescent cells [426], UTP enhanced LPS-induced NFκB activation via activation of $P2Y_6$ receptors in mouse macrophages [427, 428]. UTP also enhanced LPS-induced AP-1 activation in mouse macrophages [427]. Moreover, ATP at micromolar concentrations has been shown to potentiate TNFα-induced NFκB activation in human KBM-5 monocytes [429].

Mo/Mø are major cytokine-producing cells and by contributing to the cytokine network during immune responses, these cells form a link between innate and adaptive immunity. Extracellular nucleotides appear to have a considerable impact on cytokine production by Mo/Mø. Many studies have confirmed that ATP at millimolar concentrations stimulates production of IL-1α [164, 430-434], IL-1β [52, 162, 164, 380, 384, 389-392, 394, 424, 430-452], IL-6 [164, 393, 432, 444], IL-18 [391, 453, 454] and TNFα [393, 424, 432, 455]. These stimulatory effects are likely mediated through $P2X_7$ receptor activation [162, 164, 380, 389-394, 424, 430, 433-435, 437, 439-449, 451, 453, 454]. Recently, Denlinger et al. (2004, 2005) showed that LPS-stimulated whole blood samples of individuals with polymorphisms in $P2X_7$ alleles, which resulted in impaired monocyte $P2X_7$ pore-forming function, had lower levels of TNFα and higher levels of IL-10 relative to LPS-treated samples with normal monocyte $P2X_7$ function, *i.e.*, high pore activity [456, 457]. $P2X_7$ receptors may thus be involved in regulating the production of a wide variety of inflammatory cytokines by Mo/Mø. P2Y receptors may also contribute to cytokine production by Mo/Mø.

Production of TNFα by human monocytic U-937 cells was increased by low micromolar concentrations of UDP via activation of the $P2Y_6$ receptor subtype [421]. Hanley et al. (2004) recently demonstrated that both ATP and UTP at low micromolar concentrations increased expression of IL-6 in human macrophages, possibly via activation of $P2Y_2$ receptors. The authors suggested that extracellular ATP may contribute to inflammatory activation of macrophages during an immune response [403]. Activation of P2Y receptors was shown to also increase IL-6 expression and production by LPS-stimulated murine macrophages via a NFκB-dependent mechanism [458]. A single study in LPS-stimulated peritoneal mouse macrophages suggested that ATP at micromolar levels inhibited production of IL-12 and TNFα, and enhanced IL-10 production. However, these effects were prevented substantially by the addition of ADA, suggesting that the observed effect of ATP was predominantly due to its degradation to Ado [459]. In LPS-stimulated human monocytes, low micromolar concentrations of ATP were recently shown to inhibit TNFα and CCL2 production and to increase IL-10 production [396].

Microglial cells are resident macrophages in the central nervous system [460]. Therefore, the effects of extracellular nucleotides on cytokine production by these cells mostly resemble their effects in Mo/Mø. Microglial production of IL-1α [447, 461], IL-1β [379, 447, 461-467], IL-6 [468, 469], IL-18 [447], TNFα [253, 447, 464, 465, 468-472] and leukemia inhibitory factor (LIF) [473] is increased through activation of $P2X_7$ receptors by millimolar ATP concentrations [379, 447, 461, 462, 464, 465, 468, 469, 471, 472]. In contrast, the production of several pro-inflammatory cytokines (*i.e.* TNFα, IL-1β, IL-6, IL-12, and/or CCL3) by cultured microglial cells from rat embryos and newborn mice in response to LPS was inhibited by micromolar ATP concentrations, presumably through stimulation of P2Y receptors [470, 474]. Attenuation of IL-6 production was also observed when LPS-activated human microglial cells were incubated with 300 μM benzoyl ATP (BzATP), an agonist of $P2X_7$ and $P2Y_{11}$ receptors [447]. Recently, it was shown that LPS-stimulation of rat microglia caused the release of low levels (nM) of ATP which, in an autocrine manner putatively via P2Y receptors, induced IL-10 expression; no effect on TNFα production was observed [475]. Taken together, the effects of extracellular nucleotides on cytokine production by cells of the Mo/Mø lineage are not straightforward and may depend on the cell type studied, the agonist used, and its concentration. Several reports indicate that ATP at millimolar levels induces a pro-inflammatory cytokine profile through activation of $P2X_7$ receptors, whereas the effects of lower ATP levels, which may occur via P2Y receptor activation, have not yet been fully elucidated.

IL-1β is a potent pro-inflammatory cytokine mediating acute inflammatory responses. Mechanisms involved in ATP-mediated stimulation of IL-1β release have been extensively studied. Thus, extracellular ATP appears to be a crucial signal that triggers the synthesis and release of mature IL-1β following Mo/Mø priming by an inflammatory signal such as LPS. Binding of ATP to $P2X_7$ receptors causes opening of the $P2X_7$ channel and depletion of intracellular potassium, resulting in activation of Ca^{2+}-independent phospholipase A_2 ($iPLA_2$), which induces generation of active caspase-1 that catalyzes the formation of mature IL-1β from proIL-1β [384, 391, 392, 430, 433, 438, 441-444, 448, 449, 452]. $P2X_7$ receptor stimulation also induces a rise in intracellular Ca^{2+}, which mediates the release of mature IL-1β [434, 440, 452]. Secretion of mature IL-1β may depend on ATP-binding cassette (ABC)-1 transporters [432, 436, 476], may involve shedding of microvesicles [385, 477] and has recently been suggested to occur via Ca^{2+}-dependent activation of cytosolic PLA_2 ($cPLA_2$) which induces exocytosis of secretory

lysosomes [452, 478]. Since ATP triggers the externalization of both mature IL-1β and caspase-1 [438, 452], processing of mature IL-1β by caspase-1 probably occurs within the secretory lysosomes [452, 478]. P2Y-mediated mobilization of calcium from intracellular stores might also contribute to the secretion of IL-1β [434, 440]. ATP-induced release of IL-18 may occur via similar mechanisms [391, 453]. Recent data indicate that $P2X_7$ activation may also induce the release of IL-1ra concurrent with IL-1β by LPS-primed mouse macrophages [385]. This would suggest that extracellular ATP not only triggers IL-1β production, but also affects the balance between IL-1 and IL-1ra which is of importance in determining the outcome of inflammatory responses.

Another group of inflammatory mediators produced by Mo/Mø are eicosanoids [479]. These are lipid mediators which are synthesized from arachidonic acid (AA). AA derives from PLA_2-mediated hydrolysis of membrane phospholipids and can be further metabolized via either the 5-lipoxygenase (5-LO) or the COX pathway. The 5-LO pathway generates leukotrienes (*e.g.* LTB_4, LTC_4), while the COX pathway, which comprises constitutively expressed COX-1 as well as inducible COX-2, generates prostaglandins (*e.g.* PGE_2, PGD_2) [480, 481]. Pfeilschifter et al. (1989) showed that production of PGE_2 and LTC_4 by mouse peritoneal macrophages was increased upon stimulation of P2Y receptors [482]. Pyrimidinergic P2Y receptor subtypes (*i.e.* $P2Y_4$ and $P2Y_6$) may in fact be the P2Y receptors mediating eicosanoid synthesis. Uridine nucleotides were shown to stimulate AA release via Ca^{2+}-dependent $cPLA_2$ activation in mouse macrophages [483, 484], resulting in formation of the cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4, but not LTB_4 [485]. UTP, besides inducing production of 5-LO derivatives, also increased COX-1-dependent PGE_2 production by mouse macrophages [400]. Release of AA in response to UTP has been shown to be enhanced upon priming of macrophages with LPS [486]. Accordingly, stimulation of $P2Y_6$ receptors by UTP markedly potentiated PGE_2 production by LPS-primed murine macrophages via NFκB-dependent up-regulation of COX-2 expression [400, 458]. Since UTP enhanced LPS-induced AA release through stimulation of Ca^{2+}-independent $iPLA_2$ activity [400], Chen et al. (2000) hypothesized that extracellular uridine nucleotides may initiate divergent pathways depending on the state of cellular activation. According to these authors, PGE_2 production by unprimed macrophages may be increased by UTP through activation of $cPLA_2$ and COX-1, whereas UTP may potentiate PGE_2 production by classically activated cells via activation of $iPLA_2$ and NFκB-dependent up-regulation of COX-2 expression. Besides P2Y receptor involvement, $P2X_7$ receptors may also contribute to eicosanoid production [483]. In Mo/Mø, both LPS-induced AA release and COX-2 expression have been shown to be enhanced by stimulation of $P2X_7$ receptors [390, 487]. This might be indicative of $P2X_7$ receptor involvement in prostaglandin synthesis during inflammation, as COX-2 is an inducible enzyme that is dramatically up-regulated upon inflammatory activation [481].

Cytotoxicity. An important function of inflammatory macrophages is phagocytosis and elimination of potentially harmful pathogens. For this purpose, macrophages are equipped with a diverse array of cytotoxic effector functions, including the secretion of proteolytic enzymes and the production of ROS and reactive nitrogen species (RNS) [488-490]. However, uncontrolled cytotoxicity and cell killing may result in considerable collateral damage to healthy tissues. ATP has been shown to modulate macrophage phagocytosis bi-directionally depending on its extracellular concentration. Phagocytosis was inhibited through $P2X_7$ receptor activation at millimolar ATP concentrations, while it was stimulated

through activation of $P2Y_2$ receptors at lower ATP levels [491, 492]. Proteolytic enzyme secretion is affected in a similar bi-directional fashion. Whereas ATP at millimolar levels has been shown to increase lysosomal enzyme secretion via stimulation of $P2X_7$ receptors in macrophages [52, 442, 493], ATP as well as ADP inhibited lysosomal enzyme secretion at micromolar concentrations in one study [494].

As part of their cytotoxic and antimicrobial effector functions, macrophages generate RNS [490]. Three types of nitric oxide synthases (NOSs) are involved in RNS production, *i.e.*, NOS1 (nNOS or ncNOS), NOS2 (iNOS) and NOS3 (eNOS or ecNOS). Expression of NOS1 and NOS3 is constitutive, whereas NOS2 expression is induced following classical activation of macrophages. In general, NOS1 and NOS3 comprise the so-called low-output pathway that encompasses physiological functions of NO in the healthy host, whereas NOS2 is responsible for the high-output pathway that is engaged during inflammation and infection. NO production as a result of this latter pathway leads to sustained cytotoxic effects. Extracellular ATP at micromolar concentrations was shown to enhance LPS-induced NO production and NOS2 expression in mouse macrophages [455, 495, 496]. $P2X_7$ receptors may be involved in ATP-mediated RNS production, since the $P2X_7$ receptor antagonist oxidized ATP (oATP) has been shown to inhibit LPS- and TNFα-induced NO production and NOS2 expression [377, 422-424, 497]. Also, the potent $P2X_7$ receptor ligand BzATP was shown to enhance NO production and NOS2 expression by immunostimulated macrophages [424, 425, 496]. In addition, Guerra et al. (2003) demonstrated that mouse macrophages, in which $P2X_7$ receptors were rendered inactive by a mutation in the transmembrane domain, were hypo-responsive to BzATP with regard to LPS-induced NO production [424]. However, involvement of the $P2X_7$ receptor subtype in macrophage RNS production has been questioned, since oATP-induced inhibition of NO production was reported to be independent of $P2X_7$ receptor antagonism [377, 498, 499]. Moreover, Sikora et al. (1999) found that LPS-stimulated NO production by macrophages from both wild-type and $P2X_7$ knock-out mice was identical, suggesting that $P2X_7$ receptor activation may not be required for NO production by macrophages [497].

Besides a putative role of the $P2X_7$ receptor, P2Y receptors may contribute to macrophage RNS production. In addition to ATP, both ADP and UTP have been shown to enhance LPS-stimulated NO production and NOS2 expression in mouse macrophages [400, 427, 458, 495]. The UTP-induced effect was proposed to be mediated by $P2Y_6$ receptors [400]. Furthermore, intriguing data derive from several *in vivo* studies in which divergent purinergic modulation of NO production and NOS2 expression by macrophages has been noted. The non-selective P2 receptor agonist 2-methylthio-ATP (2-MeS-ATP) was shown to attenuate increased NO production and NOS2 expression by macrophages of mice treated with LPS [500], whereas it stimulated macrophage NO production and NOS2 expression in rats [501, 502]. These latter effects were independent of LPS-induced macrophage activation nor involved NFκB activation, suggesting an enhancement of RNS production without a concomitant inflammatory response [501, 502].

In addition to RNS, Mo/Mø are capable of producing ROS. Extracellular nucleotides at micromolar concentrations have been shown to stimulate ROS generation by rat alveolar macrophages [426, 503], guinea pig peritoneal macrophages [504] and human monocyte-derived macrophages [398]. Stimulation

of ROS generation was shown to be $P2X_7$ receptor-independent [497] and may actually involve activation of several P2Y receptor subtypes [503].

Cell death. Transient binding of ATP to $P2X_7$ receptors on Mo/Mø generates a reversible plasma membrane pore allowing influx and efflux of molecules up to 0,9 kDa in mass [160, 161, 164, 384, 388, 389, 448, 462, 466, 505-511]. Prolonged activation of Mo/Mø $P2X_7$ receptors by millimolar concentrations of extracellular ATP appears to trigger cell death, which is either apoptotic or necrotic depending on the strength and duration of receptor activation [160, 161, 164, 388, 422, 433, 443, 461, 505-507, 509, 512-516]. Interestingly, intermittent exposure of Mo/Mø to extracellular ATP at low millimolar concentrations is believed to trigger a 'suicide program' that is inevitably followed by apoptosis, even if ATP is no longer present [517-519]. This may result from activation of intracellular caspases (*e.g.* caspase-1 and -3) [433, 443]. Classical activation appears to sensitize macrophages to the lytic effects of ATP [160, 161, 388, 389, 433, 515, 520]. Taken together, sustained exposure of $P2X_7$ receptors to high ATP levels during inflammation may initiate a delayed feedback signal to terminate potentially aberrant inflammatory responses by over-activated macrophages.

4.2.2 Adenosine

Receptors. All four Ado receptors are expressed by both monocytes [170, 381, 383, 521-528] and macrophages [47, 166, 262, 381, 529-536]. Receptor expression and function appear to change during maturation. Whereas expression of the A_1, A_2 and A_3 receptor subtypes is relatively low in quiescent monocytes [521, 529], receptor expression increases during differentiation into macrophages [262, 381, 405, 527, 529, 530]. Receptor expression also appears to change upon inflammatory activation of Mo/Mø. A_{2A} receptor function in human THP-1 monocytes was shown to be up-regulated by IL-1 and TNFα, but to be down-regulated by IFNγ [170]. The IFNγ-mediated down-regulation of A_{2A} receptors may be overcome by exposure of THP-1 monocytes to LPS [383, 523]. In macrophages, LPS was shown to increase both A_{2A} and A_{2B} receptor expression [168, 531, 534, 535], whereas IFNγ selectively up-regulated A_{2B} receptors in these cells [166]. These data would suggest that anti-inflammatory A_2 receptors are gradually being up-regulated following classical activation of macrophages, possibly as an auto-regulatory mechanism to orchestrate ongoing inflammatory processes and to initiate inflammatory resolution.

Recruitment. As mentioned before, recruitment of monocytes to inflammatory foci involves both adhesive and migratory responses of these cells. Extracellular Ado may affect monocyte adhesion during inflammation by modulating adhesion molecule expression on the endothelium. Endothelial A_{2A} receptor activation has been shown to inhibit both TNFα- and LPS-stimulated monocyte adherence to human endothelial cells via NFκB-dependent down-regulation of E-selectin expression [537]. Ado has also been shown to inhibit VCAM-1 expression in immunostimulated endothelial cells via stimulation of both A_{2A} and A_{2B} receptors [292, 538]. Several *in vivo* findings support a regulatory role of extracellular Ado in adhesive interactions between monocytes and the vascular endothelium. Recent reports demonstrate that an increase in ICAM-1 expression induced by renal ischemia-reperfusion injury in mice is attenuated through activation of A_1 receptors [539, 540]. A_{2A} receptor activation has been shown to also attenuate increased expression of ICAM-1 as well as expression of VCAM-1 and P-selectin after

ischemia-reperfusion injury in mice [299, 541]. In a mouse carotid ligation model, administration of the A_{2A} agonist ATL-146e reduced recruitment of macrophages in carotid arterial segments and attenuated acute inflammatory responses after vascular injury [299]. Koszalka et al. (2004) demonstrated that monocyte adhesion induced by ischemia-reperfusion was increased in CD73 knock-out mice compared to wild-type controls [542]; the authors suggested that endogenous Ado generated by CD73 may inhibit leukocyte adhesion and thereby limit vascular inflammatory responses *in vivo*. Furthermore, by inhibiting TNFα-induced apoptosis of porcine endothelial cells and by modulating expression of cytoprotective proteins by these cells through activation of both A_{2A} and A_{2B} receptors, extracellular Ado has been shown to mediate endothelial cytoprotection [538], and may impede excessive leukocyte extravasation as such by sustaining vascular integrity.

Extracellular Ado may also prevent excessive accumulation of Mo/Mø in inflamed tissues, since Ado at high micromolar concentrations was shown to inhibit chemotactic responses of these cells [543, 544]. In addition, extracellular Ado may affect chemotactic responses of Mo/Mø indirectly via modulation of chemokine production by leukocytes and endothelial cells. High micromolar concentrations of Ado as well as of its metabolite inosine have been shown to inhibit LPS-induced production of CCL3 by macrophages via A_3 receptor activation [48, 49, 545, 546]. Similar Ado levels also inhibited CXCL8 release by immunostimulated endothelial cells [292], possibly mediated by A_{2B} receptors [547]. CXCL8 production by monocytes was also shown to be inhibited by extracellular Ado [548-550]. Sonoki et al. (2003) demonstrated that Ado inhibited glycoxidized low-density lipoprotein (LDL)-induced expression of CCL2 in human endothelial cells through stimulation of A_{2A} receptors [551]. A_{2A} receptor activation by ATL-146e has been shown to suppress expression of several inflammatory chemokines induced by ischemia/reperfusion injury *in vivo*: CCL2, CCL5 (also known as regulated upon activation normal T-cell expressed and secreted, RANTES), and CXCL10 [552]. Thus, by reducing the synthesis of potent chemoattractants during inflammatory responses, Ado may inhibit migration and recruitment of leukocytes, including Mo/Mø.

Inflammatory mediators. Extracellular Ado may be involved in the regulation of NFκB activity in Mo/Mø, even though its precise role appears to be a matter of debate. Majumdar et al. (2003) demonstrated that Ado at high micromolar concentrations inhibited TNFα-induced NFκB activation in human KBM-5 monocytic cells through activation of A_2 receptors [429]. The authors proposed that the pathway leading to NFκB activation could differ between activating agents (*e.g.* TNFα vs. LPS), which might determine whether NFκB activity is affected by Ado. Indeed, several reports suggest that LPS-mediated activation of NFκB in Mo/Mø is not affected by Ado [168, 429, 529, 553, 554]. However, it has been reported that A_{2A} receptor activation resulted in inhibition of LPS-induced NFκB-dependent transcription in human THP-1 monocytes [383]. Recently, A_3 receptor stimulation was shown to inhibit NFκB activation in LPS-stimulated mouse macrophages [536]. A_3 receptor stimulation by adenyl carbocyclic nucleoside analogues has been shown to inhibit LPS-induced nuclear translocation of NFκB in both human and mouse macrophages [47, 555]. A carbocyclic nucleoside analogue was also shown to inhibit LPS-induced transcriptional activity of NFκB in mouse macrophages [556]. Lukashev et al. (2004) provided strong evidence for an inhibitory role of the A_{2A} receptor in NFκB DNA-binding activity. In macrophages from A_{2A}-deficient mice that were activated by CpG oligodeoxynucleotide DNA *in vivo*, they

demonstrated stronger NFκB binding associated with increased pro-inflammatory cytokine transcription as compared to cells from wild-type mice [95]. Negative feedback regulation of pro-inflammatory mediator synthesis during inflammatory responses might thus be effectuated through inhibitory signaling by endogenous Ado on the level of transcription factors, such as NFκB.

Many studies have investigated effects of extracellular Ado on cytokine production by Mo/Mø. Ado inhibits production of the potent pro-inflammatory cytokines IL-12 [48, 49, 90, 95, 170, 557, 558] and TNFα [47, 90, 95, 143, 166, 168, 383, 522, 525, 526, 528, 529, 532, 535, 536, 548-550, 553-557, 559-581] by inflammatory Mo/Mø, an effect which appears to be mediated through activation of multiple P1 receptor subtypes, *i.e.*, A_{2A}, A_{2B} and A_3 receptors [47-49, 90, 95, 166, 168, 170, 383, 525, 526, 528, 529, 532, 535, 536, 545, 549, 553, 557, 558, 564, 566, 569, 572, 576, 578, 580]. As shown in murine macrophages, Ado also inhibited LPS-mediated production of IFNγ, which is central in Mo/Mø activation and cell-mediated immunity; this inhibition may occur via stimulation of A_3 receptors [49, 582]. In contrast to the essential role of extracellular ATP in the synthesis and secretion of IL-1β by immunostimulated Mo/Mø, several studies suggested that Ado does not affect IL-1β production by human and mouse macrophages [529, 555, 565, 568, 571]. In contrast, other studies have shown that Ado receptor agonists inhibited IL-1β production by immunostimulated human monocytes [562, 583]. Xaus et al. (1999) proposed the existence of a negative feedback mechanism whereby activation of A_{2B} receptors, whose function was up-regulated by IFNγ in murine bone marrow-derived macrophages, mediates macrophage deactivation by inhibiting IFNγ-induced expression of pro-inflammatory cytokines, including IL-1β [166]. Recent data from Day et al. (2004) show that increased hepatic expression of IL-1β as well as other pro-inflammatory cytokines and chemokines as a result of ischemia-reperfusion injury is suppressed by the A_{2A} receptor agonist ATL-146e in wild-type mice, but not in A_{2A} knock-out mice [552]. Ado may also be involved in IL-1ra production, since LPS-induced expression of IL-1ra mRNA in a human macrophage cell line was shown to be modestly increased by Ado agonists [529]. In addition, Ado increases IL-10 production by both human and mouse Mo/Mø through activation of A_{2A} and, as recently shown, A_{2B} receptors [170, 534, 554, 557, 558, 561, 573, 579]. Although extracellular Ado also appears to affect IL-6 production by Mo/Mø, the reported effects are contradictory. In different studies, Ado or its analogues have been shown: (i) not to effect LPS-induced IL-6 production by both human and rat macrophages [529, 555, 568], (ii) to inhibit both basal and LPS-induced IL-6 production by murine macrophages [48, 565, 571, 584], or (iii) to enhance both basal and LPS-induced IL-6 production by both rat and murine macrophages [570, 579]. Interestingly, the Ado-mediated modulation of IL-6 production by immunostimulated monocytes might depend on the inflammatory mediator used to activate monocytes, since Ado was shown to increase IL-6 production by monocytes that had been activated with IL-1β or TNFα, whereas Ado attenuated IL-6 production upon cellular activation with LPS [550].

Overall, the cytokine secretion profile exhibited by Mo/Mø upon exposure to Ado appears to be characteristic for alternatively activated Mo/Mø. This would suggest that extracellular Ado is capable of down-regulating Mo/Mø-mediated inflammatory and immune responses. Down-regulation of inflammatory and immune responses resulting from Ado-mediated signaling through P1 receptors has been confirmed in various *in vivo* models of inflammation [47-49, 90, 95, 143, 151, 526, 539, 540, 549, 552, 554, 562, 565, 566, 582, 585-587]. Of note, the Ado metabolite inosine also appears to modulate

cytokine production by Mo/Mø. Inosine has been shown to enhance production of IL-4 and inhibit production of IL-1, IL-6, IL-12, TNFα and CCL3 [56, 151, 546, 588-591], possibly via activation of A_{2A} and A_3 receptors [151].

Eicosanoid synthesis by Mo/Mø probably is not substantially affected by extracellular Ado. Stimulation of A_{2A} receptors attenuated the production of LTB_4 in fMLP-stimulated human whole blood samples that had been primed with LPS and TNFα [350]. LTB_4 production in these samples originated mostly from neutrophils and to a lesser extent from monocytes. Concomitantly, inhibition of LTB_4 synthesis by A_2 receptor agonists was less substantial in monocytes compared to the inhibition in neutrophils [350]. Release of AA as well as subsequent production of PGE_2 and LTC_4 were unaffected by Ado [482, 544, 548].

Cytotoxicity. Macrophage phagocytosis was shown to be enhanced through A_1 receptor activation [262], whereas it was inhibited by Ado via stimulation of A_2 receptors [262, 492, 530]. Ado also inhibited zymosan-induced lysosymal enzyme secretion in macrophages [494]. Extracellular Ado may also affect both RNS and ROS production by Mo/Mø. Effects of extracellular Ado on RNS production may be P1 receptor subtype-specific. NOS2 expression and NO production by both IFNγ-primed and LPS-stimulated murine macrophages may be enhanced through activation of A_1 receptors [592, 593], whereas LPS-stimulated NOS2 expression and NO production by murine macrophages is inhibited through activation of A_2 and A_3 receptor subtypes [48, 49, 536, 554, 594, 595]. Diminished NO production by immunostimulated macrophages upon exposure to extracellular Ado may be due to down-regulation of NOS2 expression via activation of low-affinity A_{2B} receptors [166]. Extracellular Ado may also be involved in controlling ROS production by Mo/Mø, since Ado-mediated stimulation of A_{2A} receptors has been shown to inhibit O_2^- generation by Mo/Mø [527, 544, 568, 596, 597]. Also, A_3 receptor activation was shown to inhibit O_2^- generation by immunostimulated monocytes [524, 527].

Furthermore, it has previously been reported that A_1 receptors promote and A_{2A} receptors inhibit multinucleated giant cell formation by phorbol myristate acetate (PMA)-stimulated human peripheral blood monocytes [381]. This function is common in granulomatous inflammation.

4.2.3 Modulation of monocyte and macrophage function by ATP and adenosine

Function of Mo/Mø in immunity and inflammation appears to be tightly regulated by extracellular nucleotides and nucleosides. Mo/Mø exert mostly pro-inflammatory and cytotoxic effector functions when exposed to ATP, whereas they exhibit anti-inflammatory and immunosuppressive functions upon exposure to Ado. This could indicate that purinergic signaling induced by endogenous ATP and Ado may actually contribute to classical and alternative activation pathways, respectively. Findings on phenotypic characteristics of Mo/Mø during inflammatory processes strengthen this notion.

First, ecto-enzyme expression and function change during cell differentiation and activation. Whereas the enzymatic activity of CD39 and CD73 is believed to be relatively low in undifferentiated monocytes, activity of CD39 and especially CD73 are up-regulated upon differentiation [177, 178], possibly implying that Mo/Mø may progressively acquire an Ado-forming phenotype. Such changes in ecto-enzyme

functionality might allow Mo/Mø to adjust the outcome of the purinergic cascade on their cell surface in order to fine-tune their effector functions during an inflammatory event.

Second, the expression profile of purinergic receptors in Mo/Mø is thought to be under control of inflammatory mediators, which may modulate the responsiveness of these cells to extracellular ATP and Ado during inflammatory processes. Expression of $P2Y_1$, $P2Y_2$ and $P2X_7$ receptor subtypes on Mo/Mø has recently been demonstrated to change during the course of an inflammatory reaction in rats [406]. Pro-inflammatory $P2X_7$ receptors appear to be up-regulated in Mo/Mø following classical activation by IFNγ, IL-1, TNFα and LPS [161, 163, 164], probably sensitizing Mo/Mø to pro-inflammatory effects of ATP at high levels. IFNγ simultaneously down-regulates A_{2A} receptors in Mo/Mø [170], which may prevent negative feedback signaling by Ado at anti-inflammatory levels during the initial phase of classical Mo/Mø activation. However, IFNγ-induced down-regulation of A_{2A} receptors may be overruled by LPS, IL-1 and TNFα, which ultimately induce up-regulation of A_{2A} as well as A_{2B} receptors in Mo/Mø [168, 170, 383, 531]. IFNγ priming also induces delayed up-regulation A_{2B} receptors in Mo/Mø [166]. Subsequent activation of A_2 receptors may then indirectly affect $P2X_7$ receptor function, either through cAMP-mediated inhibition of IFNγ- and TNFα-induced up-regulation of $P2X_7$ receptors [163], or by augmenting IL-10-mediated down-regulation of $P2X_7$ receptors [164]. Upon progression of inflammatory and immune responses, signaling through anti-inflammatory A_2 receptors may thus predominate in order to regulate or antagonize the effector functions of classically activated Mo/Mø.

Third, A_{2A} receptor activation in murine macrophages has been demonstrated to act in synergy with ligands of TLR-2, -4, -7 and -9 to increase VEGF expression and production, while simultaneously inhibiting production of TNFα and IL-12, which was suggested to allow macrophages to switch from a clasically activated to an angiogenic, alternatively activated phenotype [533, 553]. Such an angiogenic switch would be of significance in relation with wound healing and tissue repair. Indeed, stimulation of A_{2A} receptors has been shown to promote angiogenesis and wound healing in mice [533, 598, 599]. Thus, extracellular Ado appears to facilitate an angiogenic switch by macrophages that is characteristic for an alternatively activated phenotype [600, 601].

In conclusion, the above findings would suggest that classical activation of Mo/Mø during early inflammatory events may involve purinergic signaling by extracellular ATP, while Ado-mediated purinergic signaling might be involved in the switch to an alternatively activated Mo/Mø phenotype that is a prerequisite for resolution of inflammation and tissue healing. Thus, it seems that ATP and Ado are endogenous signaling molecules that may regulate Mo/Mø function and may contribute to the phenotype switching of macrophages during inflammatory and immune responses [93, 600, 602, 603].

4.3 Dendritic cells

Dendritic cells (DCs) are antigen-presenting innate immune cells whose specific function is to activate naive T cells and to initiate primary immune responses [604]. Immature DCs are sentinels of the immune system, patrolling host tissues to monitor the environment for molecular entities that are associated with pathophysiological events such as infection, cellular stress or tissue damage. Immature DCs can take up

such entities, and at the same time receive activation and instruction signals from the microenvironment. These environmental signals are translated by the DCs into an immunomodulatory message. Maturating DCs then migrate from peripheral tissues to T cell areas in order to deliver both antigen and the immunomodulatory message, which is manifested mainly by DC-derived cytokines, to naive T cells, thereby determining specific lymphocyte-effector mechanisms. In this way, DCs play a pivotal role in the initiation of cellular immunity, humoral immunity or tolerance [605-607]. Both ATP and Ado have been shown to modulate DC function [86-88, 93, 158, 608]. The outcome of purinergic signaling by extracellular ATP and Ado on DC function is affected by the presence of various ecto-enzymes expressed by DCs, including members of the E-NTPDase family as well as CD73 [609-615].

4.3.1 ATP

Receptors. Human DCs have been shown to express $P2X_{1,4,5,7}$ and $P2Y_{1,2,4,6,11,13,14}$ receptor subtypes [387, 610, 615-620]. Purinergic receptor expression may depend on degree of DC maturity, since expression of $P2Y_{11}$ and $P2Y_{14}$ receptor subtypes was shown to be down-regulated upon maturation of monocyte-derived DCs (MoDCs) [619, 620].

Antigen capture, migration and maturation. In peripheral tissues, immature DCs have the ability to migrate towards inflammatory foci where they take up and process available antigens and then emigrate through the lymphatics to draining lymph nodes. ATP at low micromolar concentrations transiently enhanced endocytotic activity of human immature DCs [621]. ATP also appears to affect migration of DCs, as originally reported by Liu et al. (1999) who demonstrated that DCs migrated towards a pipette containing ATP [622]. Subsequent studies showed that low micromolar concentrations of ATP as well as UDP and UTP stimulated migration of DCs by activating P2Y receptors [618, 619, 623, 624]. In contrast, a gradient of ATP and chemokines, which is likely to occur at sites of inflammation or tissue damage *in vivo*, resulted in inhibition of chemokine-directed migration of human MoDCs through activation of $P2Y_{11}$ receptors [619]. The authors hypothesized that ATP, after initially stimulating DC migration towards sites of inflammation, may cause an arrest in movement of DCs arriving at these inflammatory sites by activating $P2Y_{11}$ receptors, thereby prolonging exposure of DCs to antigens and factors inducing DC maturation. Extracellular ATP may in fact be one of the factors involved in DC maturation, as micromolar ATP concentrations have been shown to induce up-regulation of maturation markers (*i.e.* CD80, CD83, CD86, CD54 and major histocompatibility complex (MHC)-II) in human peripheral blood mononuclear cell (PBMC)-derived immature DCs [610, 614, 616, 621, 625, 626], possibly by stimulation of the $P2Y_{11}$ receptor subtype [616]. The $P2X_7$ receptor subtype may also contribute to DC maturation, since $P2X_7$ receptor activation in human PBMC-derived immature DCs by ATP induced rapid loss of the low-affinity immunoglobulin (Ig)-E receptor (CD23) from the cell surface [627]. CD23 is normally down-regulated in maturating DCs. The effect of ATP on DC maturation was shown to be synergistic with that of various pro-inflammatory mediators, such as TNFα, LPS and CD40L [610, 616, 621, 625, 626, 628]. Thus, in synergism with these locally released inflammatory mediators, ATP may contribute to the maturation of DCs by activating $P2Y_{11}$ and possibly $P2X_7$ receptors. Down-regulation of $P2Y_{11}$ receptors upon DC maturation may then neutralize the inhibitory effect of this receptor subtype on DC migration, thereby facilitating the migration of mature DCs towards draining lymph nodes.

Cytokine production and T cell activation. Activated mature DCs prime naive T cells. DCs are able to shape the outcome of immune responses through the secretion of cytokines that drive the deviation of T cells into T helper (Th) cells (Th_1 vs. Th_2) or regulatory T cells. Secretion of IL-12 by DCs favours the differentiation of Th_1 cells, while secretion of IL-10 together with the lack of IL-12 secretion favours the differentiation of Th_2 cells or regulatory T cells. Purinergic signaling by extracellular nucleotides is thought to modulate cytokine production by DCs, resulting in a specific cytokine profile that appears to depend on which P2 receptor subtype is being activated. The first indication of such an effect came from an experiment showing that murine fetal skin-derived DCs (FSDCs) selected for low $P2X_7$ receptor expression released less IL-1β upon co-incubation with Th lymphocytes than wild-type FSDCs, which express the $P2X_7$ receptor subtype at a high level [629]. When co-incubated with syngeneic Th cells, the ATP-resistant FSDCs also showed reduced T cell-stimulatory activity in comparison with wild-type cells, as judged by the ability to trigger IL-2 secretion by T cells [629].

P2Y receptors may also be involved in cytokine production by murine DCs. Expression and secretion of IL-1β, IL-6, IL-10 and IL-12 by murine primary DCs was initiated in response to UTP (20 μM); no response to ATP was shown at these concentrations [630]. Mizumoto et al. (2002) demonstrated that ATP (1 mM) induced IL-6 production by murine bone marrow-derived DCs, but was ineffective in DCs from CD39 knock-out mice [609]. According to the authors, the stimulatory effect of ATP on IL-6 production in wild-type mice was abrogated as a result of P2Y receptor desensitization in the DCs from CD39 knock-out mice [609, 631]. In addition, Langerhans cells (LCs; *i.e.* DCs within the epidermis) from these CD39 knock-out mice were less efficient than wild-type LCs in triggering IFNγ release by T cells [609]. Also, when bone marrow-derived DCs from CD39 knock-out mice were pulsed with hapten *in vitro* and then injected into wild-type mice, these DCs were found to be less potent than wild-type DCs in their *in vivo* ability to initiate an immune response in recipient mice [609]. Thus, CD39 knock-out DCs appeared to have an impaired T cell-stimulatory function, and the authors hypothesized that the apparent role of CD39 in ATP-mediated signaling in murine DCs might be twofold. First, CD39 could prevent desensitization of certain P2 receptor subtypes by hydrolyzing ATP and thereby optimizing receptor functionality involved in modulation of DC function (*e.g.* cytokine secretion). Second, CD39 may at the same time prevent overactivation of other P2 receptor subtypes by lowering extracellular ATP levels, thereby protecting DCs from deleterious effects of high ATP concentrations (*e.g.* $P2X_7$ receptor-mediated cytotoxic effects) [609, 631]. Recently, Granstein et al. (2005) showed that *in vivo* administration of the P2 agonist adenosine 5'-*O*-(3-thiotriphosphate) (ATPγS) augmented cutaneuos cell-mediated immunity by demonstrating that intradermal ATPγS injection enhanced both hapten- and vaccine-induced immune responsivess [632]. Furthermore, they showed in correlative *in vitro* experiments that high concentrations of ATPγS: (i) enhanced LPS/GM-CSF-induced expression of the maturation markers CD80, CD86 and MHC-II by murine-derived XS-106 dendritic cells, (ii) increased LPS/GM-CSF-induced production of IL-1β and IL-12, while decreasing IL-10 production by murine-derived XS-106 dendritic cells, and (iii) enhanced antigen-presenting activity by murine LCs, *i.e.*, increased IFNγ release by T cells [632]. Overall, P2 receptor signaling in murine DCs may amplify cytokine production as well as the T cell-stimulatory capacity of these cells.

Involvement of nucleotides in cytokine production by human MoDCs seems to depend on their extracellular concentrations. It has been shown that a high micromolar (> 100 µM) to millimolar levels: (i) ATP stimulated production of IL-1β and TNFα by LPS-stimulated MoDCs; IL-1β release was increased through activation of the $P2X_7$ receptor subtype [617], (ii) ATP stimulated production of IL-12 by LPS-, sCD40L- or TNFα-stimulated MoDCs, possibly via activation of the $P2Y_{11}$ receptor subtype [616, 621, 626], and (iii) UDP increased the release of CXCL8 by LPS-matured MoDCs, possibly via activation of the $P2Y_6$ receptor subtype [624]. At low micromolar concentrations (< 100 µM), adenine nucleotides have been shown to inhibit production of TNFα, IL-1α, IL-1β, IL-6 and IL-12 by sCD40L- or LPS-stimulated MoDCs, whereas production of IL-10 by these cells was either enhanced or inhibited by adenine nucleotides [614, 618, 625, 626, 633].

Marteau et al. (2004) proposed that multiple P2Y receptor subtypes may be involved in the effects of extracellular adenine nucleotides on cytokine production by human MoDCs [614]. They showed that both ATPγS and adenosine 5'-*O*-(2-thiodiphosphate) (ADPβS) inhibited the production of IL-12 and TNFα by LPS-stimulated MoDCs; IL-10 production, however, was potentiated by ATPγS but inhibited by ADPβS. According to the authors, ATPγS exerted its effects probably via the $P2Y_{11}$ receptor subtype, while ADPβS effects were presumably mediated via the $P2Y_{13}$ or possibly $P2Y_{12}$ receptor subtype, or via a yet uncharacterized P2Y receptor subtype [614]. A recent study by Schnurr et al. (2005) confirmed the notion that distinct P2Y receptor subtypes may be involved in the regulation of cytokine production by human MoDCs [633]. They showed that low micromolar concentrations of ATP and ATPγS inhibited production of IL-12 and IL-27 by *E. coli*-activated MoDCs, probably through stimulation of the $P2Y_{11}$ receptor subtype. In contrast, low micromolar levels of ATP and ADP increased secretion of IL-23, which appeared to be mediated by a P2Y receptor subtype other than $P2Y_{11}$ [633]. Thus, it would appear that extracellular nucleotides modulate cytokine production by human MoDCs in a concentration-dependent fashion, thereby affecting the Th cell balance (*i.e.* Th_1 or Th_2).

In accordance with their effects on cytokine production, human MoDCs exposed to high concentrations (> 100 µM to mM) of adenine nucleotides appear to initiate a Th_1 response, whereas cells exposed to lower levels (< 100 µM) of adenine nucleotides appear to initiate a Th_2 response. MoDCs primed with TNFα and ATP at high micromolar concentrations were shown to have an increased T cell-stimulatory capacity, which was suggested to depend on increased IL-12 release by these cells [621]. In this respect, high ATP levels may amplify the effects of other mediators in inducing a Th_1 immune response. In contrast, when naive Th cells were primed with MoDCs stimulated with either LPS or *E. coli* in the presence of low micromolar ATP concentrations, the Th cells exhibited reduced production of IFNγ and enhanced production of IL-4, IL-5 and IL-10 [625, 633]. The induction of this Th_2 cytokine profile depended on ATP-mediated inhibition of IL-12 production by the DCs [625, 633]. Moreover, La Sala et al. (2002) demonstrated that supernatants from MoDCs that had been treated with LPS and low concentrations of ATP had a reduced capacity to attract Th_1 cells, whereas their ability to attract Th_2 cells was not affected [623]. Thus, it seems that DCs exposed to low ATP concentrations have a diminished capacity to induce a Th_1 immune reponse, which may skew the Th cell balance to a more Th_2-like immune response and thereby prevent excessive inflammation.

Apoptosis. Prolonged exposure (> 15-30 minutes) to millimolar ATP concentrations causes morphological changes and eventually apoptosis of mouse and human DCs [609, 617, 627, 629, 634-636]. These cytotoxic effects of ATP, which were shown to be mediated by the $P2X_7$ receptor and partly depended on activation of caspase-1 and caspase-3 [635, 636], may contribute to the removal of DCs after antigen delivery to lymphocytes in lymphoid tissues.

4.3.2 Adenosine

P1 receptor expression by DCs appears to depend on their maturation state. Whereas immature human MoDCs have been shown to express functional A_1 and A_3 receptors, as well as low levels of A_{2A} receptor mRNA, mature human MoDCs predominantly express functional A_{2A} receptors [521, 637-640]. Although no expression of A_{2B} receptor mRNA had previously been demonstrated in human MoDCs [637, 639], A_{2B} receptor mRNA and protein was recently shown to be expressed in both immature and mature MoDCs [640]. However, these A_{2B} receptors may be non-functional, since they acted as ADA-anchoring proteins in human MoDCs [640]. ADA co-localizing with A_{2B} receptors on DCs interacted with CD26 on T cells and constituted a co-stimulatory signal in the immunological synapse, which resulted in increased production of the Th_1 cytokines IFNγ, TNFα and IL-6. According to the authors, ADA may enhance Th cell function by degrading extracellular Ado [640].

Immature human plasmacytoid DCs (PDCs), a subset of peripheral blood-derived DCs that are capable of producing large amounts of type I interferons in response to viruses [641], were shown to express only functional A_1 receptors [639]. Upon maturation, A_1 receptor mRNA was down-regulated, whereas A_{2A} receptor mRNA was strongly up-regulated [639]. A murine-derived dendritic cell line (XS-106), which closely resembles mature DCs, has been shown to express both functional A_{2A} and A_3 receptors; no functional expression of the A_1 receptor subtype was shown in this cell line [642].

In accordance with these apperent changes in receptor expression during DC maturation, Ado affects immature and mature DCs differently. Extracellular Ado at physiological concentrations (nM to low μM) stimulated chemotaxis of human immature MoDCs and PDCs due to A_1 receptor activation [637, 639]. Recently, Ado's breakdown product inosine was shown to provoke migration of immature MoDCs [643]. Ado also enhanced macropinocytotic activity (*i.e.* antigen uptake) of immature MoDCs [644]. In synergy with inflammatory mediators such as LPS and TNFα, Ado has been shown to contribute to maturation of human MoDCs [616, 644]. Thus, purinergic signaling by Ado at physiological levels, preferentially through A_1 receptors, appears to promote cellular functions of immature DCs.

Upon an inflammatory event, when various inflammatory mediators induce DC maturation, Ado levels in the microenvironment rise and might modulate cellular functions of maturating DCs. Ado may inhibit the emigration of mature DCs towards draining lymph nodes, since migration of human epidermal DCs and MoDCs *in vitro* as well as migration of murine LCs and dermal DCs to draining lymph nodes *in vivo* was shown to be delayed by the nonselective P1 receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) [638]. By reducing the migratory capacity of DCs, Ado may delay the arrival of mature DCs to T cell areas and thereby affect DC-T cell interactions as well as ensuing immune responses. Moreover, Ado at micromolar concentrations affected the chemokine profile of LPS-matured human MoDCs by reducing

production of the type 1 lymphocyte chemotaxin CXCL10 and increasing production of the type 2 lymphocyte chemotaxin CCL17 (also known as thymus- and activation-regulated chemokine, TARC) [644]. This could indicate that mature DCs exposed to Ado preferentially attract Th_2 lymphocytes. Ado may also affect DC-driven T cell polarization via effects on cytokine secretion by DCs. Following LPS-induced maturation, extracellular Ado at micromolar concentrations was shown to stimulate IL-10 production and inhibit the production of TNFα and IL-12 by mature MoDCs through activation of A_{2A} receptors [637, 644]. Similar Ado levels inhibited production of IL-6, IL-12 and IFNα in immunostimulated mature PDCs, which predominantly express A_{2A} receptors [639]. In addition to A_{2A} receptor involvement in human DC subsets, LPS-induced TNFα production by mouse DCs was shown to be inhibited by stimulation of both A_{2A} and A_3 receptors [642]. The cytokine profile exhibited by DCs in response to purinergic signaling by Ado may induce Th_2-mediated immune responses, as shown by down-regulated release of IFNγ and up-regulated release of IL-5 by T cells primed with LPS-matured MoDCs in the presence of Ado [644]. Ado may thus diminish the capacity of mature DCs to initiate and amplify Th_1 immune responses.

4.3.3 Modulation of dendritic cell function by ATP and adenosine

Taken together, the effects of both ATP and Ado on DCs seem to depend on a number of factors, including extracellular concentrations of ATP, Ado and inflammatory mediators as well as ecto-enzyme expression on DCs. At the onset of inflammation (*e.g.* tissue damage or pathogen encounter), rising extracellular ATP levels as well as physiological Ado levels mainly appear to facilitate the initiation of primary immune responses by stimulating chemotaxis, antigen uptake and maturation of DCs. Acute tissue inflammation leads to dramatic increases in extracellular ATP concentrations [110], which further stimulate DC-evoked immune responses by its effects on cytokine production and antigen presenting activity in synergy with inflammatory mediators that are present in the DC microenvironment at this stage (*e.g.* TNFα, LPS). Moreover, as shown by Robson et al. (1997) and by Kalsi et al. (2002) in endothelial cells [360, 361], down-regulation of diphosphohydrolase and CD73 by inflammatory mediators (*e.g.* TNFα) at sites of inflammation delays ATP hydrolysis and Ado formation, thereby contributing to the stimulatory effects of ATP. At the same time, feedback mechanisms exist to prevent tissue damage by DC-evoked immune responses. For example, excessive inflammation will lead to millimolar concentrations of extracellular ATP that eventually induce apoptosis of DCs, thereby preventing further deleterious effects of activated DCs [634]. In addition, increasing levels of DC-stimulating mediators (*e.g.* LPS and CD40L) as a result of excessive inflammation cause inhibition of DC IL-12 production by extracellular ATP [626], which might prevent excessive immune responses. Finally, extracellular ATP levels eventually decrease through the action of CD39 and CD73 on DCs resulting in increasing levels of extracellular Ado [610]. Both low ATP levels and high Ado levels then may counterbalance inflammatory stimuli by inhibiting pro-inflammatory cytokine production, impairing migration and skewing the immune response to a Th_2-like response, and may thus contribute to the resolution of inflammation.

4.4 Lymphocytes

Lymphocytes are central to humoral and cellular immune responses [645-647]. Two major lymphocyte subclasses are B and T cells expressing antigen-specific receptors, which are the hallmark of adaptive

immunity. B lymphocytes originating from the bone marrow mediate humoral immunity by producing antibodies that neutralize and eliminate extracellular pathogens. T lymphocytes are mediators of cell-mediated immunity directed against intracellular pathogens. Thymus-derived T lymphocytes are usually subclassified in CD4 expressing Th cells and CD8 expressing cytotoxic T cells (CTLs). Th cells orchestrate immune responses by secreting cytokines that activate innate killing mechanisms and contribute to humoral immune responses by helping B cells to proliferate into antibody-producing plasma cells. CTLs are able to recognize and kill infected cells through either exocytosis of cytotoxic granule proteins or apoptosis-inducing membrane-bound proteins. An additional class of lymphocytes does not express antigen-specific receptors, *i.e.*, natural killer (NK) cells, which participate in innate defense mechanisms. NK cells have cytotoxic effector functions resembling those of CTLs.

4.4.1 ATP

T lymphocytes. T lymphocyte function may be subject to autocrine or paracrine regulation by extracellular nucleotides as suggested by three lines of evidence. First, T lymphocytes are capable of releasing ATP upon activation [386, 648-651]. Second, T lymphocytes express purinergic receptors for extracellular nucleotides. Several P2X receptor subtypes are expressed in peripheral T lymphocytes, *i.e.*, $P2X_{1,4,5,7}$ [175, 203, 395, 397, 650, 652-655]. Although mRNA of $P2Y_{1,2,4,6,11,12,13,14}$ receptor subtypes has been detected in resting peripheral T lymphocytes [193, 387, 395, 656-659], P2Y receptors are thought to be non-functional in these cells [653, 656, 657, 660-662]. However, T cell activation during immune responses may induce expression of functional P2Y receptor subtypes. Somers et al. (1998) demonstrated that the $P2Y_6$ receptor subtype was up-regulated in activated T cells and responded to UDP by intracellular Ca^{2+} mobilization [657]. Additionally, IL-2 production by activated Jurkat T cells has been shown to be increased by UTP, suggesting expression of functional P2Y receptors following cellular activation [650]. The third line of evidence, which would suggest extracellular nucleotide-mediated regulation of T lymphocyte function, comprises the expression of several purine-metabolizing ecto-enzymes by T lymphocytes. Lymphocytes are thought to be characterized by an ATP-regenerating/Ado-eliminating phenotype allowing them to maintain surrounding ATP at steady-state levels [127]. T cells exhibit low or no CD73 activity, but have a high expression of E-NTPDase (CD39), the enzymatic activity of which may be up-regulated following lymphocyte activation [663-668]. E-NTPDases may also exert extra-enzymatic functions in that CD39 has been proposed to be a cell-surface signaling molecule playing a role in the regulation of effector functions of activated lymphocytes [666, 669-672].

After maturation in the thymus, naive T lymphocytes preferentially home to secondary lymphoid tissues through high endothelial venules. Continuous recirculation of naive T cells provides a mechanism for patrolling of secondary lymphoid tissues for microbial antigens by these lymphocytes. Upon encounter of antigen-presenting cells, naive cells proliferate and differentiate into effector cells when expressing a T cell receptor (TCR) specific for the presented antigen-MHC combination and when receiving the proper co-stimulatory signals. Changed adhesion molecule expression by activated T lymphocytes enables them to migrate into inflamed peripheral tissues through postcapillary venules [673, 674]. Extracellular ATP has been shown to induce shedding of L-selectin from T lymphocytes via activation of $P2X_7$ receptors [52, 203, 675-679]. Since L-selectin is primarily involved in lymphocyte homing to lymphoid

tissues and is shed upon lymphocyte activation, ATP may be involved in migration of activated lymphocytes to sites of inflammation. Purine nucleotides may also indirectly facilitate transendothelial lymphocyte extravasation by impairing vascular barrier function. Henttinen et al. (2003) demonstrated that adhesion of lymphocytes to endothelial cells suppressed endothelial purine metabolism [680]. Binding of Jurkat T cells to human endothelial cells inhibited endothelial CD73 activity, but not E-NTPDase activity. Adherent lymphocytes thereby prevented Ado formation, which interfered with Ado's ability to restrain leukocyte transmigration, and sustained extracellular ATP levels [680].

During the immune response, T cells become activated, undergo massive clonal expansion and differentiate into effector cells. Extracellular ATP may be involved in lymphocyte proliferative responses. In murine peripheral T lymphocytes, ATP at concentrations of 0.5 to 2 mM was shown to inhibit proliferation of both resting and stimulated cells [681, 682]. These inhibitory effects might be due to breakdown of ATP to Ado. Using human peripheral blood lymphocytes, ATP at micromolar levels (100-300 µM) was shown to be a co-stimulator of mitogen-induced $CD4^+$ and $CD8^+$ T lymphocyte proliferation via activation of a P2X receptor channel, possibly the $P2X_7$ receptor [662]. Budagian et al. (2003) recently confirmed the possible involvement of $P2X_7$ receptors in lymphocyte proliferation by demonstrating that millimolar ATP concentrations induced proliferation of Jurkat T cells, which was suggested to result from ATP-mediated up-regulation of IL-2 transcription [652]. However, Duhant et al. (2002) showed that proliferation of activated human peripheral blood $CD4^+$ T cells was inhibited by the ATP derivatives ATPγS and BzATP [656].

Activation of T cells eventually results in the development of several effector T cell subsets with distinct functions [683]. Effector $CD4^+$ Th cells promote cellular and humoral immune responses by producing a wide variety of cytokines. Activation of $P2X_7$ receptors on Jurkat T cells by extracellular ATP was shown to activate the transcription factor AP-1 and to increase transcription of IL-2 [650, 652]. Strikingly, ATP at millimolar concentrations inhibited binding activity of NFκB transiently in quiescent Jurkat T cells [652]. However, the effect of extracellular ATP on NFκB activity may depend on cellular activation, since micromolar levels of ATP enhanced NFκB activity in TNFα-activated Jurkat T cells [429].

Effector functions of $CD4^+$ Th cells may also be inhibited by extracellular adenine nucleotides. In mouse spleen cells, which contain both T lymphocytes and NK cells, micromolar ATP concentrations inhibited LPS- and anti-CD3-Ab-stimulated production of IFNγ [459]. Duhant et al. (2002, 2005) showed that 100 µM ATP and ADP as well as the ATP analogs ATPγS and BzATP inhibited the production of IL-2, IL-5, IL-10 and IFNγ by activated human $CD4^+$ T cells, and down-regulated expression of the high affinity IL-2 receptor (CD25) during $CD4^+$ T cell activation [656, 684]. The authors suggested that purinergic signaling through an unidentified P2Y receptor subtype mediated the anti-inflammatory effects of the ATP analogs in these cells [656]. However, Langston et al. (2003), who in murine $CD4^+$T lymphocytes also demonstrated inhibition of IL-2 and IFNγ secretion by ATPγS, proposed that these effects were independent of P2Y receptor signaling [664]. Instead, these authors suggested that E-NTPDase-mediated hydrolysis of extracellular ATP, rather than P2 receptor activation, might be required for the effects on $CD4^+$ T cells [664].

$CD8^+$ CTLs are activated and acquire effector functions by exposure to IL-2 and by recognition of class I MHC-associated antigen peptides. Effector CTLs bind to infected target cells and induce an apoptotic response by exposing the target cells to membrane proteins such as Fas ligand, and by releasing soluble secretory proteins such as perforin and granzymes [685, 686]. Extracellular ATP has been suggested to be an alternative mediator of cell-mediated cytotoxicity by CTLs or lymphokine-activated killer (LAK) cells [687-691]. CTLs release ATP upon activation (*i.e.* TCR-crosslinking) [515, 648, 649]. High local concentrations of ATP accumulating in the immunological synapse between effector cells and target cells could then activate pore-forming $P2X_7$ receptors expressed on target cells, which results in target cell lysis [515, 648, 649, 663, 688, 689, 692, 693]. ATP appears to have no effect on granule exocytosis [649, 694]. CTLs and LAK cells themselves are thought to be protected against ATP-mediated cytotoxicity by means of high ecto-ATPase activity and/or the absence of $P2X_7$ expression [663, 688, 689, 695-697]. In fact, ecto-ATPase activity was shown to be up-regulated upon TCR-mediated activation of murine CTLs [664].

Others have disputed the role of ATP and $P2X_7$ receptors in cell-mediated cytotoxicity [698-700]. An alternative explanation of the role of extracellular ATP in lymphocyte-mediated cytotoxicity may be its interaction with ecto-protein kinases [691, 701]. T lymphocytes express a casein II like ectokinase [694, 702]. By serving as a substrate for this ecto-protein kinase, ATP could constitute a phosphate donor mediating the phosphorylation of extracellular plasma membrane proteins that are involved in immunological synapse formation and cell functionality [694, 700-702].

After successful elimination of pathogens by effector T cells, the immune response extinguishes during which most lymphocytes die by apoptosis while a small number of antigen-specific cells escapes cell death becoming long-lived memory cells [703]. Extracellular ATP causes programmed cell death of lymphocytes through $P2X_7$ receptor activation [52, 132, 653, 678, 679, 695, 704-708]. Activation of lymphocyte $P2X_7$ receptors by millimolar ATP concentrations induces a plasma membrane pore that is smaller than that described for Mo/Mø (0.3 kDa vs. 0.9 kDa, respectively) [653, 662, 695, 705, 706]. Influx of calcium ions through this $P2X_7$ ion channel may trigger sustained activation of phospholipase D (PLD) resulting in a delayed permeability lesion [705]. Since functionality of $P2X_7$ receptors has been shown to be higher in murine peripheral $CD4^+$ lymphocytes than in $CD8^+$ lymphocytes, Th cells may be more susceptible to the pore-forming and lytic effects of extracellular ATP than CTLs [653].

B lymphocytes. The $P2X_7$ receptor is expressed on normal B lymphocytes [654, 709, 710], but also on $CD5^+$ B lymphocytes from chronic lymphocytic leukemia (CLL) patients [711]. CLL is a type of leukemia resulting in a progressive accumulation of mature $CD5^+$ B lymphocytes in blood and bone marrow [712]. The level of activation of the $P2X_7$ receptor can either mediate cell death or proliferation in lymphocytes. Wiley et al. (2002) showed that in individuals with CLL, activation of the $P2X_7$ receptor leads to apoptosis of lymphocytes [706]. Moreover, a reduced function of this receptor had an anti-apoptotic effect, resulting in an increase in B cell numbers. *In vitro* proliferation of B lymphocytes, isolated from evolutive B-CLL patients (with disease progression), was inhibited by extracellular ATP, but ATP had no effect on lymphocytes from patients with the indolent form of CLL (no disease progression) [397]. Baricordi et al. (1999) reported that $P2X_7$-transfection in lymphoid cells enhanced cell proliferation in the absence of

exogenous growth factors and this effect was dependent on autocrine/paracrine stimulation by released ATP [713].

B lymphocytes undergo recirculation *in vivo*, which involves continuous transendothelial migration, mediated by L-selectin expressed on the surface of B lymphocytes [675, 714]. B-CLL lymphocytes, which overexpress the $P2X_7$ receptor, have impaired ability for transendothelial migration compared to normal peripheral blood lymphocytes, leading to high concentrations of soluble CD23 and L-selectin in the serum of CLL patients [714]. Moreover, Jamieson et al. (1996) showed that ATP may have a physiological role in the loss of L-selectin since ATP was able to interact with lymphocyte $P2X_7$ purinoceptors, which caused a rapid loss of L-selectin expression from human lymphocytes [675].

Conigrave et al. (2001) reported the presence of a cAMP-linked purinoceptor for ATP, $P2Y_{11}$, on CLL lymphocytes [715]. These authors showed that ATP induced cAMP accumulation via the activation of $P2Y_{11}$ receptors in freshly isolated B lymphocytes from patients with CLL. The effect of ATP on cAMP accumulation was stronger compared to ADP and AMP. Padeh et al. (1991) showed that low concentrations of extracellular ATP (10-100 μM) triggered B cell activation, presumably due to a dose-dependent increase in cytosolic free Ca^{2+} mediated by the phospholipase C (PLC) signal transduction pathway [661], suggestive of P2Y receptor involvement. All these processes were specific for ATP and the responses of B cells to extracellular ATP were mediated by P2 purinoceptors. ATP also stimulated PLD activity in lymphocytes isolated from patients with B cell CLL in a dose-dependent way via the $P2X_7$ purinoceptor. ADP, UTP and Ado failed to show this effect [716].

Dombrowski et al. (1997) showed that ecto-ATPase can be a marker of B cell activation expressed by immortalized human and murine B cells [671]. The reason for the presence of an inducible ecto-ATPase on these B cells is not fully understood. These B cells also express an ecto-ADPase (or ecto-ATPDase) but no ecto-AMPase (CD73) [671, 717]. However, Barankiewizc et al. (1988) showed, besides the presence of ecto-ATPase and ecto-ADPase, the presence of ecto-AMPase on the external surface of B cells [717]. The expression of these ecto-enzymes seemed closely related with B cell development and may have a role in control of ATP-induced B cell activation [717].

NK cells. NK cells are a subset of lymphocytes that appear to have a specific capacity for spontaneous cytolysis of tumor and virus-infected cells [718]. Research on the effects of nucleotides on the activity of NK cells is very limited; existing knowledge derives mainly from studies done in the early 1990s. Krishnaraj et al. (1992) reported a dose-dependent inhibition of NK cell-mediated cytotoxic activity by exogenous ATP, probably by interacting with P2 purinergic receptors. It was shown that ATP (micromolar) treatment of these NK cells neither induced a non-specific decrease in the number of viable cells, nor showed specific elimination of the NK cells [719]. In human NK cells, incubation with extracellular purine nucleotides resulted in an inhibition of the IL-2 dependent NK proliferation, which was stronger for ATP than for Ado. This was not due to cell damage, but was probably mediated through a specific purinergic receptor [720]. Baijpai et al. (1993) demonstrated that the inhibition of NK cell-mediated cytotoxicity was not specific for ATP because ADP, guanine nucleotides and pentasodium triphosphate were also effective inhibitors of NK cell-mediated cytotoxicity [665], which would suggest a

P2Y receptor-mediated effect. These authors suggested that the exogenous nucleotide-mediated inhibition was neither due to the blockage of the exocytosis machinery, nor to the presence of ecto-ATPases [665]. In contrast, Schmidt et al. (1984) showed a specific inhibition of human NK activity by exogenous ATP [721]. Dombrowski. et al. (1995) demonstrated that extracellular adenine nucleotides were inhibitory to NK cell-cytolytic activities in the IL-2-dependent human NK cell line NK3.3 [672]. In an earlier study in NK3.3 cells, Dombrowski et al. (1993) demonstrated the presence of membrane-associated ectoATPases, which could mediate the effects of ATP on human NK lytic activity [722]. From earlier research, it appeared that ecto-ATPase could play an important role in regulating NK cell-cytolytic activity through calcium signaling [670]. Koziak et al. (1999) showed in human NK cells a correlation between the levels of CD39 expression and ATP-diphosphohydrolase (ATPDase) activity. The presence of mRNA specific for purinergic P2 receptors ($P2Y_1$, $P2X_4$, and $P2X_7$) in these NK cells would suggest a regulation of the purinergic signaling by the CD39/ATPDase co-expression [376].

4.4.2 Adenosine

T lymphocytes. As mentioned before, defective lymphocyte function seen in patients with the ADA-SCID syndrome may partly be attributed to accumulating intracellular and extracellular Ado levels [131, 133, 135, 137, 723]. This would suggest that Ado is an important signaling molecule affecting lymphocyte function. Extensive data on P1 receptor expression on T lymphocytes as well as evidence of changing receptor expression during cellular activation contribute to the notion that purinergic signaling by extracellular Ado may play an important part in modulation of T cell function. Both peripheral CTLs and Th cells express A_{2A}, A_{2B} and A_3 receptors [134, 267, 268, 704, 724-740], whereas these cells are thought to express little or no A_1 receptor transcript [134, 725, 729, 732, 734, 735, 739]. A_{2A} receptors are proposed to be the predominantly expressed receptor subtype in peripheral T lymphocytes [134, 726], and this receptor subtype is thought to be non-redundant in mediating the effects of extracellular Ado in these cells [90, 730, 732]. Therefore, modulation of A_{2A} receptor expression might have drastic influences on T cell responses to extracellular Ado *in vivo*. More Th cells than CTLs are thought to express A_{2A} receptors and activation of T cells increases A_{2A} receptor expression, primarily in CTLs [726, 740].

Expression of A_{2B} receptors also has been demonstrated to be up-regulated following activation of human peripheral $CD4^+$ and $CD8^+$ T cells [724, 731], but not in murine $CD4^+$ T cells [740]. Interestingly, Herrera et al. (2001) demonstrated that A_{2B} receptors on the cell surface of T lymphocytes could act as anchoring proteins for cell-surface ADA (ectoADA) [731]. In non-lymphoid cells, a similar role has been proposed for A_1 receptors [741]. ADA is known to localize to the cell surface of lymphocytes by binding to CD26, a T cell activation antigen. This ectoADA/CD26 interaction on the surface of T cells serves to attenuate the immunosuppressive receptor-mediated signaling induced by high levels of extracellular Ado [738, 742-744]. Co-ordinated up-regulation of ectoADA and A_{2B} receptors, and co-localization between ectoADA and A_{2B} receptors upon activation of CD26-negative T cells, were shown to increase ligand-binding affinity of A_{2B} receptors [731], suggesting that ectoADA/A_{2B} interaction may render subsets of activated T cells more sensitive to negative feedback signaling by extracellular Ado. In addition to up-regulated expression of A_2 receptors in activated T lymphocytes, Gessi et al. (2004)

recently provided evidence for rapid up-regulation of A_3 receptors by human peripheral Th cells upon activation [736].

It has been shown that Ado at micromolar levels (50 µM) promotes vascular barrier function and inhibits lymphocyte transmigration [680]. However, adhesion of lymphocytes to the vascular endothelium was shown to mask enzymatic activity of CD73 on endothelial cells [680], possibly constituting a mechanism to compensate for Ado-mediated inhibition of lymphocyte extravasation. During lymphocyte adhesion to endothelial cells, CD73-mediated AMP breakdown may be further reduced by engagement of the CD73 molecule itself in the adhesive processes. Engagement of lymphocyte CD73 was shown to induce clustering of LFA-1 molecules, thereby enhancing LFA-1 avidity as well as lymphocyte adhesion to the vascular endothelium [745-747]. A comparable role has been suggested for ADA during binding of lymphocytes to epithelial cells [748]. Recently, Niemelä et al. (2004) demonstrated both *in vitro* and *in vivo* that IFNα induced long-term up-regulation of CD73 with increased enzymatic activity in human endothelial cells, but not in peripheral blood lymphocytes [749]. The observation that this effect was a late event which was associated with enhanced vascular barrier function, would suggest that progressive up-regulation of endothelial CD73 during prolonged inflammatory conditions may eventually lead to increased Ado levels, limiting lymphocyte extravasation and thereby controlling the extent of lymphocyte-mediated inflammation. Ado has also been shown to suppress the adhesion molecules lymphocyte Peyer's patch adhesion molecule (LPAM)-1 ($\alpha_4\beta_7$-integrin) and ICAM-1 on lymphocytes [750, 751]. Thus, extracellular Ado may inhibit ongoing accumulation of lymphocytes at inflammatory sites by limiting lymphocyte adhesion and transmigration to extravascular sites [752].

Antigen-triggered TCR signaling in naive Th cells induces production of IL-2 and up-regulation of cell-surface IL-2 receptors (CD25). IL-2 and CD25 are crucial for activation and proliferation of antigen-specific $CD4^+$ and $CD8^+$ T lymphocytes [753]. Extracellular Ado at low micromolar concentrations (5-10 µM) may interfere with this important phase of the immune response by attenuating proliferative responses of activated T cells through stimulation of A_{2A} and/or A_{2B} receptors [134, 548, 681, 682, 734, 739, 742, 754]. In mouse T cells, Ado has been shown to inhibit TCR-triggered up-regulation of CD25 through stimulation of A_{2A} receptors [131, 134, 740]. Ado has also been shown to inhibit TCR-triggered production of IL-2 via A_{2A} receptor activation in T cells [682, 725, 742, 743, 755, 756]. A_{2B} receptors may also contribute to the Ado-mediated inhibition of IL-2 secretion by activated human peripheral lymphocytes [724]. Mirabet et al. (1999) demonstrated up-regulation of A_{2B} receptors following lymphocyte activation and proposed that this receptor subtype plays a role in lymphocyte deactivation [724]. NFκB may be the target for A_{2B} receptor-mediated lymphocyte deactivation. Ado at high micromolar concentrations (50-200 µM) was shown to inhibit TNFα-induced activation of NFκB in Jurkat T cells [429, 757, 758], probably via stimulation of low-affinity A_{2B} receptors [757, 759]. A_{2B} receptors are the main functional Ado receptor subtype in Jurkat T cells [737, 738].

Cytokine production by activated effector Th cells may also be modulated by extracellular Ado, since A_{2A} receptors are thought to be predominantly expressed in human cytokine-producing Th cells [726]. Lappas et al. (2005) recently demonstrated that TCR-mediated activation of murine Th cells induced rapid up-regulation of functional A_{2A} receptors; signaling through the up-regulated A_{2A} receptors inhibited

IFNγ release by these cells [740]. Strikingly, the inhibitory effect of A_{2A} agonism on IFNγ production was abolished in Th cells from A_{2A} $^{-/-}$ mice and attenuated by approximately 50% in Th cells from A_{2A} $^{+/-}$ mice, indicating a gene-dose effect as well as suggesting that there may be no receptor reserve for the A_{2A}-mediated inhibition of IFNγ production by murine Th cells [740]. Another recent study by Erdmann et al. (2005) showed that A_{2A} receptor stimulation inhibited IL-2 secretion, but not IFNγ secretion, by a mixed population of murine effector $CD4^+$ Th_1 and $CD8^+$ Tc_1 cells [756]. IL-10 secretion, but not secretion of IL-4, by a mixed population of murine effector $CD4^+$ Th_2 and $CD8^+$ Tc_2 cells was also moderately inhibited through A_{2A} receptor stimulation. A_{2A} agonism *in vivo* by intraperitoneal administration of 2-[p-(2-carboxyethyl)phenylethylamino]-5'-N-ethylcarboxamido-adenosine (CGS-21680) to mice was shown to inhibit IL-2-dependent antigen-driven expansion of effector Th_1 and Tc_1 cells, while not influencing T cells not responding to antigen [756]. Thus, activation of lymphocyte A_{2A} receptors appears to selectively inhibit T cells undergoing TCR-triggered activation and may thereby inhibit lymphocyte-driven inflammatory responses. Inhibition of lymphocyte-driven chronic inflammation by A_{2A} receptor activation was recently demonstrated *in vivo* by Odashimi et al. (2005) [58]. *In vivo* administration of ATL-146e suppressed the production of IFNγ, TNFα and IL-4 by mucosal Th lymphocytes, and also diminished disease severity of Th lymphocyte-induced chronic ileitis in mice [58].

Furthermore, it has been shown that ectoADA expression on the surface of Th cells is regulated by cytokines. IL-2 and IL-12 were shown to increase ectoADA expression in activated Jurkat T cells, whereas IL-4 decreased ectoADA expression [744]. Up-regulation of ectoADA may favor Th_1 immune responses by evading suppressive signaling by high Ado levels. In contrast, increasing Ado levels due to IL-4-mediated down-regulation of ectoADA may be associated with A_2 receptor-mediated shift to a more anti-inflammatory Th_2-like response. Interestingly, in human disorders with an immunological status reflecting Th_1-shifted immunity (*e.g.* chronic heart failure, pre-eclampsia, hyperemesis gravidarum), up-regulated expression of A_{2A} receptors on peripheral lymphocytes as well as increased plasma Ado levels have been demonstrated *in vivo* [105, 733, 760-762]. Increased plasma Ado levels were associated with altered ADA and CD73 activities [763-766]. These findings would suggest the existence *in vivo* of an autoregulatory mechanism whereby compensatory increases of extracellular Ado levels redress Th_1/Th_2 imbalances via A_{2A} receptor activation. In addition, the recent finding that A_3 receptors are selectively up-regulated in activated $CD4^+$ T cells, but not in activated $CD8^+$ T cells [736], would suggest that this receptor subtype may also contribute to the Ado-mediated autoregulatory effects on activated Th cells. A carbocyclic nucleoside analogue, which mediates its action by binding to the A_3 receptor, was shown to inhibit T cell-derived TNFα production by human PBMCs [548].

CTLs are MHC-I-restricted effectors of anti-viral and anti-tumor immunity. Tumors may resist attack by CTLs by producing immunosuppressive soluble molecules. It was suggested that Ado may be such a tumor-derived molecule [767]. Extracellular Ado levels are believed to rise to micromolar levels within the microenvironment of tumors [768]. At these levels, Ado has been shown to prevent CTL activation and proliferation by inhibiting the inducible expression of the co-stimulatory molecules CD2 and CD28, as well as by inhibiting the expression of CD25 [734, 769, 770]. Ado has also been shown to interfere with the adhesion of activated murine killer T cells to tumor cells through A_3 receptor-mediated inhibition of LPAM-1 interaction with VCAM-1 on target cells [750, 771-773].

In addition to attenuating CTL proliferation and binding to tumor cells, Ado has also been shown to inhibit lethal hit delivery by activated CTLs [770, 774]. Both granule-mediated and Fas-mediated cytotoxicity were inhibited by stimulation of either A_{2A} or A_3 receptors on activated murine killer T cells [725, 734]. However, the A_{2A} receptor agonist CGS-21680 did not affect either Fas-based or exocytosis-based cytotoxicity in murine effector CTLs at doses that potently affected cytokine production by these cells [756]. Moreover, low micromolar levels of Ado inhibited production of IL-2 and IFNγ by murine activated killer T cells via A_3 receptor activation [734, 769]. Overall, CTL effector functions appear to be substantially repressed by Ado-mediated purinergic signaling. The purinergic modulation of CTL function may be especially effective in the microenvironment of tumors where high extracellular Ado levels might represent an immunological barrier to CTL-mediated oncolysis.

B lymphocytes. With regard to receptor expression by B lymphocytes, Conigrave et al. (2001) showed an induced cAMP accumulation by Ado via the activation of A_{2A} receptors in freshly isolated B lymphocytes from patients with CLL, which may influence the development fate of normal B lymphocytes [715]. In a human B cell line, a co-ordinated regulation of the expression of CD73 and the A_{2A} receptor was shown [775]. Recently, Minguet et al. (2005) found that Ado blocks NFκB activation in primary murine B cells [776]. This effect was receptor-mediated, associated with elevating cAMP levels and activating protein kinase A (PKA), leading to the inhibition of IκB phosphorylation. ADA is expressed on B cells in peripheral mononuclear blood cells and functions as a regulator of the levels of extracellular Ado and deoxyadenosine, which are toxic compounds to lymphocytes [742]. Ginès et al. (2002) showed that the expression of ADA-free CD26 on lymphocytes is correlated with the adhesion of these lymphocytes to Caco-2 cells, which express high levels of cell-surface ADA [748]. B cells, which express 90-95% of ADA on their surface, adhered less to Caco-2 cells than T cells, which had a pool of ADA-free CD26. These authors suggested a new role for the A_1 receptor as an anchoring molecule, able to present ADA to CD26 on the surface of contacting cells. ADA deficiency in humans causes a profound depletion of B lymphocytes and results in SCID syndrome. Apasov et al. (2001) showed a severe depletion in B lymphocytes and defects in B cell development in ADA-deficient mice, as was reflected by the expression of cell surface markers and localization in different zones of lymphoid organs [131]. Some purine nucleosides, which are analogs of Ado and 2'-deoxyadenosine, such as tubercidin (Tub), were able to inhibit the proliferative response of purified B cells [770].

NK cells. A_1 receptor agonists, at extremely low concentrations, have been shown to enhance NK cell-mediated cytolysis of target cells, while A_2 receptor agonists are potent inhibitors of NK cell-cytolytic function [777]. Little is known about the A_3 receptor in the regulation of NK cell activity, even though adhesion of NK-like CTLs to adenocarcinoma cells was inhibited by Ado through an A_3 receptor [773]. Harish et al. (2003) showed that oral administration of 2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-methylcarboxamide (Cl-IB-MECA), an A_3 receptor agonist, to naive and tumor-bearing mice induced increased levels of serum IL-12, which was followed by elevated NK cell activity and tumor growth inhibition [778]. The authors suggested that activation of the A_3 receptor-potentiated NK cell activity could be mediated by inhibition of cAMP production. Nishida et al. (1984) found that NK cell activity was partly inhibited by high concentrations of 2-chloroadenosine. A possible mechanism responsible for this inhibition could be either a partly inhibition of all NK cells or alternatively the activation of suppressor

cells [754]. NK cell activity was also inhibited by Tub (an A_2 receptor agonist) and stimulated by 2-fluoro-1-β-D-arabinofuranosyladenine 5'-monophosphate (an A_1 receptor agonist) in spleen lymphocytes from mice treated with these drugs *in vivo* [770, 774], and in murine and human lymphocytes *in vitro* [777]. In summary, results obtained with these Ado receptor agonists suggest roles for A_1 and A_2 receptors in regulating murine NK cell activity. Granule exocytosis is a crucial event in NK cell-mediated cytotoxicity since cytoplasmic granules contain various cytolytic effector molecules, such as perforin and granzymes. Ado (5-25 μM), in contrast to 2-chloroadenosine [779], is known to suppress the cytotoxicity of stimulated mouse NK cells by interfering with the process of granule exocytosis, but nothing is known about the possible receptor involved [780]. It is thought that cytotoxicity, mediated by NK cells, plays an important role in host anti-cancer defense. Ohana et al. (2003) showed that activation of the A_3 receptor induced anti-cancer activity, concomitantly with a myeloprotective effect in mice bearing colon carcinomas [781]. This anti-cancer activity was attributed to a direct anti-proliferative effect and to an indirect effect via up-regulation of IL-12 production and NK cell activity.

5. ATP and adenosine are endogenous signaling molecules in immunity and inflammation

The extensive available data on the immunologic effects of purinergic signaling by extracellular ATP and Ado, as presented in the previous paragraphs, gives overwhelming evidence that these endogenous signaling molecules and their purinergic receptors play a major role in immunity and inflammation. However, the presented data also suggest that the role of ATP and Ado in immunity and inflammation is extremely complex and interdependent. Existing knowledge derives mainly from *in vitro* studies, which are directed towards unravelling biochemical mechanisms at molecular receptor and post-receptor levels, rather than providing a general picture of physiological or pathological conditions *in vivo*. Therefore, in this paragraph, we will attempt to provide a conceptual framework for the reviewed information.

We suggest that the immunological role of ATP and Ado is best viewed within the scope of a model which provides a functional explanation for the initiation of inflammatory and immune responses as well as their resolution, in short, for the regulation of immunity. One theory, which has gained considerable empirical support over the past decade and that complements the classical infectious-nonself theory of immunity, is the so-called Danger theory, which proposes that the immune system is principally occupied with detecting 'danger', defined as anything causing tissue damage or cellular stress [11, 782-785].

Figure 2A. *Pro-inflammatory and immunostimulatory effects of high-level purinergic signaling by ATP. Upon injury or infection, injurious or infectious agents damage healthy tissue cells. These damaged tissue cells then release intracellular ATP into their extracellular microenvironment, where ATP constitutes a 'danger' signal. High-level ATP-mediated purinergic signaling through P2 receptors expressed by neutrophils (PMN) and monocytes/macrophages (Mo/Mø) contributes to the onset of acute inflammation. ATP stimulates cell migration, cytotoxicity and pro-inflammatory cytokine secretion by these cells. Purinergic signaling is also involved in the initiation of primary immune responses by its effects on dendritic cells (DC) and T lymphocytes. ATP induces maturation of dendritic cells into a Th_1-skewing mature phenotype, promoting cell-mediated immunity.*

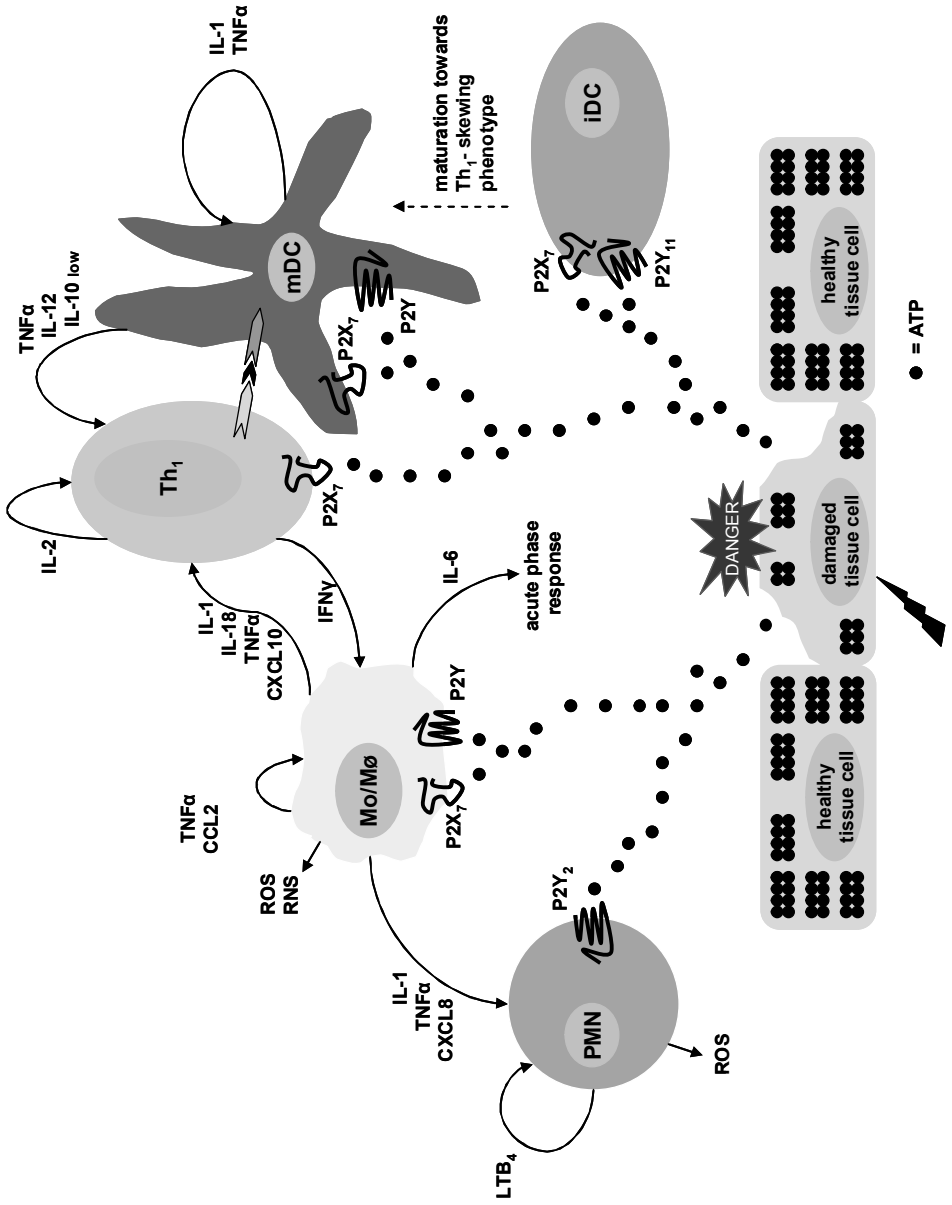
IL-1
TNFα
maturation towards
Th1- skewing
phenotype
iDC
mDC
TNFα
IL-12
IL-10 low
P2X7
P2Y
P2X7
P2Y11
Th1
IL-2
IL-1
IL-18
TNFα
CXCL10
IFNγ
IL-6
acute phase
response
DANGER
damaged
tissue cell
healthy
tissue cell
healthy
tissue cell
= ATP
P2Y
TNFα
CCL2
Mo/Mø
P2X7
ROS
RNS
P2Y2
IL-1
TNFα
CXCL8
PMN
ROS
LTB4

Figure 2A.

According to this line of thought, substances causing damage or emerging during, after or because of damage constitute danger signals that alert and instruct the immune system [81, 83, 84]. A distinction is made between on the one hand exogenous danger signals, which are typically the pathogen-associated molecular patterns (PAMPs; *e.g.* LPS) triggering responses through ligation of pattern-recognition receptors (PRRs; *e.g.* Toll-like receptors), and on the other hand endogenous danger signals, which can be produced by activated immune cells (*e.g.* cytokines) or can derive from stressed or damaged tissue cells. These latter tissue-derived distress signals have recently also been called damage-associated molecular patterns (DAMPs) [786].

DAMPs initiate and regulate immune responses in co-operation with other danger signals, and should ideally be entities that: (i) are constitutively present at high intracellular concentrations, (ii) are normally present at negligible extracellular concentrations, (iii) are easily released in response to injury, infection or other inflammatory stimuli, (iv) are able to activate selective and specific cellular receptors responsive over a wide range of concentrations, and (v) are quickly degraded following their release [86, 88]. Both ATP and Ado meet the above prerequisites, and may therefore be considered as DAMP molecules.

For the purpose of clarity, inflammatory and immune responses are divided into three stages, which partly overlap [10, 85, 787]. The first stage encompasses the onset of acute inflammation and the initiation of primary immune responses upon encounter with infectious or injurious agents. The second stage comprises the modulation and fine-tuning of ongoing inflammatory and immune responses by endogenous immunoregulatory substances. Finally, the third stage encompasses the down-regulation of immune responses, the induction of inflammatory resolution and the restoration of damaged tissues to preserve cellular homeostasis. These three stages will be used as a steppingstone to positioning purinergic signaling by extracellular ATP and Ado in immunity and inflammation (Fig. 2).

In the first stage, extracellular ATP mainly functions as a pro-inflammatory and immunostimulatory mediator in the microenvironment of damaged cells (Fig. 2A). ATP may be part of a group of endogenous molecules that have recently been termed ‘alarmins’ [788]. These multifunctional molecules appear to be an unique subgroup of endogenous danger signals since they exhibit both chemotactic and activating effects on leukocytes, particularly DCs, thereby displaying potent innate immuno-enhancing activity [788].

Figure 2B. *Immunomodulation and immunosuppression by ATP and Ado. During the course of inflammatory and immune responses, extracellular ATP concentrations decrease while levels of extracellular Ado increase through breakdown of ATP. Chronic low-level ATP signaling as well as Ado signaling fine-tune ongoing inflammatory and immune responses by contributing to alternative macrophage activation and by skewing the immune response towards a Th_2-like response. At the same time, uncontrolled immune responses and inflammation by overactive immunocytes results in collateral damage to healthy tissue cells. This secondary 'danger' induces the release and formation of extracellular Ado, which inhibits virtually all effector functions of neutrophils (PMN), monocytes/macrophages (Mo/Mø), dendritic cells (DC) and T lymphocytes. In this way, Ado acts as an immunosuppressant that contributes to down-regulation of immune responses, to resolution of inflammation and to the initiation of tissue repair mechanisms.*

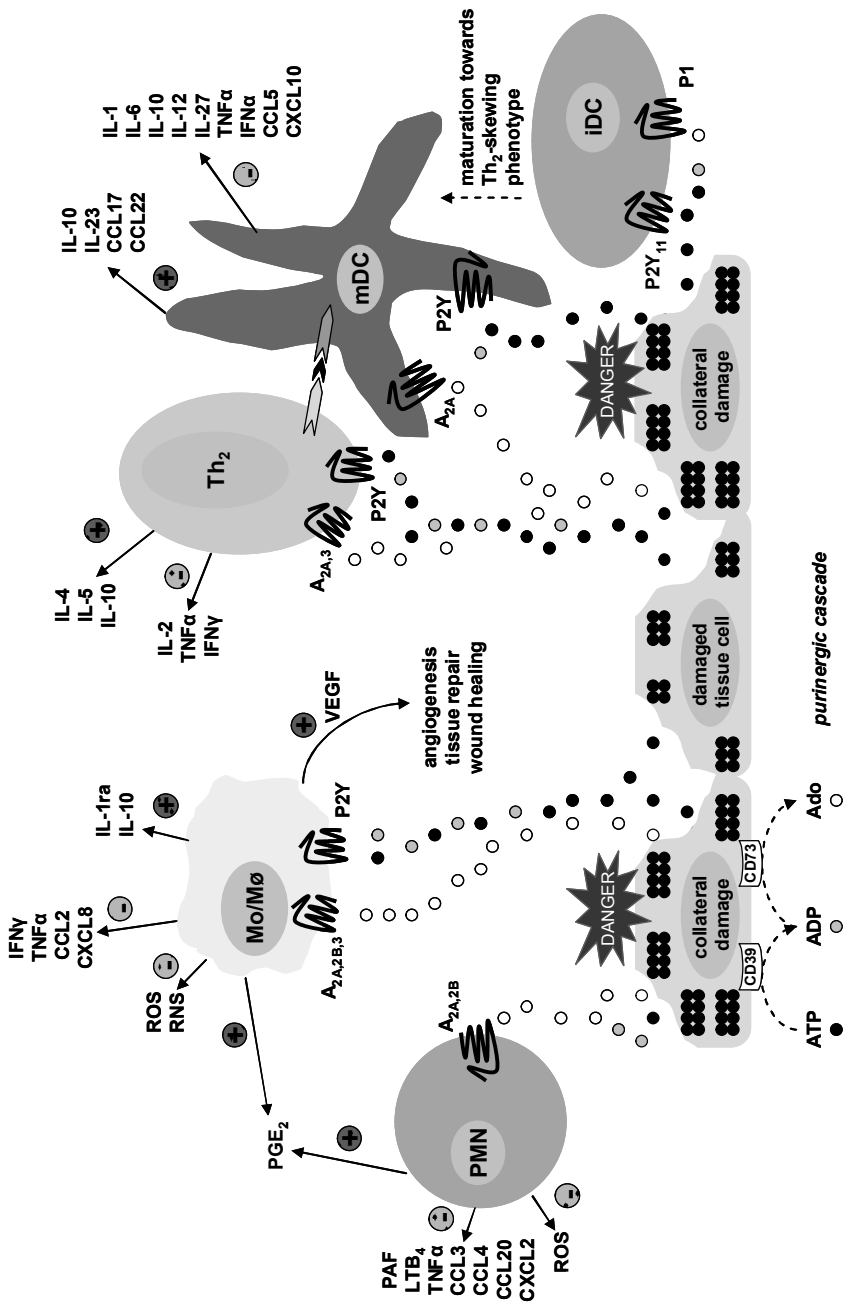
IL-10
IL-23
CCL17
CCL22
IL-1
IL-6
IL-10
IL-12
IL-27
TNFα
IFNα
CCL5
CXCL10
maturation towards
Th2-skewing
phenotype
mDC
iDC
P1
P2Y11
P2Y
A2A
DANGER
collateral damage
damaged tissue cell
Th2
IL-4
IL-5
IL-10
IL-2
TNFα
IFNγ
A2A,3
VEGF
angiogenesis
tissue repair
wound healing
IL-1ra
IL-10
IFNγ
TNFα
CCL2
CXCL8
ROS
RNS
Mo/Mø
A2A,2B,3
PGE2
A2A,2B
PMN
PAF
LTB4
TNFα
CCL3
CCL4
CCL20
CXCL2
ROS
CD39
CD73
ATP
ADP
Ado
purinergic cascade

Figure 2B.

ATP is present in the cell cytosplasm at millimolar concentrations and is released from the intracellular compartment upon cellular stress or non-physiological necrotic cell death. Extracellular ATP concentrations in the local microenvironment of damaged cells can rise considerably, marking the damaged site and contributing to the promotion of inflammation and the initiation of primary immune responses. From this point of view, ATP at high extracellular concentrations appears to be a natural endogenous adjuvant released from injured and dying cells, which initiates inflammation and has an augmenting effect to amplify and sustain cell-mediated immunity through P2 receptor-mediated purinergic signaling.

At an early phase following cell damage, when ATP levels are highest, the receptor subtype most likely involved in sensing the purinergic danger is the $P2X_7$ receptor [52, 433, 444, 628, 679]. Also, this receptor subtype appears to be up-regulated by pro-inflammatory mediators, thereby making inflammatory cells more responsive to the imminent danger [161, 163, 164]. Via activation of $P2X_7$ receptors, ATP induces the production of cytokines such as IL-1β, IL-2, IL-12, IL-18 and TNFα by residing immune cells, triggering the inflammatory response and inducing type 1 lymphocyte polarization. High-level signaling through other P2 receptors probably also contributes to the pro-inflammatory and immunostimulatory role of ATP. Recruitment of leukocytes to damaged sites is promoted by ATP as well as other adenine and uridine nucleotides, which also induce inflammatory activation of neutrophils, classical activation of macrophages, maturation and Th_1 cell-stimulatory capacity of DCs, and proliferation and activation of lymphocytes.

In the second stage, the immunological role of ATP appears to shift gradually from being mostly immunostimulatory to being more immunomodulatory. In addition, extracellular Ado mainly appears to predominate as an anti-inflammatory mediator at this stage. Over-activation of the immune system may lead to uncontrolled or chronic inflammation resulting in collateral cell damage and destruction of healthy tissues. Therefore, inflammatory and immune responses must be tightly regulated to protect the host. For this purpose, the immune system disposes of several regulatory molecules that orchestrate host responses by controlling inflammatory and immune responses [82, 789]. Extracellular ATP and Ado could be endogenous regulatory molecules comprising a purinergic feedback system (Fig. 2B). Crucial components of such a purinergic feedback system are the purinergic receptors mediating the immunological effects of extracellular nucleotides and nucleosides, as well as the ecto-enzymes mediating a purinergic cascade that leads to a progressive decrease in nucleotide concentrations and an increase in nucleoside concentrations.

The expression profile of purinergic receptors as well as of ecto-enzymes by immune cells changes under inflammatory conditions, allowing for the progressive acquisition of an immunomodulatory purinergic repertoire expressed by the cells involved in inflammatory and immune responses. The ecto-enzymes CD39 and CD73 control extracellular nucleotide concentrations and thereby regulate the extent of purinergic signaling, as evidenced by several studies using ecto-enzyme knock-out models [191, 192, 212, 416, 542, 609]. It appears that low-level purinergic signaling by P2 receptors, induced by nucleotides at decreased concentrations, modulates ongoing inflammatory and immune responses.

Activation of P2 receptors, most probably P2Y receptors, attenuates pro-inflammatory cytokine production by Mo/Mø, diminishes Th_1 cell-stimulatory capacity of DCs and inhibits lymphocyte effector functions.

Thus, upon progression of the immune response, nucleotide-mediated purinergic signaling may switch from being predominantly pro-inflammatory to being mostly immunomodulatory, depending on the extracellular concentrations of the nucleotides as well as the P2 receptor subtype(s) ligated by these nucleotides. This switch could be part of a mechanism by which the effector class of an immune response is being fine-tuned and re-directed by microenvironmental signals according to the tissue in which the response occurs [86, 87, 606]. Evidence for such an immunomodulatory role of low-level ATP-mediated purinergic signaling is very limited. Our group recently showed that ATP at low micromolar concentrations markedly inhibited the inflammatory reaction by inhibiting TNFα release and stimulating IL-10 release in immunostimulated whole blood, even under conditions of severe oxidative stress [67, 68]. Since natural cell-to-cell interactions are preserved in this diluted whole blood system and blood components are present in *in vivo* ratios with non-cellular components, this *ex vivo* system is a good reflection of the natural environment.

Furthermore, Ado-mediated P1 receptor signaling during the second stage down-regulates neutrophil effector functions, contributes to alternative activation of macrophages, stimulates Th_2 cell-stimulatory capacity of DCs and inhibits lymphocyte effector functions.

At the final stage, extracellular Ado appears to be an important immunosuppressive and tissue-healing factor (Fig. 2B). Like ATP, Ado can also be considered as a danger molecule because its extracellular levels rise markedly in response to tissue damage. However, unlike ATP, rising extracellular Ado levels in response to damage inflicted by overactive host immune cells mediate an autoregulatory immunosuppressive loop to protect healthy tissues from these 'dangerous' immune cells. Damage-associated increases in extracellular Ado thus represent a 'second' danger signal that is sensed by P1 receptors [100, 601]. Inflammatory mediators progressively up-regulate the expression of the P1 receptor subtypes through which Ado mediates its immunosuppressive effects [166, 170, 269, 534, 535, 581, 726, 733, 736, 740, 790]. Using receptor knock-out models, the A_{2A} receptor subtype has been considered to play a non-redundant role in down-regulating cell-mediated immunity and in activating pro-resolution pathways [90, 95, 97, 354, 368, 537, 552, 598, 740, 756, 791, 792]. A_1, A_{2B} and A_3 receptors also contribute to the Ado-mediated negative feedback signaling [34, 151, 166, 539, 540, 578, 793-795]. Ado-mediated signaling by these purinergic receptors induces an angiogenic switch in macrophages or deactivates macrophages, suppresses proliferation and effector functions of lymphocytes, and promotes angiogenesis and tissue regeneration.

In conclusion, although it is still far from clear how extracellular ATP and Ado exert their immunologic roles, they appear to be crucial endogenous signaling molecules in immunity and inflammation. Because ATP and Ado are unstable molecules with a short half-life, they probably operate only transiently in the local microenvironment of cells in an autocrine or paracrine manner. Purinergic signaling constitutes one

of the mechanisms by which the immune system tailors inflammatory and immune responses according to the host's need for protection against danger. Purinergic signaling molecules may be beneficial in the treatment of immune-related diseases, as signaling by extracellular Ado may contribute to the anti-inflammatory effects of low-dose methotrexate, which probably is one of the most prescribed anti-inflammatory drug in the treatment of patients with rheumatoid arthritis [26]. Thus in view of the pivotal role of the immune system in health and disease, current research on endogenous immunomodulatory molecules, ATP and Ado included, may help devise novel strategies for the treatment of human diseases.

CHAPTER 3

Local effect of adenosine 5'-triphosphate on indomethacin-induced permeability changes in the human small intestine

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Abstract

Background: Nonsteroidal anti-inflammatory drug (NSAID) use is associated with an elevated risk of gastrointestinal damage. As adenosine 5'-triphosphate (ATP) may play a protective role in the small intestine, our objective was to determine the local effect of ATP on small intestinal permeability changes induced by short-term challenge of the NSAID indomethacin in healthy humans.

Methods: Mucosal permeability of the small intestine was assessed by the lactulose/rhamnose permeability test, that is, ingestion of a test drink containing 5 g lactulose and 0.5 g L-rhamnose followed by total urine collection for 5 h. Urinary excretion of lactulose and L-rhamnose was determined by fluorescent detection high-pressure liquid chromatography (HPLC). Basal small intestinal permeability was assessed as a control condition. As a model of increased small intestinal permeability, two doses of indomethacin were ingested before ingestion of the test drink (75 mg and 50 mg at 10 h and 1 h before the test drink, respectively). Concomitantly with indomethacin ingestion, placebo or 30 mg/kg ATP was administered through a naso-intestinal tube.

Results: Median urinary lactulose/rhamnose ratio (g/g) in the control condition was 0.023 (interquartile range: 0.013-0.041). Compared with the control condition, urinary lactulose/rhamnose ratio after ingestion of indomethacin and administration of placebo was significantly increased (0.042 (0.028-0.076); $P<0.01$). In contrast, urinary lactulose/rhamnose ratio after indomethacin ingestion plus ATP administration (0.027 (0.020-0.046)) was significantly lower than the lactulose/rhamnose ratio in the placebo condition ($P<0.01$).

Conclusions: Topical ATP administration into the small intestine during short-term challenge of the NSAID indomethacin attenuates the NSAID-induced increase in small intestinal permeability in healthy humans.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most prescribed anti-inflammatory and analgesic drugs worldwide [796]. It has, however, become clear over the past decades that use of NSAIDs is associated with an elevated risk of mucosal damage in the gastrointestinal tract, which can eventually result in pathological conditions such as perforations, ulcers or strictures [797]. Specific detrimental effects of NSAIDs in the small intestine, which encompass small intestinal inflammation accompanied by blood and protein loss, are collectively called small intestinal enteropathy [798, 799]. The development of small intestinal enteropathy is probably closely related to mucosal permeability changes in the small intestine, as several NSAIDs (*e.g.* indomethacin, naproxen, ibuprofen) have been shown to cause significant changes in mucosal permeability [800-802].

The mucosal epithelial cells lining the gut allow selective permeation of luminal factors [803, 804]. By controlling transport across the epithelium, the small intestinal mucosa functions as a barrier to a wide variety of luminal aggressors to which the gastrointestinal tract is constantly exposed, for instance from ingested food or residing bacteria. Intercellular cytoskeleton-associated tight junctions, which interconnect adjacent enterocytes, play an important role in intestinal permeability [804]. It is now thought that NSAID-induced permeability changes are a causal factor for the development of small intestinal enteropathy [805].

Adenosine 5'-triphosphate (ATP) is a naturally occurring nucleotide that is normally present in every living cell of the human body. Moreover, in the extracellular compartment, ATP constitutes a signaling molecule, which is capable of modulating a variety of physiological processes including cardiac function, neurotransmission, vasodilatation and inflammation [1]. ATP signaling has also been implicated in the control of epithelial function, including gastrointestinal epithelia [806]. In the small intestine, ATP stimulates epithelial Cl^- secretion and thereby contributes to luminal fluid accumulation, which is a nonspecific epithelial defence reaction to flush away luminal irritants [807, 808]. ATP may thus play a protective role in the small intestine.

The potential of ATP in NSAID-induced small intestinal permeability changes is relatively unexplored. The aim of the present study was to investigate the potential of topical ATP administration in an NSAID-induced increase in mucosal permeability of the small intestine in healthy humans. Owing to instability of ATP in the acidic environment of the stomach, we administered ATP via a naso-intestinal tube directly into the small intestine.

2. Subjects and methods

2.1 Subjects

A total of 19 non-smoking men and women between 18 and 30 years of age were recruited for participation at Maastricht University, The Netherlands, by way of information pamphlets. Criteria for

exclusion from participation were: (i) history of gastrointestinal disease or current gastrointestinal disorder (*e.g.* Crohn's disease, celiac disease), (ii) current use of NSAIDs (*e.g.* aspirin, ibuprofen) and (iii) current use of medication that could interfere with effects of ATP, including nucleoside transport inhibitors (*e.g.* dipyridamole, lidoflazine), nonselective adenosine receptor antagonists (*e.g.* theophylline, aminophylline) and xanthine oxidase inhibitors (*e.g.* allopurinol). All the participants received oral and written information about the aim and protocol of the study, and gave their written informed consent before participation. The study protocol was approved by the Ethics Committee of Maastricht University, The Netherlands.

2.2 Protocol

The protocol of the current study was based on a protocol described previously in detail by Troost et al. (2003) [809]. Briefly, the study was carried out according to a double-blind randomized cross-over design. Each individual participated in three experiments in a randomized order and with wash-out periods of 1 week in between: (i) control (no indomethacin, no placebo/ATP), as a measure of basal permeability of the small intestine, (ii) indomethacin plus placebo and (iii) indomethacin plus ATP. As alcohol intake is known to increase intestinal permeability to larger molecules (*e.g.* lactulose) [810], and as caffeine is a nonselective adenosine receptor antagonist [147], participants were requested to abstain from alcohol and caffeine-containing beverages or foods for 4 days preceding and during each experiment. The participants were also asked not to perform any kind of prolonged strenuous physical exercise (*e.g.* long-distance run, cycle race) during 2 days preceding each experiment, as this has been shown to increase permeability of the small intestine [811].

The control experiment comprised only the assessment of basal permeability without any intervention: after an overnight fast and after voiding, participants ingested a test drink containing 5 g lactulose (Centrafarm Services BV, Etten-Leur, The Netherlands) and 0.5 g L-rhamnose (MP Biomedicals, Aurora, OH, USA) dissolved in 100 mL water. Subsequently, total urine was collected for 5 hours. Only during the last 2 hours of urine collection, consumption of water *ad libitum* was allowed. Total urine volume over the 5-hour period was determined, and urine aliquots were taken and stored at -80°C until analysis. In the experiments with indomethacin plus placebo/ATP, at 14 hours before the permeability assessment, a Bengmark-type naso-intestinal tube (Flocare, Zoetermeer, The Netherlands) was inserted into the stomach. After insertion, participants had to remain on the right-hand side in the supine position until the tube tip entered the small intestine, following peristaltic movements. During the remaining hours of the experiment, participants were instructed to remain in the supine position on the back. Four hours later, when the tube tip had moved into the duodenum (as verified by pH determination and observation of characteristics of gastrointestinal juices sampled through the tube), participants ingested 75 mg indomethacin (Genfarma, Zaandam, The Netherlands), immediately followed by administration of either placebo or 30 mg/kg ATP (BUFA BV, Uitgeest, The Netherlands) directly into the duodenum through the inserted tube, using a 20-mL syringe. After an overnight fast, at 1 hour before the permeability assessment, participants again ingested indomethacin (50 mg) followed by administration of either placebo or ATP (30 mg/kg). One hour later, after voiding, participants ingested the lactulose/rhamnose test drink followed by collection of total urine for 5 hours.

2.3 Intestinal permeability

Intestinal permeability was assessed using the lactulose/rhamnose (L/R) gut permeability test. This test is based on the comparison of intestinal permeation of molecules of different sizes. The urinary L/R excretion ratio is considered to be an accurate parameter of small intestinal permeability [812]. Lactulose and L-rhamnose in collected urine samples were determined by fluorescent detection high-pressure liquid chromatography (HPLC). The method for assaying lactulose and L-rhamnose has been described previously [813]. In short, cellobiose was added to urine samples as an internal standard, and the urine was filtered through a 0.4-µm filter and diluted as necessary. Samples were deionized and then injected onto a Dionex MA-1 ion exchange column. Sugars were eluted with NaOH at a flow rate of 0.4 mL/min with concentrations ranging from 400 to 600 mmol/l. Peaks were detected using pulsed amperometric detection on a Dionex HPLC and quantified as peak areas. Calibration was performed on a daily basis with authentic standards at multiple concentrations, and the experimental standards were diluted so that the areas of all peaks fell within the calibration range.

2.4 Statistics

Five-hour urinary excretion levels of lactulose and L-rhamnose are presented as L/R ratios (g/g). Differences in urinary L/R ratios between different conditions, primarily ATP vs. placebo and secondarily ATP/placebo vs. control, were assessed using Wilcoxon signed ranks test. P-values below 0.05 were regarded as statistically significant. Data are presented as Box-Whisker plots.

3. Results

A total of 19 participants were recruited for the study. Four participants did not complete the study owing to problems related to insertion of the naso-intestinal tube, and one participant was excluded from analysis because of vomiting after duodenal administration of the placebo solution. Fourteen participants (12 women, 2 men; age (mean ± SD) 20.9 ± 1.9 years; range 18-25 years) completed the study. Two participants reported minor gastrointestinal discomfort after duodenal administration of the ATP solution (*i.e.* light stomachache and nausea). Otherwise, no side effects were reported.

Figure 1 shows urinary L/R ratios (g/g) in the control, indomethacin-plus-placebo and indomethacin-plus-ATP condition. The median L/R ratio observed in the control condition, representing basal permeability of the small intestine, was 0.023 (interquartile range: 0.013-0.041). In comparison with the control condition, urinary L/R ratio after ingestion of indomethacin and administration of placebo was significantly increased (0.042 (0.028-0.076); $P<0.01$). Urinary L/R ratio after ingestion of indomethacin and administration of ATP was significantly lower than the L/R ratio observed in the placebo condition (0.027 (0.020-0.046); $P<0.01$).

Table 1 shows urinary recovery of ingested lactulose and L-rhamnose in the experimental conditions. Although not reaching statistical significance, urinary lactulose recovery in the indomethacin-plus-placebo condition (0.127% (0.072-0.212)) tended to be higher in comparison with the control condition

(0.082% (0.054-0.152)). In the indomethacin-plus-ATP condition, lactulose recovery tended to be lower than in the indomethacin-plus-placebo condition, with values similar to the control condition (0.087% (0.044-0.212)). In the indomethacin-plus-placebo condition, median rhamnose recovery (3.152% (2.540-4.258)) was slightly lower compared to the control condition (4.218% (3.332-6.425)). Rhamnose recovery in the indomethacin-plus-ATP condition (3.600% (2.654-4.269)) was similar to the control condition.

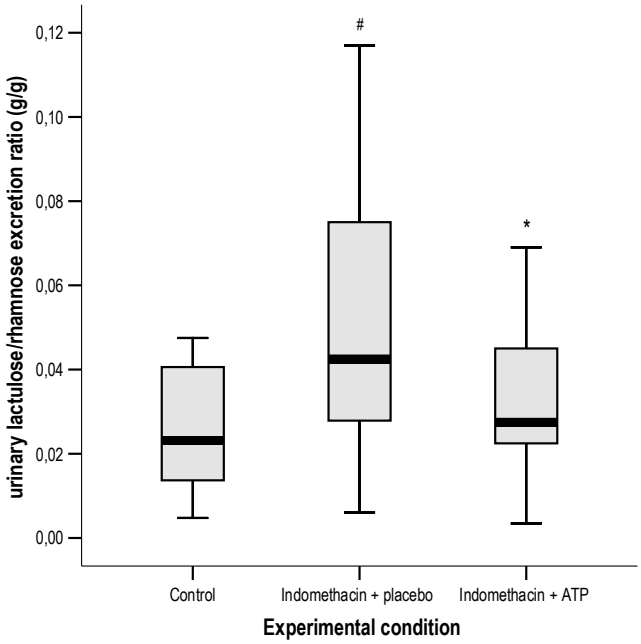

Figure 1. *Box-Whisker plot of urinary lactulose/rhamnose (L/R) ratios observed in three experimental conditions. The control condition represents basal permeability of the small intestine, as indicated by the urinary L/R ratio (g/g) after ingestion of a test drink containing 5 g lactulose and 0.5 g L-rhamnose with no prior indomethacin ingestion and no placebo/ATP challenge. Urinary L/R ratios after ingestion of two indomethacin dosages concomitant with intraduodenal administration of placebo (indomethacin + placebo) or 30 mg/kg ATP (indomethacin + ATP) at 10 h and 1 h before ingestion of the lactulose/rhamnose test drink (#P<0.01 vs. control, n=14; *P<0.01 vs. placebo, n=14, Wilcoxon signed ranks test).*

Table 1. *Five-hour urinary lactulose and rhamnose recovery (%) of orally ingested lactulose (5 g) and L-rhamnose (0.5 g) by healthy participants (n=14) in three experimental conditions.*

Experimental condition	Recovery (%) Median (interquartile range)	
	Lactulose	**L-rhamnose**
Control[a]	0.082 (0.054-0.152)	4.218 (3.332-6.425)
Indomethacin + placebo	0.127 (0.072-0.212)	3.152 (2.540-4.258)*
Indomethacin + ATP	0.087 (0.044-0.212)	3.600 (2.654-4.269)

[a]*No indomethacin, no ATP*

**P=0.01 vs. control (Wilcoxon signed ranks test)*

4. Discussion

In this study, early-stage small intestinal enteropathy was induced by administering two doses of the NSAID indomethacin to healthy human volunteers. Indomethacin causes a rapid (within 8-10 h) and temporary (< 48 h) increase in intestinal permeability [800, 809, 814, 815], which can be assessed by the urinary excretion ratio of lactulose and L-rhamnose permeating the gut mucosa through a paracellular and a transcellular pathway, respectively [816]. Using this model, we demonstrated that duodenal administration of ATP concomitant with indomethacin ingestion prevented the increase in urinary L/R ratio induced by indomethacin challenge plus placebo, indicating that ATP attenuated an indomethacin-induced increase in small intestinal permeability in healthy humans. Urinary L/R ratio from the indomethacin plus ATP condition was similar to that of the control condition, that is, a measure of basal small intestinal permeability without any intervention. Hence, ATP may be an effective compound in attenuating NSAID-induced small intestinal enteropathy.

The gastrointestinal toxicity of NSAIDs is widely recognized. It is thought that increased permeability of the gut mucosa is the earliest event in NSAID enteropathy. The main mechanism, which is responsible for increasing mucosal permeability, is thought to be uncoupling of oxidative phosphorylation in enterocytes by NSAIDs [817]. This topical effect during NSAID absorption leads to intracellular ATP deficiency, which causes loss of control over intercellular tight junctions resulting in increased paracellular permeability [818, 819]. In addition, inhibition of cyclooxygenases (*i.e.* COX-1 and COX-2) by NSAIDs prevents the production of reparative prostaglandins, thereby prolonging the permeability changes [805]. As a result of disruption of the intestinal barrier, the mucosa is exposed to luminal aggressive factors that attract and activate neutrophils, which evoke a local inflammatory response and damage small intestinal tissues associated with blood and protein loss [797].

Gastroduodenal endoscopy is the gold standard for assessing damage to the gastrointestinal tract, but it is generally time consuming, expensive and mostly confined to the upper gastrointestinal tract. More recently, capsule endoscopy has been introduced as a useful tool for assessing more distal gastrointestinal damage [820]. Although such endoscopic imaging techniques are useful for assessing anatomical lesions in the gastrointestinal tract (*e.g.* ulcers, strictures), they do not allow the assessment of compromised mucosal integrity. For this reason, measurement of urinary excretion of orally administrable permeability markers was developed as a non-invasive and safe method to detect site-specific changes in gastrointestinal permeability [821]. For example, sucrose can be used to specifically assess gastric permeability changes [822]. To assess changed permeability of the small intestinal mucosa, use of the differential excretion ratio of two permeability markers is preferred (*e.g.* lactulose/rhamnose, lactulose/mannitol, ^{51}Cr-EDTA/rhamnose) [821]. The intestinal permeability increase attributed to NSAIDs is reported to be two-fold to five-fold [76], which corresponds to the about two-fold increase in small intestinal permeability induced by indomethacin in the present study.

To our knowledge, no earlier report on the effect of ATP on an indomethacin-induced increase in small intestinal permeability exists. The mechanism by which ATP exerts beneficial effects on indomethacin-

induced permeability changes is presently unknown. At least two independent mechanisms for the effect of ATP may exist. First, ATP that is administered topically in the small intestine may be salvaged by enterocytes and in this way attenuate the intracellular ATP deficiency which is induced by NSAID ingestion [823, 824]. Second, ATP may exert beneficial effects through ligation of the so-called purinergic receptors (P2 receptors), which have been shown to be expressed by mucosal epithelial cells [825, 826]. Via stimulation of these receptors, ATP was shown to decrease tight-junctional permeability in cultured human cervical epithelial cells [827, 828]. ATP has also been shown to promote ion secretion and luminal fluid accumulation in the gastrointestinal tract by P2 receptor stimulation [829, 830], thereby contributing to epithelial defence mechanisms by accelerating the removal of exogenous harmful particles that may cause local damage to the intestinal epithelium. Furthermore, as ATP was shown to stimulate epithelial cell restitution by activation of P2 receptors [831], it may modulate intestinal epithelial repair mechanisms [832, 833]. In addition, we recently found that ATP has anti-inflammatory and immunoregulatory properties *ex vivo*, which may also be beneficial in NSAID enteropathy [67, 68].

Besides NSAID-induced changes, permeability of the intestinal mucosa may be affected under several other conditions, including intestinal disease and infection with *Helicobacter pylori* [834, 835]. For the present study, participants with current gastrointestinal disorder and/or history of gastrointestinal disease were excluded. However, since *H. pylori* status was not evaluated in our study we cannot completely disregard the possibility that a subclinical *H. pylori* infection may have been present in some of the participants, even though at the time of the experiments none of the participants showed any clinical signs of *H. pylori* infection or any other disorder of the gastrointestinal tract. Importantly, because of the cross-over design of the present study, any current gastrointestinal disorder, including possible infection with *H. pylori*, would have randomly affected intestinal permeability in all three experimental conditions (control, indomethacin + placebo, indomethacin + ATP); consequently, the highly significant effect of ATP on intestinal permeability in the present study would appear to rule out the possibility of confounding by *H. pylori* or any other infection. It is also important to note that *H. pylori* infection and NSAIDs independently affect mucosal permeability and that *H. pylori* infection does not interact with NSAID-induced mucosal injury [836, 837].

The potential benefit of ATP in intestinal permeability changes demonstrated in the present study may also be relevant to disease-related mucosal barrier dysfunction. Disruption of intestinal barrier function is thought to contribute to the etiology and pathogenesis of several intestinal diseases, including celiac disease and Crohn's disease [70, 834]. In Crohn's disease, permeability changes of the small intestine are assumed to play an important role [838]. It has been shown that increased permeability of the gut mucosa is associated with increased disease activity in Crohn's disease [839] and also is an early predictor of relapse in Crohn's disease [840, 841]. Therefore, attenuating barrier dysfunction may be of importance in the treatment of intestinal diseases.

In conclusion, we show that intraduodenal administration of ATP, concomitantly with ingestion of the NSAID indomethacin, attenuates an indomethacin-induced increase in small intestinal permeability in healthy humans. Our finding suggests that ATP may also be beneficial in the treatment of intestinal disorders in which intestinal permeability changes are involved.

CHAPTER 4

Effects of oral adenosine 5'-triphosphate and adenosine in enteric-coated capsules on indomethacin-induced permeability changes in the human small intestine: a randomized cross-over study

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Abstract

Background: It is well-known that nonsteroidal anti-inflammatory drugs (NSAIDs) can cause damage to the small bowel associated with disruption of mucosal barrier function. In healthy human volunteers, we showed previously that topical administration of adenosine 5'-triphosphate (ATP) by naso-intestinal tube attenuated a rise in small intestinal permeability induced by short-term challenge with the NSAID indomethacin. This finding suggested that ATP may be involved in the preservation of intestinal barrier function. Our current objective was to corroborate the favourable effect of ATP on indomethacin-induced permeability changes in healthy human volunteers when ATP is administered via enteric-coated capsules, which is a more practically feasible mode of administration. Since ATP effects may have been partly mediated through its breakdown to adenosine, effects of encapsulated adenosine were tested also.

Methods: By ingesting a test drink containing 5 g lactulose and 0.5 g L-rhamnose followed by five-hour collection of total urine, small intestinal permeability was assessed in 33 healthy human volunteers by measuring the urinary lactulose/rhamnose excretion ratio. Urinary excretion of lactulose and L-rhamnose was determined by fluorescent detection high-pressure liquid chromatography (HPLC). Basal permeability of the small intestine was assessed as a control condition (no indomethacin, no ATP/adenosine). As a model of increased small intestinal permeability, two dosages of indomethacin were ingested at 10 h (75 mg) and 1h (50 mg) before ingesting the lactulose/rhamnose test drink. At 1.5 h before indomethacin ingestion, two dosages of placebo, ATP (2 g per dosage) or adenosine (1 g per dosage) were administered via enteric-coated hydroxypropyl methylcellulose (HPMC) capsules with Eudragit© L30D-55.

Results: Median urinary lactulose/rhamnose excretion ratio (g/g) in the control condition was 0.032 (interquartile range: 0.022-0.044). Compared to the control condition, lactulose/rhamnose ratio after ingestion of indomethacin plus placebo was significantly increased to 0.039 (0.035-0.068); $P<0.01$). The indomethacin-induced increase was neither affected by administration of encapsulated ATP (0.047 (0.033-0.065)) nor adenosine (0.050 (0.030-0.067)). Differences in L/R ratios between the conditions with indomethacin plus placebo, ATP or adenosine were not significant.

Conclusions: In this study, either ATP or adenosine administered via enteric-coated capsules had no effect on indomethacin-induced small intestinal permeability changes in healthy human volunteers. The observed lack of effect of encapsulated ATP/adenosine may have been caused by opening of the enteric-coated supplement at a site distal from the indomethacin-inflicted site. Further studies on site-specific effectiveness of ATP/adenosine on intestinal permeability changes are warranted.

1. Introduction

The intestinal mucosa on the luminal side of the gut is continuously exposed to an immense load of antigens, for instance from ingested food or resident bacteria. As a crucial part of intestinal defence mechanisms, the mucosa is involved in protecting the host against pathogenic substances. This protective function is called the intestinal barrier function [804, 842]. The mucosal enterocytes are of considerable importance to this barrier function by controlling translocation of pathogenic substances.

In general, it has been proposed that there are two distinct pathways in the intestine through which translocation occurs, that is, a transcellular and a paracellular (*i.e.* intercellular) pathway [843]. The functional integrity of the paracellular pathway can be assessed by measuring gastrointestinal permeability with small saccharide markers. The use of a monosaccharide-disaccharide mixture (such as rhamnose and lactulose) is particularly useful since this provides information regarding villus tip 'damage' as a function of villus surface area [75, 834].

It has been shown that increased mucosal permeability of the small intestine is associated with several gastrointestinal disorders, including inflammatory bowel disease and celiac disease [834, 844]. In Crohn's disease, small intestinal permeability is thought to be positively associated with disease activity [845, 846] and to be an early predictor of relapse [840, 847-849]. In addition to disease-related changes in intestinal barrier function, several factors have been shown to negatively affect intestinal permeability, including smoking [850], alcohol intake [810, 851] and use of nonsteroidal anti-inflammatory drugs (NSAIDs) [798, 821, 852].

Frequent use of NSAIDs is associated with an elevated risk of damage to the mucosal epithelium that lines the gastrointestinal tract lumen, thereby compromising integrity of the mucosal barrier. One of the earliest events in NSAID toxicity is uncoupling of oxidative phosphorylation within enterocytes resulting in depletion of cellular energy stores in the form of adenosine 5'-triphosphate (ATP), which leads to an increase in mucosal permeability in the intestine [817]. It has been demonstrated in previous experiments by Bjarnason and co-workers that mucosal permeability of the small intestine is increased within 8-10 hours after ingestion of two subsequent doses of the NSAID indomethacin (75 and 50 mg); the permeability increase is rapidly reverted, being no longer evident 48 hours after indomethacin ingestion [800, 814, 815]. Utilizing this human model of increased intestinal permeability induced by short-term challenge with indomethacin, we recently showed that topical administration of ATP into the upper small intestine attenuated the indomethacin-induced increase in intestinal permeability in healthy human volunteers [853]. In this randomized cross-over study, fasting subjects received two subsequent indomethacin dosages (75 and 50 mg) concomitant with administration of ATP or placebo directly into the upper small intestine via a naso-intestinal tube. Intestinal permeability was measured by the lactulose/rhamnose (L/R) sugar absorption test, which is a widely used and sensitive permeability measure of the small intestine [812]. Results showed that indomethacin induced an approximately two-fold increase in median urinary L/R excretion ratio relative to the basal L/R ratio in the control condition (*i.e.* no indomethacin, no ATP). Administration of ATP concomitant with indomethacin ingestion

completely prevented the indomethacin-induced increase in L/R ratio [853]. This finding suggested that ATP might be a beneficial compound in alleviating detrimental NSAID effects in the small intestine.

The aim of the present study was to confirm the favourable effect of ATP on the indomethacin-induced increase in intestinal permeability, when ATP is administered via enteric-coated capsules. In addition, since the effect of ATP may have been partly mediated through its breakdown to adenosine, and since adenosine has well-known anti-inflammatory and tissue-protective effects in the intestine [58], we evaluated the effect of adenosine administered via enteric-coated capsules in the same human model of indomethacin-induced permeability changes in the small intestine.

2. Subjects and methods

2.1 Subjects

Non-smoking males and females between 18 and 30 years of age were recruited for participation at Maastricht University, The Netherlands, by way of information pamphlets. Criteria for exclusion from participation, as assessed by a short questionnaire, were: (i) history of gastrointestinal disease or current gastrointestinal disorder (*e.g.* Crohn's disease, celiac disease), (ii) current use of NSAIDs (*e.g.* aspirin, ibuprofen), and (iii) current use of medication which could interfere with effects of ATP/adenosine, including nucleoside transport inhibitors (*e.g.* dipyridamole, lidoflazine), non-selective adenosine receptor antagonists (*e.g.* theophylline, aminophylline), xanthine oxidase inhibitors (*e.g.* allopurinol) and antidepressant drugs. All participants received oral and written information about the aim and protocol of the study, and gave their written informed consent before participation. The study protocol was approved by the Ethics Committee of Maastricht University, The Netherlands, and carried out in compliance with the Helsinki Declaration.

2.2 Sample size

Sample size calculation for the present randomized cross-over study was based on the results of our previous study [853], in which subjects showed an average attenuation in the indomethacin-induced increase in L/R ratio of 0.016 (*i.e.* a reduction of 33%) in response to topical ATP. In the cross-over experiments of our previous study, a standard deviation of 0.021 and correlation between paired measures of 0.74 were observed.

To be able to detect at least half of the previously observed effect, that is ~15% reduction in L/R ratio, based on the standard deviation of 0.021 and a correlation between paired measures of 0.60 (*i.e.* a conservative estimate relative to the correlation of 0.74 observed in our previous study), it was calculated that 31 subjects would be sufficient to detect a significant effect of ATP/adenosine on an indomethacin-induced increase in L/R ratio with a power of 90% and two-tailed alpha of 0.05. Accounting for potential dropout during experiments, a total of 35 participants were recruited for the present study.

2.3 Protocol

The protocol of the present study was based on our previous study in which ATP was administered topically into the upper small intestine via a naso-intestinal tube [853]. As a model of early-stage small intestinal enteropathy, a number of experiments was performed; in each experiment, two subsequent dosages of the NSAID indomethacin (75 and 50 mg) were administered to fasting healthy human subjects. In a double-blind cross-over designed study, each subject participated in four experiments, in a randomized order and with wash-out periods of one week in between: (i) control (no indomethacin, no ATP/adenosine) as a measure of basal permeability of the small intestine, (ii) indomethacin + placebo, (iii) indomethacin + ATP, and (iv) indomethacin + adenosine. Since alcohol intake is known to increase intestinal permeability to larger molecules (*e.g.* lactulose) [810], and since caffeine is a non-selective adenosine receptor antagonist [147], participants were requested to abstain from alcohol and caffeine-containing beverages or foods for four days preceding and during each experiment. Also, participants were asked not to perform any kind of prolonged strenuous physical exercise (*e.g.* long-distance run, cycle race) during two days preceding each experiment, since this has been shown to increase permeability of the small intestine [811]. Antidepressants have been shown to affect activity of the enzyme adenosine deaminase [854, 855], which catalyzes the breakdown of adenosine to inosine. Current antidepressant use may therefore interfere with effects of ATP/adenosine.

The control experiment comprised only the assessment of basal permeability without any intervention: after an overnight fast and after voiding, participants ingested a test drink containing 5 g lactulose (Centrafarm Services BV, Etten-Leur, The Netherlands) and 0.5 g L-rhamnose (MP Biomedicals, Aurora, OH, USA) dissolved in 100 mL water. Subsequently, total urine was collected for five hours. During the last two hours of urine collection, subjects were allowed unlimited intake of water, which stimulates adequate urine production without influencing urinary recovery of lactulose and L-rhamnose as well as the lactulose/rhamnose excretion ratio [856]. Total urine volume over the five-hour period was determined, and urine aliquots were taken and stored at -80°C until analysis.

For the experiments with indomethacin plus placebo/ATP/adenosine, at 11.5 h prior to the permeability assessment (*i.e.* ingestion of the lactulose/rhamnose test drink), participants ingested five capsules containing placebo, 2 g ATP or 1 g adenosine. One and a half hour later, participants ingested 75 mg indomethacin (Genfarma, Zaandam, The Netherlands). After an overnight fast, 2.5 h before permeability assessment, participants again ingested five capsules containing placebo, 2 g ATP or 1 g adenosine, followed by a second dose of indomethacin (50 mg) at 1 h prior to the permeability assessment. One hour later, after voiding, participants ingested the lactulose/rhamnose test drink followed by collection of total urine for five hours.

2.4 Experimental supplements

ATP, adenosine and placebo were administered via enteric-coated hydroxypropyl methylcellulose (HPMC) Vcaps capsules, which are two-piece capsules consisting of a body and cap (a kind gift from the Laboratory of Pharmaceutical Technology, Ghent University, Ghent, Belgium). HPMC caps and bodies (capsule size 00: average weight 118 mg, volume capacity 0.91 mL and closed length 23.3 mm) were

coated with Eudragit® L30D-55 [857]. The enteric coating of the HPMC capsules generally dissolves within approximately 60 minutes after gastric stage at pH 6.0 and within approximately 40 minutes at pH 6.5, that is, in the proximal small intestine [857]. Using an automatic capsule-filling machine (Blokland Medical Supplies BV, IJsselstein, The Netherlands), the coated bodies were filled with lactose (BUFA BV, Uitgeest, The Netherlands), which was used as placebo as well as inert excipient, and/or ATP or adenosine and closed with the coated caps. Each participant ingested ATP by taking five capsules twice, containing a dosage of 0.4 g ATP per capsule, that is, 4 g ATP in total. Preliminary experiments demonstrated the safety of this ATP dosage (H.J. Bos, P.C. Dagnelie, unpublished observations). Adenosine was administered at an equimolar dosage: each participant ingested adenosine by taking five capsules twice, containing 0.2 g adenosine per capsule, that is, 2 g adenosine in total. The dosage of lactose that was administered does not produce symptoms of lactose intolerance, which are reported to occur at dosages of about 12 to 18 g [858].

2.5 Intestinal permeability

Intestinal permeability was assessed using the lactulose/rhamnose (L/R) sugar absorption test: ingestion of 5 g lactulose and 0.5 g L-rhamnose dissolved in 100 mL water followed by five-hour collection of total urine. This test is based on the comparison of intestinal permeation of molecules of different sizes. The urinary L/R excretion ratio is considered to be an accurate parameter of small intestinal permeability [812]. Lactulose and L-rhamnose in collected urine samples were determined by fluorescent detection high-pressure liquid chromatography (HPLC). The method for assaying lactulose and L-rhamnose has been described previously [813]. In short, cellobiose was added to urine samples as an internal standard, and the urine was filtered through a 0.4-µm filter and diluted as necessary. Samples were deionized and then injected onto a Dionex MA-1 ion exchange column. Sugars were eluted with NaOH at a flow rate of 0.4 mL/min with concentrations ranging from 400 to 600 mmol/L. Peaks were detected using pulsed amperometric detection on a Dionex HPLC and quantified as peak areas. Calibration was performed on a daily basis with authentic standards at multiple concentrations, and the experimental standards were diluted so that the areas of all peaks fell within the calibration range.

2.5 Statistics

Five-hour urinary excretion levels of lactulose and L-rhamnose are presented as recovery (%) of ingested lactulose and L-rhamnose, and as L/R ratios (g/g). Differences in urinary L/R ratios between different conditions were assessed using Wilcoxon signed ranks test. P-values below 0.05 were regarded statistically significant. Data are presented as Box-Whisker plots.

3. Results

For the present study, a total of 35 participants were recruited, of whom two subjects did not complete all four experiments. One subject had to stop after completion of one experiment because of newly diagnosed celiac disease, and one subject only participated in two experiments due to limitation of time. Thirty-three participants (7 males, 26 females; age (mean ± SD) 22 ± 3.3 years; range 18-30 years)

completed all four experiments and were included in the analyses. No side effects were reported during the experiments.

Figure 1 shows L/R ratios of the four experimental conditions. Median L/R ratio (g/g) in the control condition (no indomethacin, no ATP/adenosine) was 0.032 (interquartile range: 0.022-0.044). After ingestion of indomethacin plus placebo, the median L/R ratio was significantly increased to 0.039 (0.035-0.068; P<0.01 vs. control). Intake of enteric-coated capsules with either ATP or adenosine at 1.5 h prior to indomethacin ingestion had no effect on the indomethacin-induced increase in L/R ratio. Median L/R ratio after ingestion of indomethacin plus ATP was 0.047 (0.033-0.065; P=0.22 vs. placebo), and median L/R ratio after ingestion of indomethacin plus adenosine was 0.050 (0.030-0.067; P=0.49 vs. placebo). Median L/R ratios after indomethacin ingestion with administration of ATP or adenosine remained significantly increased compared to the L/R ratio in the control condition (P<0.01, Fig. 1).

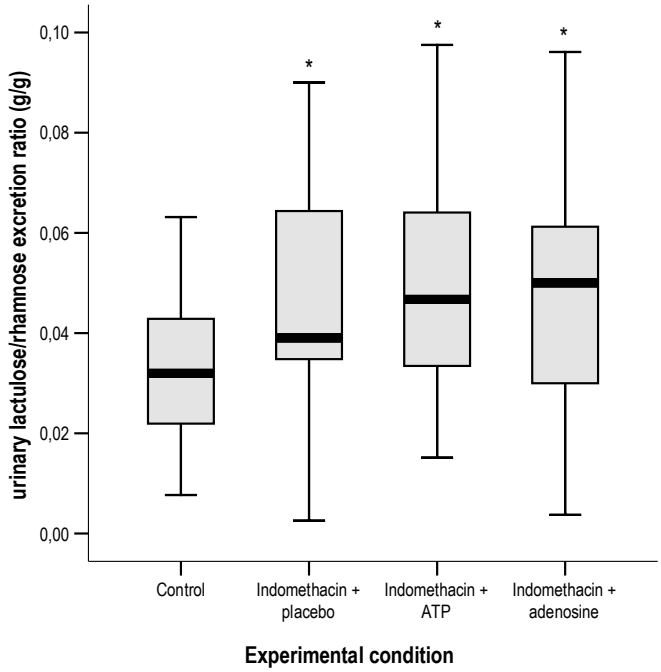

Figure 1. *Box Whisker plot of urinary lactulose/rhamnose (L/R) excretion ratios (g/g) observed in four experimental conditions. The control condition represents basal permeability of the small intestine, as indicated by the urinary L/R ratio after ingestion of a test drink containing 5 g lactulose and 0.5 g L-rhamnose with no prior indomethacin ingestion and no placebo/ATP/adenosine challenge. The other conditions represent urinary L/R ratios after ingestion of two subsequent indomethacin dosages at 10 h (75 mg) and 1 h (50 mg) before ingestion of the test drink; at 1.5 h prior to the indomethacin dosages, two dosages of placebo (indomethacin + placebo), ATP (indomethacin + ATP, 2 g ATP per dosage) or adenosine (indomethacin + adenosine, 1 g adenosine per dosage) were administered via enteric-coated capsules (*P<0.01 vs. control; n=33, Wilcoxon signed ranks test). Differences in L/R ratios between the conditions with indomethacin plus placebo, ATP or adenosine were not significant.*

Table 1 shows total urine volumes (mL) and five-hour urinary recovery (%) of orally ingested lactulose (5 g) and L-rhamnose (0.5 g) in four experimental conditions. Five-hour total urine volumes were similar in all experimental conditions (Table 1). Compared to lactulose recovery in the control condition (0.14% (0.08-0.22%)), urinary lactulose recovery was significantly increased by ingestion of indomethacin plus placebo (0.20% (0.11-0.31%); P<0.01). Neither administration of ATP nor of adenosine affected the indomethacin-induced increase in lactulose permeation. Lactulose recovery after ingestion of capsules with ATP was 0.18% (0.12-0.32%) and was 0.23% (0.11-0.28%) after ingestion of capsules with adenosine; both these values remained significantly increased compared to the control condition (P<0.01). Basal urinary recovery of L-rhamnose was not significantly affected by ingestion of indomethacin plus placebo, ATP or adenosine (Table 1).

Table 1. *Five-hour total urine volumes (mL) and urinary recovery (%) of orally ingested lactulose (5 g) and L-rhamnose (0.5 g) in four experimental conditions (n=33).*

Experimental condition	**Recovery (%)** Median (interquartile range)		**Urine volume (mL)** Median (interquartile range)
	Lactulose	**Rhamnose**	
Control[a]	0.14 (0.08-0.22)	4.41 (3.31-5.88)	298 (182-613)
Indomethacin + placebo	0.20 (0.11-0.31)*	4.60 (3.15-5.91)	324 (193-493)
Indomethacin + ATP	0.18 (0.12-0.32)*	4.12 (3.23-4.90)	371 (165-518)
Indomethacin + adenosine	0.23 (0.11-0.28)*	4.52 (3.90-5.26)	307 (213-563)

[a]*No indomethacin, no placebo/ATP/adenosine*

**P<0.01 vs. control (Wilcoxon signed ranks test)*

Differences in lactulose/rhamnose recovery and urine volumes between the conditions with indomethacin plus placebo, ATP or adenosine were not significant

In all experimental conditions, no differences were found between male and female subjects regarding urinary L/R excretion ratios, total urine volumes and urinary recovery of lactulose and L-rhamnose (data not shown).

4. Discussion

We previously reported that topical administration of ATP into the human small intestine by naso-intestinal tube attenuated an indomethacin-induced increase in mucosal permeability in healthy human volunteers [853]. This finding indicated that ATP administration may be of clinical use by alleviating early adverse effects of NSAIDs and by preserving small intestinal barrier function. Like in the present study, early-phase small intestinal permeability changes were induced by administering two subsequent dosages of the NSAID indomethacin (75 and 50 mg). Short-term indomethacin challenge causes a rapid (within 8-10 h) and temporary (< 48 h) increase in intestinal permeability, which can be assessed non-invasively by calculating the urinary excretion ratio of orally ingested lactulose and L-rhamnose as markers of small intestinal permeability [800, 809, 814, 815].

To administer ATP experimentally in the upper small intestine, the ATP in our previous study was administered via a naso-intestinal tube. Since a naso-intestinal tube is a useful way of administration in an experimental setting but not in daily practice as it can cause great discomfort to sensitive subjects or patients, the aim of the present study was to corroborate the previously observed favourable effect of ATP on indomethacin-induced permeability changes when ATP was administered via enteric-coated capsules, which is a more practically feasible mode of administration. We also evaluated the effect of adenosine administration via enteric-coated capsules, since we hypothesized that the previously observed favourable effect of ATP may have been partly mediated by its breakdown product adenosine, which has well-known anti-inflammatory and tissue-protective properties in the intestine [58].

Results of the present study show that, like in our previous study, ingestion of two subsequent indomethacin dosages significantly increases urinary L/R excretion ratio compared to the L/R ratio in the control condition, which is a measure of basal small intestinal permeability. The rise in intestinal permeability was probably due to enhanced permeation of lactulose, since urinary recovery of lactulose, but not of rhamnose, was increased by indomethacin ingestion. Unexpectedly, neither administration of encapsulated ATP nor adenosine affected the indomethacin-induced rise in small intestinal permeability, suggesting that ATP and adenosine administered via enteric-coated capsules are ineffective in attenuating an indomethacin-induced increase in paracellular permeability of the small intestinal mucosa in healthy humans.

Several potential explanations of the unexpected ineffectiveness of both enteric-coated ATP and adenosine on indomethacin-induced permeability changes in the present study can be put forward. First, the possibility should be considered that the present study results would suggest a false-positive finding in our previous study, meaning that the favourable effect of topically administered ATP on small intestinal permeability changes induced by indomethacin in our previous study was random. However, the highly significant P-value ($P<0.01$) observed in our previous study, which was conducted according to a double-blind, randomized cross-over design, argues against the possibility of a false-positive finding.

Second, it could be argued that subject-related differences between the present and our previous study could be responsible for the different results. This explanation is also unlikely since all study participants were recruited from the same study base (*i.e.* students at Maastricht University, The Netherlands), and age and sex ratio of the participants were quite similar in both studies. Moreover, permeability of the small intestinal mucosa of healthy subjects, as assessed by urinary excretion ratio of two different-sized test molecules, is thought to be independent of age and gender [859, 860]. Nevertheless, it must be noted that intestinal permeability may vary considerably under normal conditions, since basal permeability in the present study appeared to be somewhat higher than in our previous study (median L/R ratio of 0.032 vs. 0.023, respectively) despite identical study design and methods.

A third possibility is that the ATP and adenosine within the capsules were chemically unstable and therefore degraded before application. However, this is unlikely since capsules filled with ATP or adenosine in crystalline form were stored dry at 4°C, at which storage conditions both substances are stable for 24 months according to the manufacturer's specifications (BUFA BV, Uitgeest, The

Netherlands). To confirm stability of the ATP used in the present study, which had been stored at 4°C for several months, we measured its quality by HPLC and found no signs of any ATP degradation (data not shown). Also, the enteric properties of the Eudragit© L30D-55 coating polymer have been shown to be maintained even after 6 months of storage at different conditions [857].

A fourth potential explanation of the ineffectiveness of ATP and adenosine in the present study could be relative insolubility of ATP and adenosine. In our previous study, we administered ATP as an aqueous solution (30 mg/kg ATP dissolved in 100 mL water), whereas in the present study ATP and adenosine were administered in crystalline form within the enteric-coated HPMC capsules. However, since both ATP and adenosine are substances which are freely soluble in water [861], both are likely to dissolve within minutes in the liquid environment of the gut upon opening of the capsules.

Finally, a possible explanation may be a discrepancy between the intestinal site of indomethacin-inflicted mucosal damage relative to the site of ATP/adenosine delivery from the enteric-coated capsules in the small intestine. It is believed that a short-term NSAID challenge induces permeability changes mainly in the upper small intestine (duodenum and proximal jejunum) [819, 862]. This would imply that the timing of the pH-dependent release of ATP/adenosine by the enteric-coated capsules might be an important factor limiting the effectiveness of these compounds. In our previous study, in which ATP was administered as an aqueous solution via a naso-intestinal tube directly into the upper small intestine, which is the intestinal site where indomethacin-inflicted damage would occur, ATP attenuated the indomethacin-induced increase in small intestinal permeability [853]. In the present study, ATP and adenosine were administered via HPMC capsules coated with Eudragit© L30D-55 [857]. Huyghebaert et al. (2004) showed by *in vitro* dissolution tests that the Eudragit© L30D-55 enteric-coated capsules released 80% of their contents within 60 minutes at pH 6.0, with a lag-phase of 20 minutes after simulated gastric stage. At pH 6.5, 80% of the capsule content was released within 30 minutes after simulated gastric stage without lag-time [857]. In additional experiments by our research group, in which lithium was used as a marker to evaluate the timing of contents release by identical enteric-coated capsules ingested by healthy subjects, we observed rising lithium concentrations in plasma between approximately 90 to 200 minutes following capsule ingestion, suggesting that opening of the capsules *in vivo* may be subject to considerable variation (H.J. Bos, P.C. Dagnelie, unpublished observations). This would suggest that the lack of effect of ATP and adenosine in the present study might be explained by missing the target area of indomethacin-inflicted upper-small-intestinal damage when administering ATP and adenosine via the Eudragit© L30D-55 enteric-coated capsules.

In conclusion, we were not able to corroborate the previously shown beneficial effects of topical ATP administration on indomethacin-induced permeability changes in the human small intestine, when using enteric-coated capsules containing either ATP or adenosine as a more practically feasible mode of administration. The most likely explanation for the present finding is that the enteric-coated supplement may have opened at an intestinal site different (probably more distal) from the site where mucosal damage by indomethacin occurs. Further studies on site-specific effectiveness of encapsulated ATP and adenosine on intestinal permeability changes are warranted.

CHAPTER 5

Effects of adenosine 5'-triphosphate and adenosine on cytokine production and ICAM-1 expression by human enterocytes

Submitted for publication

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Abstract

Background: Effective mucosal immunity in the small intestine requires co-operative efforts of leukocytes and enterocytes. It is well-established that inflammatory processes are modulated by co-ordinate actions of ATP and adenosine on various leukocyte functions through P2 and P1 receptor signaling, respectively. In the present study, our objective was to explore effects of ATP and adenosine on immunological functions mediated by human enterocytes.

Methods: Two experiments with human enterocyte-like Caco-2 cells were performed: (i) time-dependent metabolism of ATP and adenosine was analyzed by HPLC and mRNA expression for P2 and P1 receptors was determined by RT-PCR, (ii) effects of ATP and adenosine on ICAM-1 expression were assessed by flow cytometry and effects on cytokine production by ELISA.

Results: Both ATP and adenosine were metabolized by Caco-2 cells with subsequent formation of their metabolites. Caco-2 cells expressed mRNA for $P2Y_1$, $P2Y_2$, $P2Y_{11}$, $P2Y_{12}$, A_{2A}, A_{2B} and A_3 receptor subtypes. Basal ICAM-1 expression was decreased by adenosine at 500 μM but increased at 2500 μM adenosine. ATP did not affect ICAM-1 expression. ATP and adenosine increased basal levels of IL-8. ATP at 500 μM and 2500 μM enhanced the production of IL-8 and TNFα that was induced by 16-hour stimulation of cells by IFNγ and IL-1β, whereas the IFNγ/IL-1β-stimulated production of IL-6 was inhibited by ATP. The ATP analogue and P2 receptor agonist ATPγS had similar effects on IL-8 and IL-6, but did not affect TNFα production. Adenosine at 2500 μM increased IFNγ/IL-1β-stimulated production of IL-8 and decreased IFNγ/IL-1β-stimulated production of IL-6 by Caco-2 cells. EHNA, intended as an adenosine breakdown inhibitor, exerted direct effects on cytokine production by Caco-2 cells.

Conclusions: The present results indicate that (i) Caco-2 cells exhibit ecto-enzyme activity enabling them to control extracellular ATP and adenosine levels, (ii) Caco-2 cells express both P2 and P1 receptor mRNA, and (iii) generation of inflammatory mediators, especially cytokines, by Caco-2 cells is affected by extracellular ATP and adenosine. Our findings warrant further investigation on the role of purinergic signaling in inflammatory responses mediated by human enterocytes.

1. Introduction

The epithelial cells that line the inner wall of the small intestine are crucial actors of mucosal defence. On the one hand, these enterocytes constitute a cellular barrier that protects the sensitive immune system from continuous contact with the abundance of potentially harmful substances in the gut lumen (*e.g.* food-borne antigens or bacterial products). On the other hand, the enterocytes are part of an immunological barrier by their capability to initiate local inflammatory responses upon pathogen penetration into underlying mucosal tissues. Enterocytes have been shown to generate a variety of pro-inflammatory mediators [73, 863], which promote recruitment and activation of different types of leukocytes that establish a local inflammatory reaction. It is believed that efficient pathogen elimination and protection of mucosal tissues in the intestine requires regulated interaction between leukocytes and enterocytes. Imbalanced immunological interaction between leukocytes and enterocytes contributes to the pathophysiology of chronic intestinal disorders, which are associated with uncontrolled inflammation and persistent damage to mucosal tissues, such as inflammatory bowel disease (IBD). Therefore, identification of regulatory molecules involved in the process of mucosal inflammation is imperative.

Adenosine 5'-triphosphate (ATP) and its metabolite adenosine are ubiquitous signaling molecules in the extracellular compartment. They are the most important physiological ligands for a broadly expressed family of purinergic receptors of which numerous receptor subtypes have been identified to date. The family comprises P2 receptors for ATP ($P2X_{1\text{-}7}$ and $P2Y_{1,2,4,6,11\text{-}14}$) and P1 receptors for adenosine (A_1, A_{2A}, A_{2B} and A_3) [148, 156, 864]. Extracellular ATP and adenosine are thought to be involved in the complex network of immune regulation in a way that co-ordinated activation of P2 and P1 receptors modulates a variety of leukocyte functions, thereby affecting the outcome of inflammation and immune responses [93, 175, 865]. It is generally assumed that the nature of purinergic immunoregulation is determined to a great extent by concentration-dependent activation of specific P2 and P1 receptor subtypes by ATP and adenosine, respectively.

In conditions of acute cellular stress, ATP at high extracellular concentrations (> 500 µM) acts as a danger signal inducing pro-inflammatory and immunostimulatory effects, mainly through $P2X_7$ receptor activation. ATP at lower extracellular levels may mediate anti-inflammatory and immunomodulatory effects that are involved in the control of ongoing inflammatory processes, possibly via simultaneous activation of different P2Y receptor subtypes. In contrast to ATP, extracellular adenosine has been shown to predominantly mediate potent anti-inflammatory and immunosuppressive effects through activation of different P1 receptor subtypes, thereby contributing to the resolution of acute inflammatory processes [865].

Changed expression profiles of P2 and P1 receptor subtypes found in the gut mucosa of patients with active IBD suggest that purinergic signaling might play a role in the inflamed intestine [55, 657, 866]. This has fuelled the notion that ATP and adenosine, in addition to their modulatory effects on various leukocyte functions, may be involved in the modulation of inflammatory processes mediated by enterocytes. One approach to studying enterocyte function is the use of Caco-2 cells, which are human-

derived intestinal cells that in long-term culture are known to differentiate into enterocyte-like small intestinal epithelial cells [867]. Caco-2 cells have been shown to exhibit a variety of immunological properties *in vitro* by secreting interleukin (IL)-6, IL-8 and TNFα [868-873] and by up-regulating apical expression of intercellular adhesion molecule (ICAM)-1 in response to inflammatory stimuli [874, 875]. To our knowledge, effects of ATP and adenosine on the generation of inflammatory mediators by human enterocyte-like Caco-2 cells have not been investigated to date.

In the present study, two experiments with Caco-2 cells were performed to explore the role of extracellular ATP and adenosine in an enterocyte-driven inflammatory response. In the first experiment, we determined the time-dependent metabolism of ATP and adenosine in our Caco-2 cell culture as an indication of ecto-enzyme activity, and assessed the presence of mRNA for a number of P2 and P1 receptor subtypes. In the second experiment, we evaluated effects of ATP and adenosine on ICAM-1 expression and cytokine production by the Caco-2 cells. Specifically, we hypothesized that ATP at high extracellular concentrations would enhance the generation of inflammatory mediators by human enterocyte-like Caco-2 cells, whereas adenosine would have mostly inhibitory effects. This hypothesis was only partly confirmed by our findings.

2. Materials and methods

2.1 Reagents

Adenosine and adenosine 5'-triphosphate (ATP) disodium salt were purchased from Calbiochem (EMD Biosciences, Inc., San Diego, CA, USA). The P2 receptor agonist adenosine 5'-O-(3-thiotriphosphate) (ATPγS) was purchased from Sigma Chemical Company (St. Louis, MO, USA). 6-N,N-Diethyl-D-β,γ-dibromomethylene ATP trisodium salt (ARL-67156; an ecto-ATPase inhibitor) and erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA; an adenosine deaminase (ADA) inhibitor) were purchased from A.G. Scientific, Inc. (San Diego, CA, USA). Bovine serum albumin (BSA; endotoxin and fatty acid-free) was obtained from Sigma Chemical Company (St. Louis, MO, USA). Recombinant human IL-1β and interferon (IFN)-γ were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Trypsine, penicillin streptomycin (PS), sodium pyruvate (SP), non-essential amino acids (NEAA) and all cell culture media were obtained from Invitrogen Corporation (Paisley, UK). Fetal calf serum (FCS; South-American) was obtained from Greiner Bio-one (Frickenhausen, Germany).

2.2 Intestinal cell culture

The human Caco-2 cell line was purchased from the American Tissue Type Collection (ATTC). Caco-2 cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% heat-inactivated FCS and 1% PS, 1% SP and 1% NEAA. Cells were cultured at 37°C in a 5% CO_2 humidified atmosphere, refreshed every second day, and separated by trypsine-0.03% ethylenediaminetetraacetate (EDTA) when reaching 70-90% confluency. Two experiments were performed with the cultured Caco-2 cells.

2.2.1 Experiment 1

Caco-2 cells were plated in 24-well tissue culture plates at an initial density of $0.3x10^6$ cells/mL in a total volume of 0.5 mL. Medium was replaced every other day for 20 days until cells were fully differentiated. To evaluate metabolism of ATP and adenosine in the Caco-2 cell culture over a 24-hour period, cells were incubated with ATP or adenosine at different concentrations by replacing the culture medium by medium containing the compound of interest with (n=2) or without (n=1) an inflammation-inducing cocktail consisting of the pro-inflammatory cytokines IFNγ (100 U/mL) and IL-1β (50 U/mL). In part of the incubations, effects of ecto-enzyme inhibitors on ATP and adenosine metabolism were evaluated by co-incubating ATP with ARL-67156 (100 μM) and adenosine with EHNA (100 μM). At 15, 30, 60, 120, 360 and 1440 minutes, samples from the culture medium were collected for analysis of levels of ATP, adenosine and their metabolites; levels of IL-8 were also determined in these culture medium samples. Moreover, total RNA was isolated in order to determine mRNA expression of P2 and P1 receptor subtypes.

2.2.2 Experiment 2

Caco-2 cells were plated in 6-well tissue culture plates at an initial density of $0.3x10^6$ cells/mL in a total volume of 1.5 mL. Medium was replaced every other day for 18 days until cells were fully differentiated. To evaluate effects of ATP and adenosine on cytokine production and ICAM-1 expression by Caco-2 cells, the cells were incubated for 16 hours with different concentrations of ATP or adenosine with (n=2) or without (n=2) the pro-inflammatory cytokines IFNγ (100 U/mL) and IL-1β (50 U/mL). Since the HPLC data of our first experiment indicated that ARL-67156 did not inhibit ATP breakdown when co-incubated with ATP, we applied a different incubation protocol in the second experiment: ARL-67156 (100 μM) was added 30 minutes before ATP in an attempt to inhibit breakdown of ATP. Similarly, to inhibit breakdown of adenosine, cells were co-incubated with adenosine and EHNA (25 μM; we found that EHNA at this concentration was equally effective as EHNA at 100 μM in minimizing adenosine breakdown). Effects of the stable ATP analogue and P2 receptor agonist ATPγS were also evaluated. After 16 hours of incubation, Caco-2 cell culture medium was collected for analysis of cytokine levels, and living cells were used to measure cell surface ICAM-1 protein expression. Levels of ATP and adenosine in 16-hour culture medium samples were determined also.

2.3 HPLC analysis

Levels of ATP, adenosine and their metabolites (ADP, AMP, inosine, hypoxanthine, adenine and uric acid) in Caco-2 cell culture medium were analyzed by high-pressure liquid chromatography (HPLC) according to the method described by Schweinsberg et al. [876], with minor modifications. Briefly, cell culture medium samples were deproteinized with 8% (v/v) perchloric acid and centrifuged (12,000 rpm; 10 min; 4°C). The supernatant was neutralized (pH 6-7) with 2M K_2CO_3 in 6M KOH and centrifuged (14,000 rpm; 10 min; 4°C). In a single run, ATP, adenosine and their metabolites were quantified using a HPLC system (Agilent, Palo Alto, CA, USA) equipped with a UV/VIS detector (254 nm). Separation was achieved with a 3 μm ODS Hypersil C18 RP column (150 × 4.6 mm i.d.; Thermo Electron Corp., Waltham, MA, USA) protected by a 5 μm Hypersil C18 guard column (10 × 4 mm i.d.; Alltech BV, Breda, The Netherlands). Concentrations of ATP, adenosine and their metabolites were calculated by

comparing peak areas with appropriate standards. The molar balance, which is the sum of the molar concentrations of ATP/adenosine and their metabolites in the samples, was calculated at the different time points.

2.4 Analysis of mRNA expression

Total RNA was isolated using Trizol reagent (Invitrogen Corporation, Paisley, UK) according to the manufacturer's instructions. RNA (100 ng) was reversed transcribed using the ACCESS reverse transcription-polymerase chain reaction (RT-PCR) kit (Promega Corporation, Madison, WI, USA), and then amplified for 30 cycles. Glyceroaldehyde-3-phosphate dehydrogenase (G3PDH) was used as a positive control. Two RT-PCR reactions were done for each P2 and P1 receptor subtype. RT-PCR primers are shown in Table 1. After RT-PCR, 5 µL of each sample was loaded onto 1.5% agarose gel. Densitometric analysis was done using Kodak 1D Image Analysis Software. Density of the bands was expressed as a net intensity, that is, the sum of the background-subtracted pixel value within the region of interest.

Table 1. *RT-PCR primers.*

Transcript	Primers forward and reverse	Annealing conditions	Product size (bp)
A_{2A}	GGG GTA CCA GTG GAG GGA GTG C AAG CCG CGG AGA AAG ATA AAG A	58.6°C, 30s	208
A_{2B}	ACG GTA CCA CAA GAA ACA AAG AGG AC AAG CCG CGG AGC CTA CTA ATG ACA	54°C, 30s	153
A_3	ACG GTG AGG TAC CAC AGC TTG TG ATA CCG CGG GAT GGC AGA CC	59.5°C, 30s	156
$P2Y_1$	GGC AGG CTC AAG AAG AAG AAT ATC ACA CAT TTC TGG GGT CTG	56°C, 1min 50s	260
$P2Y_2$	CCC TGC CGC TGC TGG TTT A GAT GGC GTT GAG GGT GTG G	56°C, 1min 50s	340
$P2Y_{11}$	GTG GTT GAG TTC CTG GTG GC CCA GCA GGT TGC AGG TGA AG	62°C, 30s	238
$P2Y_{12}$	TTT GCC CGA ATT CCT TAC AC ATT GGG GCA CTT CAG CAT AC	57°C, 30 s	201
G3PDH	CCT CTG ACT TCA ACA GCG AC CAT GAC AAG GTG CGG CTC CC	60°C, 1 min	356

2.5 Flow cytometry analysis of ICAM-1

Cell surface ICAM-1 protein expression on living Caco-2 cells was quantified by a flow cytometry assay. Briefly, after 16-hour incubation, cells were washed three times with phosphate buffer saline (PBS) and detached with trypsine-0.03% EDTA. Next, culture medium was added and cell suspensions were centrifuged for 5 min at 1,200 rpm at room temperature, followed by re-suspending the pellets in 500 µL PBS-1% BSA. Cells were counted and diluted to 10^6 cells/mL in PBS-1% BSA. Recombinant-phycoerythrin (R-PE)-conjugated mouse-anti-human CD54 monoclonal antibody (anti-ICAM-1) or

isotype-matched control antibody (Becton Dickinson Biosciences, San Diego, CA, USA) 20 µL/10^6 cells was added and incubated for 30 minutes on ice in the dark. Next, cell suspensions were centrifuged for 5 min at 1,500 rpm and pellets were resuspended in 500 µL PBS-1% BSA. The amount of fluorescence of 10,000 living cells was counted and analyzed with the FACSort (Becton Dickinson, Franklin Lakes, NJ, USA) and CellQuest analysis software.

2.6 Measurement of cytokines by ELISA

IL-6 and IL-8 were quantified by means of specific PeliKine Compact™ human enzyme-linked immunosorbent assay (ELISA) kits (Sanquin, Amsterdam, The Netherlands) based on appropriate and validated sets of monoclonal antibodies. IL-10 and TNFα were quantified by specific Quantikine® HS human ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA). Assays were performed according to the manufacturers' instructions, in duplicate (IL-6, IL-10, TNFα) or in triplicate (IL-8). Sensitivities for IL-6, IL-8, IL-10 and TNFα were 0.5-1 pg/mL, 4-8 pg/mL, < 0.5 pg/mL and 0.06-0.32 pg/mL, respectively.

2.7 Statistical analysis

Effects of ATP and adenosine at different concentrations were assessed by Student's Independent-Samples T test in case of comparison between two conditions or concentrations, and One-Way ANOVA with Tukey HSD multiple comparisons test in case of comparison between more than two conditions or concentrations. Values are expressed as mean ± SD or SEM. P-values below 0.05 were regarded statistically significant.

3. Results

3.1 Experiment 1

3.1.1 Metabolism of ATP and adenosine in the Caco-2 cell culture

Neither ATP/adenosine nor their metabolites were detected by HPLC in culture medium before incubation of Caco-2 cells. In all incubations, the molar balance remained stable at any of the different time points.

As shown in Fig. 1, ATP at 500 µM (Fig. 1A) and 2500 µM (Fig. 1B) was metabolized in a time-dependent fashion with sequential formation of its metabolites ADP, AMP, adenosine, inosine and hypoxanthine. Other known metabolites of ATP, such as adenine and uric acid, were not detected in Caco-2 cell culture medium. At both concentrations (500 µM and 2500 µM), ATP was completely degraded within 6 hours of incubation. In an attempt to minimize ATP degradation, Caco-2 cells were also incubated with ATP in the presence of ARL-67156 (100 µM); however, this ecto-ATPase inhibitor had no effect on the ATP degradation profile in Caco-2 cell culture medium, regardless of the ATP concentration (Fig. 1C and 1D).

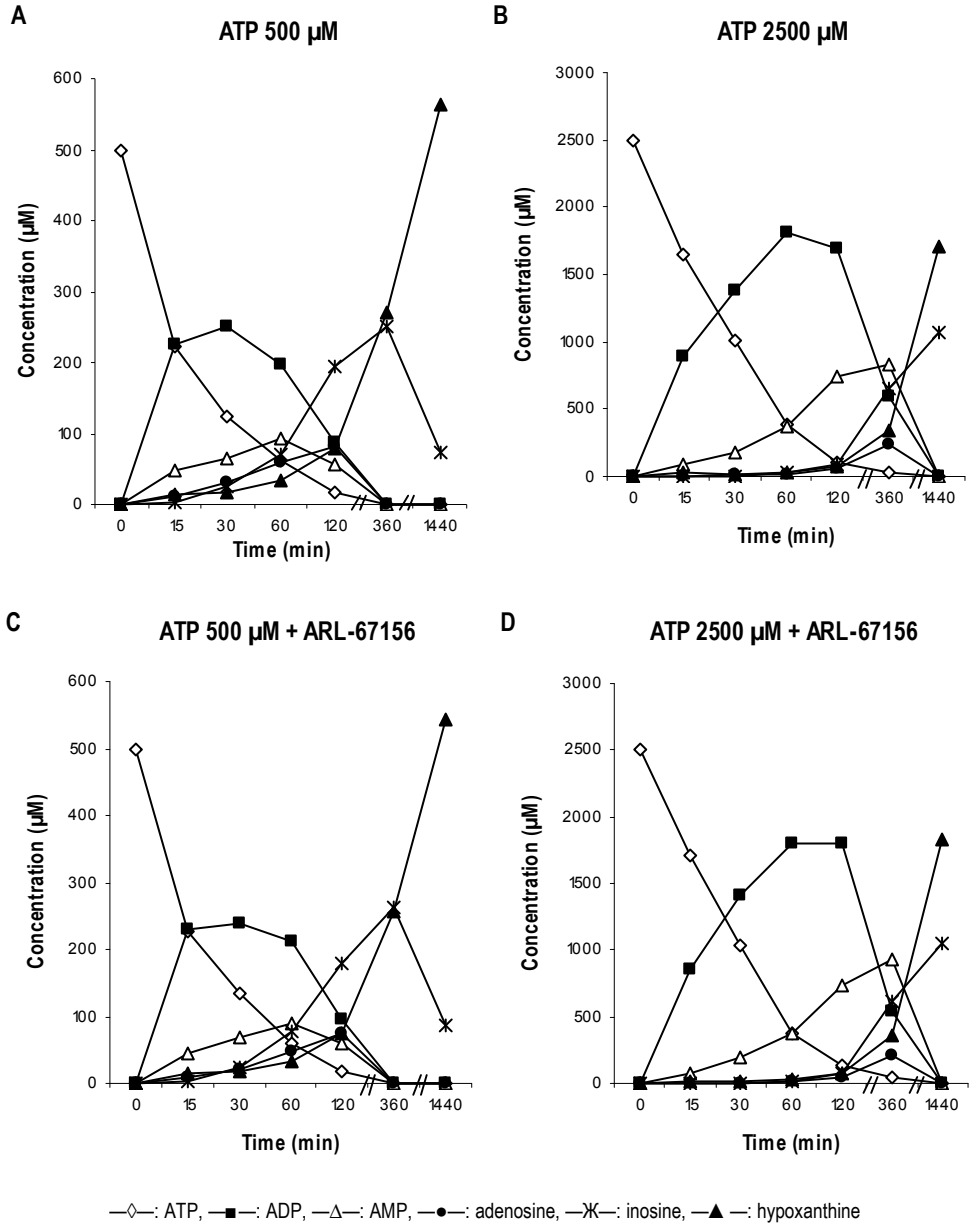


Figure 1. *ATP metabolism by Caco-2 cells. Cells were incubated for 24 hours with ATP at concentrations of 500 µM **(A)** and 2500 µM **(B)** in the presence of IFNγ (100 U/mL) and IL-1β (50 U/mL). In part of the incubations, ATP was co-incubated with ARL-67156 at a concentration of 100 µM **(C, D)** to evaluate the effect of ecto-ATPase inhibition on ATP metabolism by Caco-2 cells. All incubations were performed in duplicate. Values are expressed as mean concentrations of two measurements.*

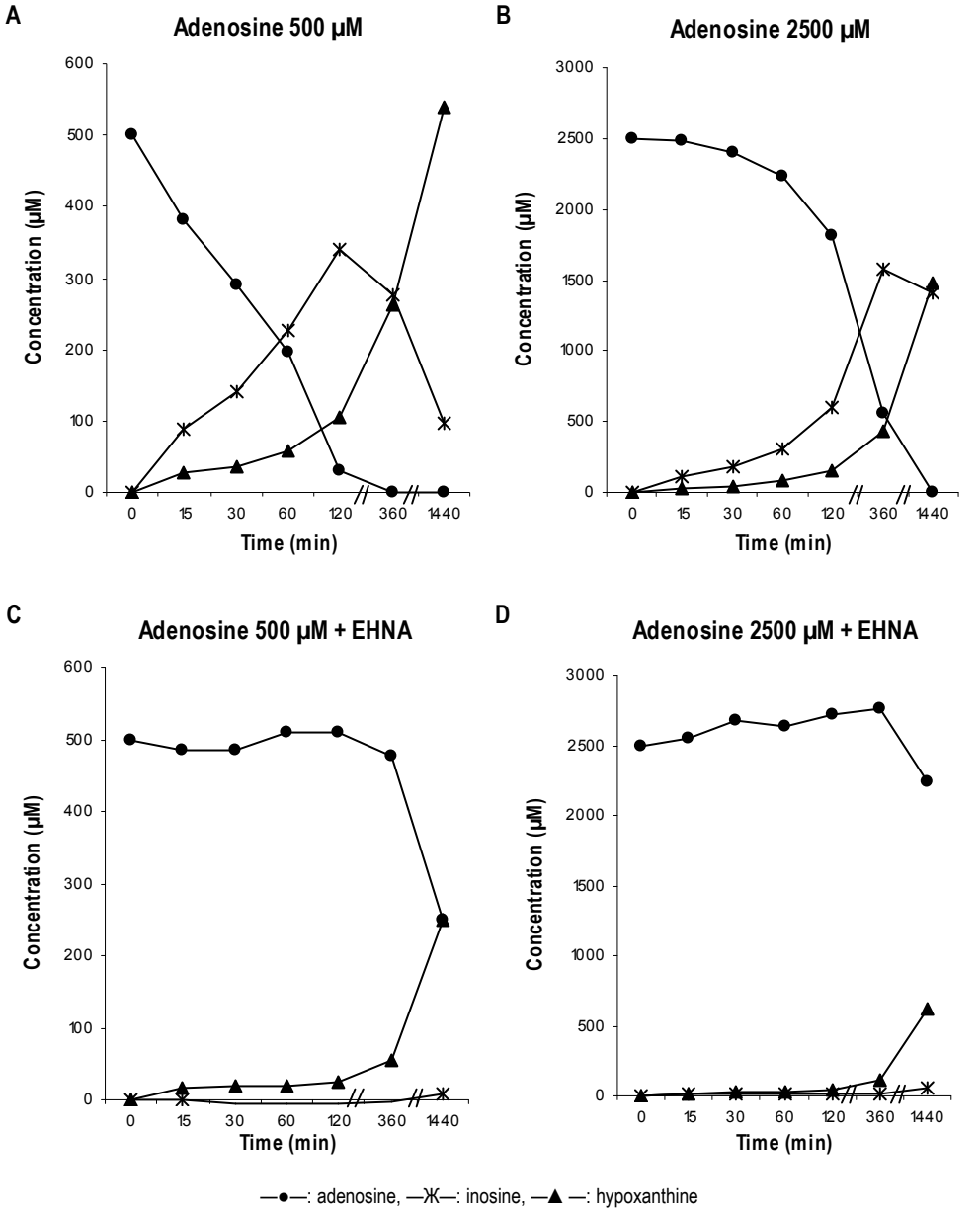


Figure 2. *Adenosine metabolism by Caco-2 cells. Cells were incubated for 24 hours with adenosine at concentrations of 500 µM* ***(A)*** *and 2500 µM* ***(B)*** *in the presence of IFNγ (100 U/mL) and IL-1β (50 U/mL). In part of the incubations, adenosine was co-incubated with EHNA at a concentration of 100 µM* ***(C, D)*** *to evaluate the effect of ADA inhibition on adenosine metabolism by Caco-2 cells. All incubations were performed in duplicate. Values are expressed as mean concentrations of two measurements.*

As shown in Fig. 2, adenosine at 500 µM (Fig. 2A) and 2500 µM (Fig. 2B) was also metabolized in a time-dependent fashion with sequential formation of its metabolites inosine and hypoxanthine. No other metabolites of adenosine, such as adenine and uric acid, were detected in Caco-2 cell culture medium. Adenosine at an initial concentration of 500 µM was completely degraded within 6 hours of incubation. After incubation of cells with adenosine at 2500 µM, a concentration of ~500 µM adenosine remained after 6 hours. In an attempt to inhibit adenosine degradation, Caco-2 cells were also incubated with adenosine in the presence of EHNA (100 µM). As shown in Fig. 2C and 2D, this ADA inhibitor minimized adenosine breakdown.

In all incubations, hypoxanthine was the end product in culture medium after 24-hour incubation of Caco-2 cells with IFNγ and IL-1β in the presence of either ATP or adenosine with or without ecto-enzyme inhibitors (Fig. 1 and 2). Cells not exposed to IFNγ and IL-1β showed similar degradation profiles of ATP and adenosine (data not shown).

Since several cell types are known to release ATP and/or adenosine in response to an inflammatory stimulus, we also stimulated Caco-2 cells by the pro-inflammatory cytokines IFNγ and IL-1β in the presence of ARL-67156 or EHNA, but without adding ATP or adenosine. Results of the HPLC analysis showed that neither ATP nor adenosine, or any of their metabolites for that matter, were detected in samples of the Caco-2 cell culture after 6-hour, 16-hour and 24-hour stimulation.

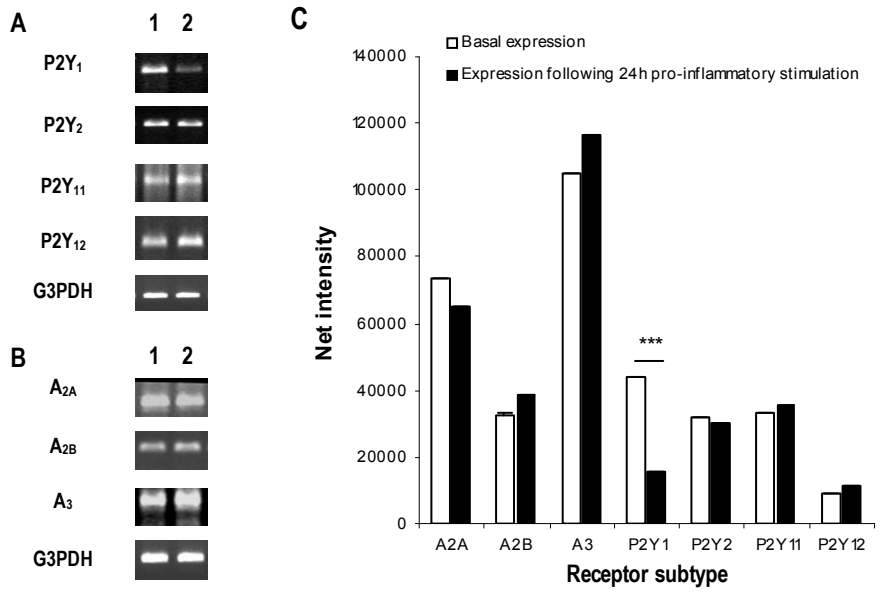


Figure 3. *Expression of P2 and P1 receptor mRNA by Caco-2 cells. RT-PCR products for $P2Y_{1,2,11,12}$* ***(A)*** *and $A_{2A,2B,3}$* ***(B)*** *receptor subtypes were obtained. Basal mRNA expression by Caco-2 cells* ***(lane 1)*** *and mRNA expression by cells stimulated by IFNγ (100 U/mL) and IL-1β (50 U/mL) for 24 hours* ***(lane 2)*** *are shown. As a quantification of basal receptor expression* ***(C, open bars)*** *and expression following 24-hour stimulation of cells by IFNγ and IL-1β* ***(C, closed bars)****, band density was calculated and expressed as mean ± SD net intensity (***: P<0.001). All reactions were done in duplicate with G3PDH as positive control.*

3.1.2 P2 and P1 receptor mRNA expression by Caco-2 cells

RNA from Caco-2 cells was isolated and analyzed by RT-PCR to determine P2 and P1 receptor expression. As shown in Fig. 3, mRNA transcripts for several P2 and P1 receptor subtypes were present in the Caco-2 cells: RT-PCR products were obtained for the $P2Y_1$, $P2Y_2$, $P2Y_{11}$ and $P2Y_{12}$ receptor subtypes (Fig. 3A), and for the A_{2A}, A_{2B} and A_3 receptor subtypes (Fig. 3B). No mRNA transcript for the $P2X_7$ receptor subtype was detected (data not shown). We observed the following order of band intensity in Caco-2 cells that had not been exposed to pro-inflammatory stimulation (Fig. 3C, open bars): $A_3 > A_{2A} > P2Y_1 > P2Y_{11} > A_{2B} > P2Y_2 > P2Y_{12}$.

Pro-inflammatory stimulation of Caco-2 cells by IFNγ and IL-1β for 24 hours had no significant effects on mRNA expression of P2 and P1 receptors, except for the $P2Y_1$ subtype (Fig. 3C, closed bars). Following 24-hour incubation of cells with IFNγ/IL-1β, band intensity of $P2Y_1$ mRNA decreased by ~64% ($P<0.001$). The following order of band intensity was observed after 24-hour exposure of cells to pro-inflammatory stimulation (Fig. 3C, closed bars): $A_3 > A_{2A} > A_{2B} > P2Y_{11} > P2Y_2 > P2Y_1 > P2Y_{12}$.

3.2 Experiment 2

In the second experiment, effects of ATP and adenosine on ICAM-1 expression and cytokine production by Caco-2 cells were assessed, with simultaneous measurements of ATP and adenosine levels in the cell culture medium. No ATP was detected in culture medium following 16-hour incubation of cells with ATP at initial concentrations of 500 μM and 2500 μM. Like in the first experiment, breakdown of ATP was not inhibited by ARL-67156 (100 μM), even though ARL-67156 was added 30 minutes before ATP. In contrast, breakdown of adenosine was again minimized by EHNA. In the presence of EHNA (25 μM), adenosine was detected in culture medium at concentrations of ~8, ~60, ~500 and ~2200 μM following 16-hour incubation of Caco-2 cells with adenosine at initial concentrations of 20, 100, 500 and 2500 μM, respectively. No adenosine was detected in 16-hour culture medium when cells were incubated with adenosine in the absence of EHNA.

3.2.1 ICAM-1 expression

Neither ARL-67156 nor EHNA as single agents affected ICAM-1 expression by Caco-2 cells. Basal expression of ICAM-1 was not affected by ATP (Fig. 4A, open bars). In contrast, adenosine at a concentration of 500 μM slightly reduced the basal expression of ICAM-1 by 8 ± 0.4% (mean ± SEM, $P<0.01$), whereas basal ICAM-1 expression increased by 22 ± 1% ($P<0.001$) at an adenosine concentration of 2500 μM (Fig. 4B, open bars). Relative to basal expression, the expression of ICAM-1 by Caco-2 cells increased ~2.5-fold after 16-hour stimulation of cells by IFNγ and IL-1β ($P<0.001$; Fig. 4A and 4B, open vs. closed bars). The IFNγ/IL-1β-induced expression of ICAM-1 was not significantly affected by either ATP or adenosine at any of the concentrations tested (Fig. 4A and B, closed bars).

We also evaluated effects of the stable ATP analogue and P2 receptor agonist ATPγS, but observed no effects on either basal expression or IFNγ/IL-1β-induced expression of ICAM-1 by Caco-2 cells (data not shown).

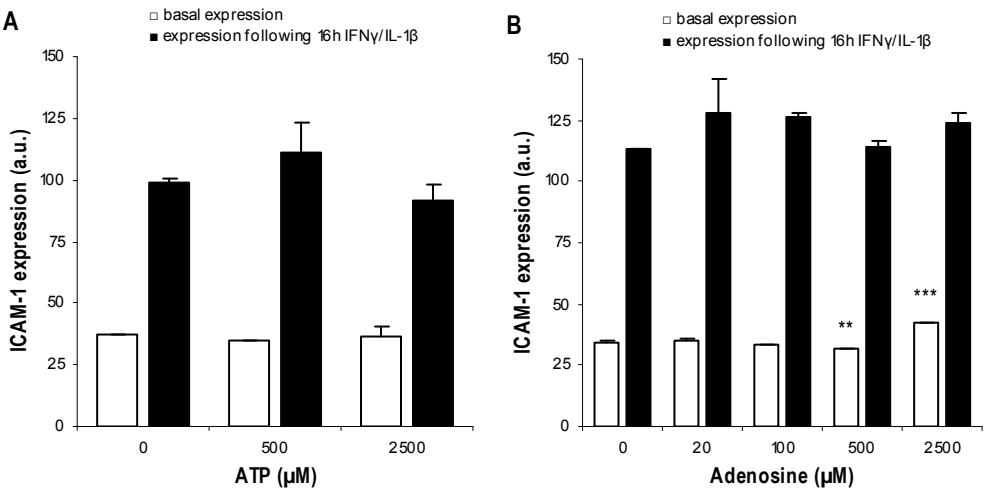


Figure 4. *Effects of ATP and adenosine on ICAM-1 expression by Caco-2 cells. Effects on basal ICAM-1 expression (open bars) were assessed by incubating cells for 16 hours with ATP **(A)** or adenosine **(B)** at different concentrations. Effects on ICAM-1 expression following pro-inflammatory stimulation (closed bars) were assessed by incubating Caco-2 cells for 16 hours with ATP **(A)** or adenosine **(B)** in the presence of IFNγ (100 U/mL) and IL-1β (50 U/mL). ARL-67156 (100 μM) was added 30 minutes before incubation of cells with ATP and adenosine was co-incubated with EHNA (25 μM). Values are means ± SEM (arbitrary units) of duplicate measurements (**: P<0.01, ***: P<0.001 vs. no adenosine).*

3.2.2 Cytokine production

IL-10 was not detected in culture medium using a High Sensitivity ELISA kit. Relative to basal levels in culture medium without added stimuli, 16-hour stimulation of Caco-2 cells by IFNγ and IL-1β increased levels of TNFα ~4-fold (1.3 ± 0.1 vs. 0.32 ± 0.01 pg/mL, P<0.05), levels of IL-6 ~20-fold (49 ± 2 pg/mL vs. 2.4 ± 0.1 pg/mL, P<0.001), and levels of IL-8 ~90-fold (3983 ± 249 pg/mL vs. 45 ± 1 pg/mL, P<0.001).

Effects of ATP and adenosine on cytokine levels in 16-hour culture medium samples were assessed under both basal and IFNγ/IL-1β-stimulated conditions; results are shown in Table 2. In addition, to gain insight into effects of ATP and adenosine on IL-8 production over time, levels of IL-8 were determined in culture medium samples from both Caco-2 cell experiments. Time-dependent effects of ATP and adenosine on IL-8 production are shown in Fig. 5, 6 and 7.

Effects of ATP (Table 2): Basal levels of IL-8 increased following incubation of cells with 500 μM and 2500 μM ATP in the presence of ARL-67156. The production of IL-8 induced by incubation of Caco-2 cells with IFNγ and IL-1β was significantly enhanced by ATP only at a concentration of 2500 μM in the presence of ARL-67156. Also, the IFNγ/IL-1β-induced production of TNFα was enhanced at 500 μM and 2500 μM ATP in the presence of ARL-67156. In contrast, the production of IL-6 induced by incubation of cells with IFNγ and IL-1β was inhibited by ATP at 500 μM and 2500 μM in the presence of ARL-67156. The IFNγ/IL-1β-induced production of IL-6 was slightly inhibited by ARL-67156 as a single agent.

In the absence of ARL-67156, 16-hour incubation of cells with ATP at a concentration of 2500 µM had similar effects on cytokine levels, that is, increased levels of IL-8 and decreased levels of IL-6; levels of TNFα were not determined.

As an indication of P2 receptor involvement in the effects mediated by ATP, we also evaluated the effects of the P2 receptor agonist ATPγS on cytokine production. Basal levels of IL-8 increased following 16-hour incubation of cells with 300 µM ATPγS. No significant effect of ATPγS on levels of IL-8 and TNFα was observed when cells were stimulated by IFNγ and IL-1β in the presence of ATPγS at a concentration of 500 µM. In contrast, the IFNγ/IL-1β-induced production of IL-6 was inhibited by 500 µM ATPγS.

Table 2. *Effects of ATP and adenosine on basal levels of IL-8, and on levels of IL-8, IL-6 and TNFα following 16-hour stimulation of Caco-2 cells by IFNγ and IL-1β.*

	IL-8 mean ± SEM (pg/mL)		**IL-6** mean ± SEM (pg/mL)	**TNFα** mean ± SEM (pg/mL)
Conditions	**basal levels**[a]	**levels following 16-hr stimulation by IFNγ and IL-1β**		
Control[b]	45 ± 1	3983 ± 249	49 ± 2.4	1.3 ± 0.1
No ATP (100 µM ARL-67156)	40 ± 3	4301 ± 308	40 ± 1.4 †	1.2 ± 0.1
ATP 500 µM + ARL-67156	70 ± 1 ***	4858 ± 272	23 ± 1.7 ***	1.9 ± 0.1 *
ATP 2500 µM + ARL-67156	74 ± 9 **	5550 ± 280 *	25 ± 1.8 ***	2.2 ± 0.1 **
ATP 2500 µM (no ARL-67156)	85 ± 3 †††	6259 ± 962 †	13 ± 0.6 †††	n.d.
ATPγS[c]	110 ± 9 †††	4044 ± 233	24 ± 0.4 ††	1.3 ± 0.1
No adenosine (25 µM EHNA)	49 ± 2	10882 ± 516 †††	26 ± 0.5 †††	3.2 ± 0.3 †
Adenosine 20 µM + EHNA	58 ± 4	6377 ± 319 ***	25 ± 0.3	1.9 ± 0.3
Adenosine 100 µM + EHNA	62 ± 2 **	6301 ± 130 ***	29 ± 0.7	2.2 ± 0.3
Adenosine 500 µM + EHNA	69 ± 2 ***	7722 ± 300 ***	36 ± 1.0 ***	4.8 ± 0.1
Adenosine 2500 µM + EHNA	91 ± 8 **	9015 ± 695	32 ± 1.3 **	5.7 ± 1.2
Adenosine 2500 µM (no EHNA)	58 ± 4 ††	5140 ± 312 †	16 ± 0.1 †††	n.d.

*Basal levels (no stimulation) and levels following 16-hour stimulation by 100 U/mL IFNγ and 50 U/mL IL-1β are presented with data expressed as mean ± SEM (pg/mL). All incubations were performed in duplicate and cytokine levels were determined by ELISA; assays were done in triplicate for IL-8 and in duplicate for IL-6 and TNFα. (†: P<0.05, ††: P<0.01, †††: P<0.001 vs. control; *: P<0.05, **: P<0.01, ***: P<0.001 vs. no ATP/adenosine; n.d.: not determined).*

[a]*Basal levels of IL-6 and TNFα were too low to detect any evident effects of ATP/adenosine and are therefore not shown.*

[b]*Control represents cytokine production by Caco-2 cells in the absence of either ATP/adenosine or the ecto-enzyme inhibitors ARL-67156/EHNA.*

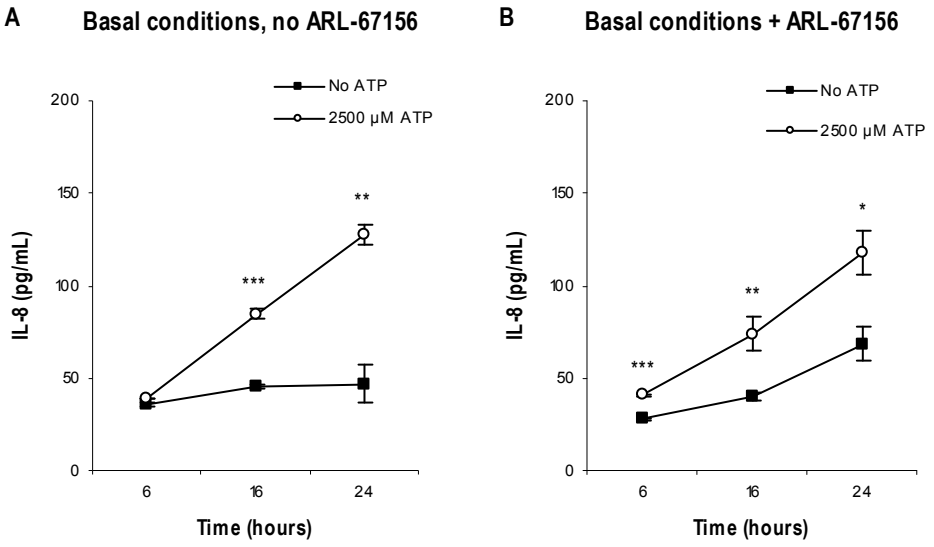
[c]*ATPγS was incubated at 300 µM in basal conditions and at 500 µM in IFNγ/IL-1β-stimulated conditions.*

Effects of adenosine (Table 2): Basal levels of IL-8 increased significantly following incubation of cells with adenosine at 100 µM, 500 µM and 2500 µM in the presence of EHNA. Under IFNγ/IL-1β-stimulated conditions, incubation of cells with EHNA in the absence of adenosine significantly affected the IFNγ/IL-

1β-induced production of cytokines by Caco-2 cells. The IFNγ/IL-1β-induced production of IL-8 and TNFα were enhanced by incubation of cells with EHNA as a single agent, whereas EHNA inhibited the IFNγ/IL-1β-induced production of IL-6. The increased production of IL-8 under IFNγ/IL-1β-stimulated conditions in the presence of EHNA alone was attenuated if adenosine was also added at a concentration of 20 µM, 100 µM and 500 µM. Similarly, the decreased production of IL-6 in the presence of EHNA alone was partly reversed by adding adenosine at a concentration of 500 µM and 2500 µM. The EHNA-induced stimulation of TNFα production in response to IFNγ and IL-1β was not significantly affected by adding adenosine at any of the concentrations tested.

In the absence of EHNA, basal levels of IL-8 without added stimuli significantly increased following incubation of Caco-2 cells with 2500 µM adenosine. Also, the IFNγ/IL-1β-induced production of IL-8 was enhanced by incubation of cells with 2500 µM adenosine in the absence of EHNA. In contrast, adenosine at 2500 µM without EHNA significantly inhibited the IFNγ/IL-1β-induced production of IL-6. Levels of TNFα in the presence of adenosine without EHNA were not determined.

Time-dependent effects on IL-8 production (Fig. 5, 6 and 7): Effects of ATP and adenosine on the production of IL-8 over time were assessed by measuring IL-8 levels in culture medium samples collected at different time points (15, 30, 60, 120, 360, 960 and 1440 min) in the two experiments with the Caco-2 cell culture. The data shown in Fig. 5 demonstrate that the ATP effects on IL-8 production by Caco-2 cells were consistent over time under both basal and IFNγ/IL-1β-stimulated conditions. As shown in Fig. 5A and 5B, basal levels of IL-8 increased at 6, 16 and 24 hours following incubation of cells with 2500 µM ATP either in the absence or presence of ARL-67156. At these time points, ATP at 2500 µM also enhanced the IFNγ/IL-1β-induced production of IL-8, regardless of the presence of ARL-67156 (Fig. 5C and 5D).



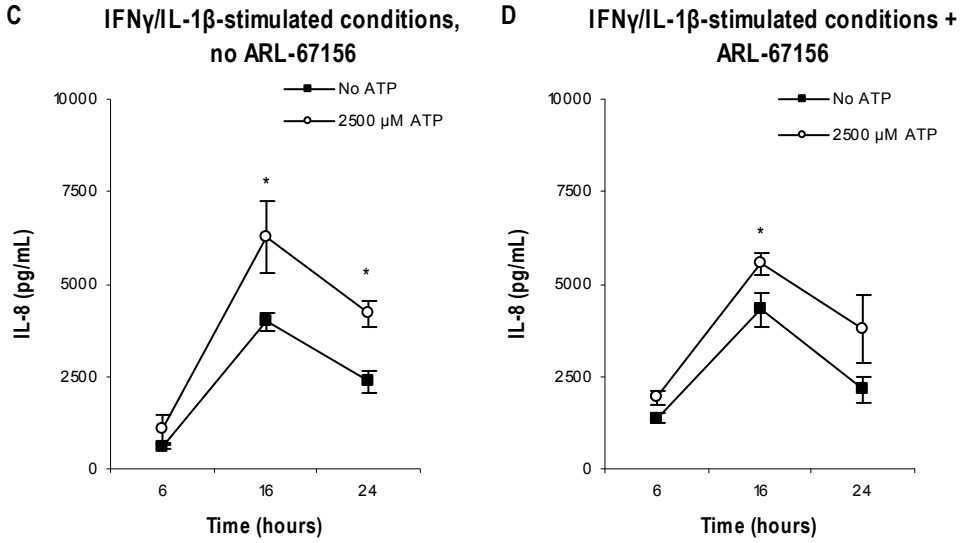
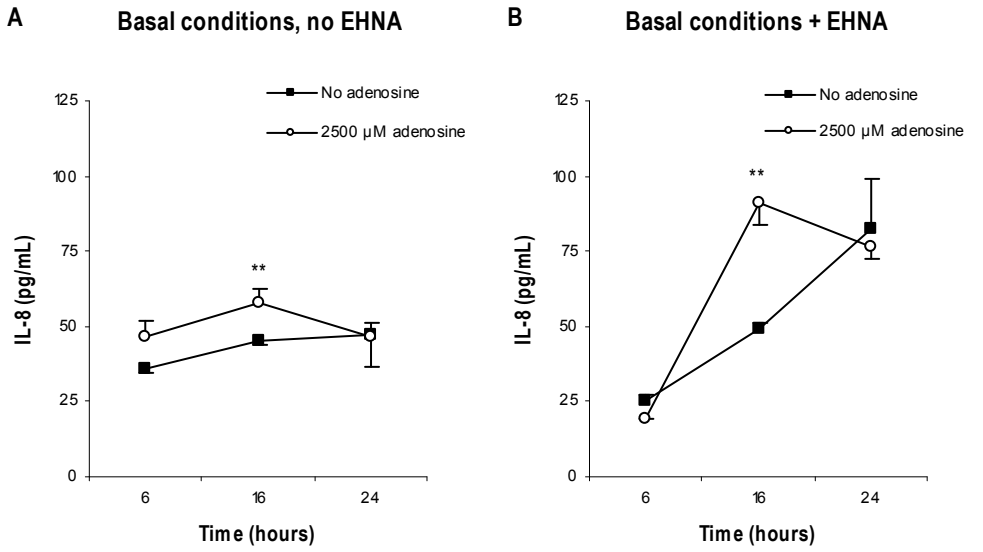


Figure 5. *Time-dependent effects of ATP on IL-8 production by Caco-2 cells. Effects on basal levels of IL-8 were assessed by incubation of Caco-2 cells with 2500 µM ATP without* ***(A)*** *or with ARL-67156* ***(B)****. Effects on IL-8 production during an inflammatory reaction were assessed by stimulation of cells by IFNγ (100 U/mL) and IL-1β (50 U/mL) in the presence of 2500 µM ATP without* ***(C)*** *or with ARL-67156* ***(D).*** *Cell medium was collected after 6, 16 and 24 hours of incubation. Values are means ± SEM of duplicate measurements (*: P<0.05, **: P<0.01, ***: P<0.001 vs. no ATP).*

The data shown in Fig. 6 demonstrate that the effects of adenosine on IL-8 production by Caco-2 cells were consistent over time only when the cells were incubated with adenosine in the absence of EHNA.



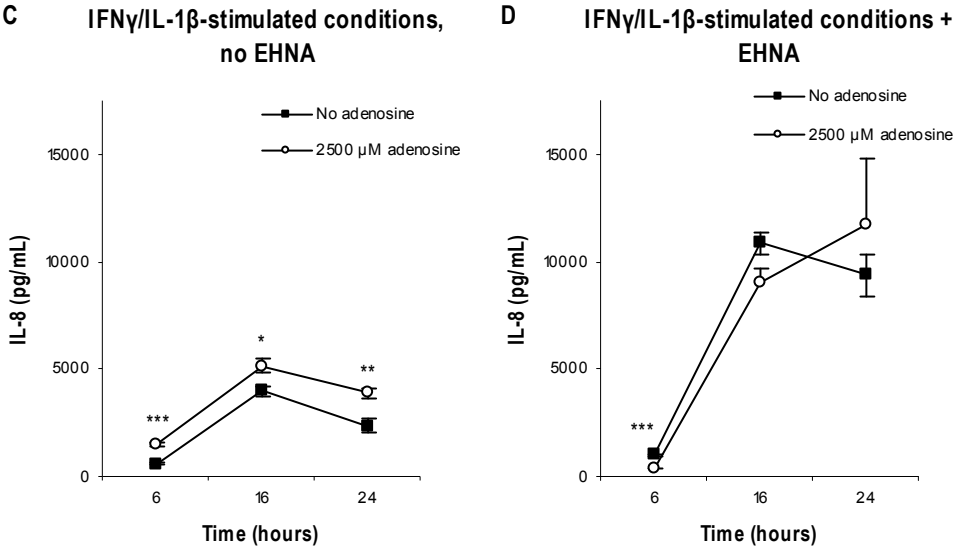


Figure 6. *Time-dependent effects of adenosine on IL-8 production by Caco-2 cells. Effects on basal levels of IL-8 were assessed by incubation of Caco-2 cells with 2500 µM adenosine without* ***(A)*** *or with EHNA* ***(B)****. Effects on IL-8 production during an inflammatory reaction were assessed by stimulation of cells by IFNγ (100 U/mL) and IL-1β (50 U/mL) in the presence of 2500 µM adenosine without* ***(C)*** *or with EHNA* ***(D).*** *Cell medium was collected after 6, 16 and 24 hours of incubation. Values are means ± SEM of duplicate measurements (*: P<0.05, **: P<0.01, ***: P<0.001 vs. no adenosine).*

As shown in Fig. 6, incubation of Caco-2 cells with 2500 µM adenosine in the absence of EHNA led to an increase in IL-8 levels at 6, 16 and 24 hours under both basal (Fig. 6A) and IFNγ/IL-1β-stimulated conditions (Fig. 6C). It is also shown in Fig. 6 that the interfering effect of EHNA on IL-8 production, which was already shown in Table 2, was consistently observed at different time points. The presence of EHNA in the Caco-2 cell culture interfered with the effects of adenosine on IL-8 production at 6, 16 and 24 hours under both basal (Fig. 6B) and IFNγ/IL-1β-stimulated conditions (Fig. 6D). Although less clear-cut, similar results on IL-8 production over time were observed at a concentration of 500 µM ATP and adenosine (data not shown).

In Fig. 7, the influence of the ecto-enzyme inhibitors ARL-67156 and EHNA on the effects of ATP and adenosine, respectively, is further illustrated. Whereas ARL-67156 did not influence the effects of ATP on IL-8 production by Caco-2 cells at any time point (Fig. 7A and 7B), it is again clearly shown that the presence of EHNA under both basal and IFNγ/IL-1β-stimulated conditions significantly distorted the effects of adenosine on the production of IL-8 over time in a non-directive fashion (Fig. 7C and 7D).

4. Discussion

Over the past decades, ATP and adenosine have been established as important signaling molecules in the extracellular compartment. Through activation of P2 and P1 receptors, respectively, ATP and

adenosine are believed to contribute to the regulation of immunity by modulating a diversity of leukocyte functions [865]. In addition to the obvious role of leukocytes in immunity, the enterocytes contribute to the initiation of mucosal immune responses in the intestine [863]. The present study was aimed at exploring effects of ATP and adenosine on immunological functions mediated by human enterocytes.

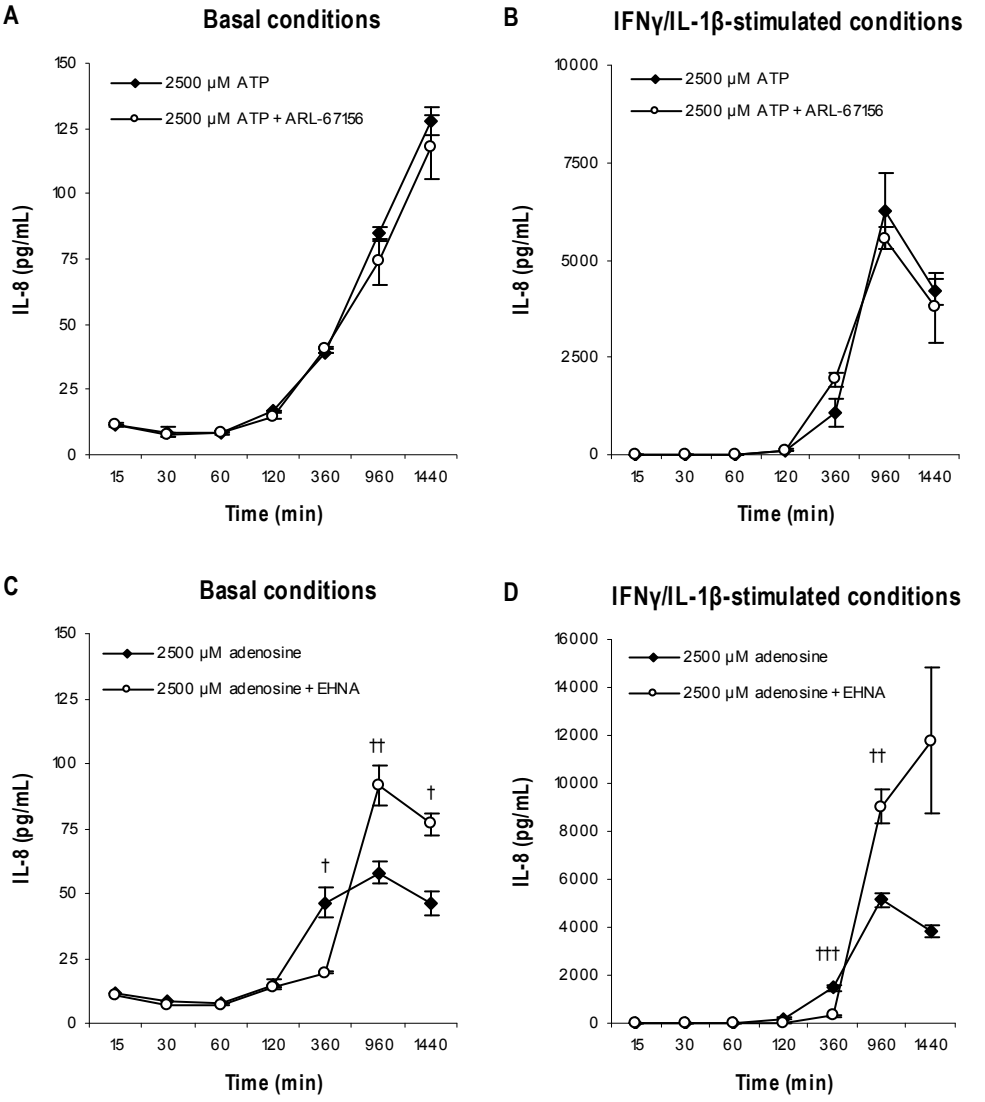


Figure 7. *Influence of ecto-enzyme inhibitors on the effects of ATP and adenosine on IL-8 production by Caco-2 cells under basal and IFNγ/IL-1β-stimulated conditions. Samples from culture medium were collected at different time points following incubation of cells with ATP* ***(A, B)*** *or adenosine* ***(C, D)*** *at 2500 µM in the presence or absence of ARL-67156 or EHNA, respectively. Values are expressed as means ± SEM of duplicate measurements (†: P<0.05, ††: P<0.01, †††: P<0.001).*

Two experiments with human enterocyte-like Caco-2 cells were performed to analyse ATP and adenosine metabolism and assess the presence of P2 and P1 receptor mRNA (experiment 1), and to evaluate effects of ATP and adenosine on ICAM-1 expression and cytokine production (experiment 2). We hypothesized that ATP at high extracellular concentrations would enhance the generation of inflammatory mediators by the Caco-2 cells, whereas adenosine would have mostly inhibitory effects. This hypothesis was only partly confirmed by our findings, as will be discussed below.

Extracellular purines are metabolized by a cascade of ecto-enzymes that control extracellular concentrations of ATP and adenosine [116]. Our results show that ATP and adenosine were metabolized in the Caco-2 cell culture, which indicates that these cells exhibit significant ecto-enzyme activity. Although ecto-enzyme expression by Caco-2 cells is relatively unexplored, earlier studies have reported the presence of several ecto-enzymes on Caco-2 cells. Ecto-5'-nucleotidase and alkaline phosphatase, which contribute to the conversion of extracellular ATP to adenosine, have been detected on the apical cell surface of Caco-2 cells [364, 877-879]. In addition, ADA, which catalyzes the breakdown of adenosine to inosine, was shown to be highly expressed by Caco-2 cells [748]. In an attempt to minimize breakdown of ATP and adenosine in our Caco-2 cell culture, we assessed effects of the ecto-enzyme inhibitors ARL-67156 and EHNA on ATP and adenosine degradation, respectively. Whereas EHNA almost completely blocked adenosine breakdown as expected, it is noteworthy that ARL-67156 did not inhibit ATP breakdown to even the slightest extent. Since this ecto-ATPase inhibitor has been reported to inhibit enzymatic activity of several members of the E-NTPDase family (ecto-nucleoside triphosphate diphosphohydrolases) [880-882], the observed lack of inhibition of ARL-67156 in our experiments would suggest that ecto-enzymes other than E-NTPDases are to be responsible for the metabolism of ATP in our Caco-2 cell culture; possible candidates would be alkaline phosphatases, whose presence on Caco-2 cells has previously been reported [878, 879].

The present study also demonstrates the expression of $P2Y_1$, $P2Y_2$, $P2Y_{11}$ and $P2Y_{12}$ receptor mRNA and of A_{2A}, A_{2B} and A_3 receptor mRNA, thus showing co-existence of P2 and P1 receptors in the Caco-2 cell culture, which suggests that these cells may be subject to purinergic receptor-mediated signaling. Although previous studies have reported the presence of binding sites for A_1 and A_2 receptors on Caco-2 cells based on pharmacological binding experiments with adenosine analogues [748, 883, 884], to our knowledge, this is the first time that the presence of mRNA for A_{2A}, A_{2B} and A_3 receptors in Caco-2 cells is demonstrated. Our findings with regard to the presence of P2Y receptor mRNA correspond to recent findings by Coutinho-Silva et al. (2005) who, in addition to detecting mRNA for $P2Y_{1,2,11,12}$ receptors, also demonstrated the expression of protein for $P2Y_1$ and $P2Y_2$ receptors on the surface of Caco-2 cells [826]. Furthermore, these authors detected high-level expression of $P2X_7$ receptor protein on Caco-2 cells in spite of a faint mRNA expression for this receptor subtype [826]; therefore, the lack of detection of mRNA for the $P2X_7$ receptor subtype in the present study does not completely rule out the presence of active $P2X_7$ receptors in our Caco-2 cell culture.

Since expression of purinergic receptors has been shown to be modulated under inflammatory conditions [163, 170, 790], we also assessed the influence of inflammatory stimuli on receptor expression. Following stimulation of cells by the pro-inflammatory cytokines IFNγ and IL-β, we observed

only a marked decrease in $P2Y_1$ mRNA. Since it has previously been observed that expression of $P2Y_6$ and $P2X_3$ receptors was up-regulated in colonic tissue extracts from patients with active IBD [657, 866], it might be suggested that P2 receptor expression by enterocytes in an inflamed intestine may be subject to transcriptional regulation in a subtype-specific fashion.

Knowing that mRNA for P2 and P1 receptors was present in the Caco-2 cell culture, it was expected that ATP and adenosine could influence immunological functions of these enterocyte-like cells. Therefore, we examined effects of ATP and adenosine on ICAM-1 expression and cytokine production by the Caco-2 cells. Whereas ATP had no effects on ICAM-1 expression, adenosine at relatively high extracellular concentrations (500 and 2500 μM) had small effects only on the basal expression of ICAM-1. This finding contrasts with previous data showing that adenosine did not alter basal nor TNFα-stimulated ICAM-1 expression by human umbilical vein endothelial cells [292]. Another study by Seifert et al. (2006) recently showed that ICAM-1 expression on human dermal endothelial cells was increased by ATPγS [885]. In our experiments, only a small but insignificant (P=0.067) increase of ~23% in IFNγ/IL-1β-induced expression of ICAM-1 was observed in response to ATPγS. Taken together, ICAM-1 expression by Caco-2 cells appears not to be substantially affected by ATP and adenosine.

Although generally not affecting ICAM-1 expression, our data show that ATP and adenosine affected cytokine production by Caco-2 cells. Basal levels of IL-8 increased by incubation of cells with ATP at high extracellular concentrations (500 and 2500 μM). At these concentrations, ATP also enhanced the IFNγ/IL-1β-induced production of IL-8 as well as TNFα, but inhibited the IFNγ/IL-1β-induced production of IL-6. The effect of ATP on IL-8 production under both basal and IFNγ/IL-1β-stimulated conditions was consistent over time. These results show that ATP at high extracellular concentrations appears to predominantly mediate immunostimulatory effects on cytokine production, confirming our hypothesis regarding ATP. However, because of the lack of inhibition of ATP breakdown by ARL-67156, it remains to be proven that the observed ATP effects are actually being mediated via extracellular ATP and its receptors.

The observed stimulatory effect of ATP on TNFα production by Caco-2 cells contrasts with recent findings by our group in human LPS/PHA-stimulated whole blood, which showed that TNFα production was inhibited by micromolar concentrations (100-300 μM) of ATP via $P2Y_{11}$ activation [67, 69]. In the present study, we also assessed effects of ATPγS, which is generally considered to be a $P2Y_{11}$ ligand [864]. ATPγS did not alter TNFα production in our experiments, which might suggest that the observed stimulatory effect of ATP on TNFα may occur via breakdown to ADP or another metabolite of ATP. In contrast to its lack of effect on TNFα, ATPγS affected the production of IL-6 and IL-8 in a way similar to ATP. Although it remains uncertain which receptor(s) is (are) relevant to the effects of ATP on cytokine production, the observed effects of ATPγS do suggest involvement of P2 receptors in IL-8 and IL-6 production by Caco-2 cells. This issue warrants further investigation using specific P2 receptor agonists and antagonists.

In contrast to ATP which affected cytokine production regardless of the presence of ARL-67156, we found that the ADA inhibitor EHNA as a single agent affected cytokine production by IFNγ/IL-1β-

stimulated Caco-2 cells and thereby substantially interfered with effects of adenosine. In view of these unexpected effects of EHNA, the data on cytokine production following incubation of cells with adenosine in the presence of EHNA under IFNγIL-1β-stimulated conditions should be interpreted with some caution. In the absence of EHNA, both the basal and the IFNγIL-1β-induced production of IL-8 were stimulated by adenosine at a concentration of 2500 µM. This effect of adenosine on IL-8 production under both basal and IFNγ/IL-1β-stimulated conditions was consistent over time. In contrast, adenosine at 2500 µM without EHNA inhibited the IFNγIL-1β-induced production of IL-6. These findings do suggest that cytokine production by Caco-2 cells is affected by adenosine.

Direct effects of EHNA on cytokine production have been reported previously and were attributed to endogenous adenosine being released upon cellular stimulation [292, 886]. In our experiments, we did not measure immediate adenosine release following stimulation of Caco-2 cells by IFNγ and IL-1β. However, we did not detect elevated levels of adenosine (or any of its metabolites) in culture medium by HPLC following 6-hour, 16-hour and 24-hour incubation of Caco-2 cells with IFNγ and IL-1β in the presence of EHNA. Nevertheless, since it is believed that extracellular concentrations of adenosine can rise to concentrations up to 100 µM in inflamed tissues [93], the possibility cannot be excluded that this lack of detection in our study is due to a threshold issue of the HPLC assay in cell culture medium. We can therefore not rule out the local release of ATP and/or adenosine following stimulation of Caco-2 cells. It thus remains unclear whether the endogenous release of adenosine (or ATP) was involved in the observed effects of EHNA in the present study.

In conclusion, our results give support to the notion that extracellular ATP and adenosine play a role in inflammatory responses mediated by human enterocytes. Both ATP and adenosine affect the generation of inflammatory mediators by enterocyte-like Caco-2 cells, especially cytokines, even though further investigation is warranted on the precise role of purinergic signaling in enterocyte-driven mucosal inflammation.

CHAPTER 6

General Discussion

The main objective of the research presented in this thesis was to explore the role of ATP and adenosine in immunity and inflammation, and to evaluate their effects in mucosal defence in the small intestine, focusing on two epithelial defence mechanisms: mucosal barrier function and enterocyte-driven inflammatory response. In this chapter, main findings and implications will be discussed in light of the explored concepts. Finally, overall conclusions and directions for future research will be suggested.

1. Immunity and inflammation

In **chapter 2** of this thesis, we have elaborated on the role of ATP and its metabolite adenosine in immunity and inflammation. Overwhelming evidence is provided indicating that both extracellular ATP and adenosine are involved in the regulation of immunity and inflammation by signaling through purinergic receptors.

To our knowledge, the compilation of evidence presented in chapter 2 is the first to focus in such detail on the intertwined actions of ATP and adenosine in immunoregulation. The complex regulatory actions of these two molecules comprise several aspects, which together contribute to shaping the outcome of immune responses and inflammation. The essentials of purinergic immunoregulation can be summarized as follows:

i. Acute high-level P2 receptor signaling by ATP is pro-inflammatory and immunostimulatory (high extracellular concentrations of ATP resulting from its acute release in situations of cellular stress are considered to be a danger signal);
ii. ATP mediates anti-inflammatory and immunomodulatory effects through low-level P2 receptor signaling;
iii. P1 receptor signaling by adenosine is mostly anti-inflammatory and immunosuppressive;
iv. Immunoregulatory actions of ATP and adenosine may shift during the course of inflammation due to changed cellular expression of both purinergic receptors and ecto-enzymes in response to accumulating inflammatory mediators (*e.g.* cytokines).

It is thus illustrated that in an inflammatory microenvironment ATP and adenosine contribute in an interdependent and multifaceted fashion to the fine-tuning of immune-mediated processes at multiple levels, possibly being involved in redirecting detrimental inflammatory and immune responses when normal tissue intergrity comes into play.

The majority of reviews that have been published to date on purine-mediated immune regulation mainly deal with specific cellular effects of either ATP or adenosine, generally addressing just one of the aspects of purinergic immunoregulation [[87, 88, 93, 94, 98, 100, 175, 887-893]. The empirical data compiled in chapter 2 of this thesis contribute to unraveling the process of purinergic immunoregulation as a whole and may thereby open potential new avenues for future research and development of purine-based treatment modalities. It is increasingly understood that ATP and adenosine are endogenous immune mediators, which in combination with the arsenal of purinergic receptors and ecto-enzymes seem to be inextricable allies during their contribution to the regulation of immunity and inflammation.

Hence, future research on purinergic immunoregulation should ideally encompass this broad purinergic arsenal, considering ATP and adenosine, P2 and P1 receptors, as well as ecto-enzymes.

Since specific immunomodulatory effects of ATP and adenosine appear to be mediated by different purinergic receptor subtypes in a concentration-dependent fashion, it would be of major relevance to assess expression patterns of purinergic receptors and ecto-enzymes *in vivo*. For instance in chronic inflammatory diseases that are characterized by dysregulated inflammatory processes and abnormal cytokine profiles (*e.g.* inflammatory bowel disease (IBD) and rheumatoid arthritis), changed expression patterns of purinergic receptors and/or ecto-enzymes may be expected. Disease-related changes in the purinergic arsenal would lead to selective up-/down-regulation of downstream purinergic signaling events and may thereby contribute to the uncontrolled inflammatory processes seen in these diseases. Insight in such changes could thus provide important opportunities for targeted immunomodulatory treatment. A relevant example of targeted purine-based treatment is illustrated by the management of patients with adenosine deaminase-deficient severe combined immunodeficiency (ADA-SCID). ADA-SCID is a rare disorder of purine metabolism with devastating consequences in the immune system, partly resulting from elevated extracellular adenosine levels due to lacking ADA expression [135, 137]. One of the options for management of ADA-SCID is enzyme replacement therapy by administration of polyethylene glycol (PEG)-ADA. PEG-ADA maintains high ectopic ADA activity in plasma, which eliminates harmful over-accumulation of the ADA substrates adenosine and deoxyadenosine, thereby minimizing infectious complications. Recent data on long-term treatment efficacy suggest that although some immune defects may persist in spite of PEG-ADA treatment, it can still be considered as a valuable life-saving treatment modality providing adequate immune protection in the management of ADA-SCID [894, 895].

2. The mucosal barrier

In order to explore the role of ATP and adenosine in mucosal barrier function, we performed two human experiments in which effects of ATP and adenosine on altered epithelial permeability of the small intestine were evaluated. In the first experiment (**chapter 3**), we showed that ATP may have a role in the maintenance of epithelial integrity of the small intestinal mucosa when applied topically by naso-intestinal tube. In contrast, when either ATP or adenosine were administered into the small intestine via enteric-coated capsules in the second experiment (**chapter 4**), we observed no effects on compromised epithelial integrity. Before further discussing the results of these experiments, I will first discus the concept of mucosal barrier function and its relevance in health and disease.

2.1 Relevance of an intact mucosal barrier

On the luminal side of the small intestine, the mucosal epithelial cells constitute a large interface between a person's internal and his or her external environment. This interface fulfills a dual function. On the one hand, it functions as a selective permeable filter allowing uptake of needed nutrients, fluids and electrolytes. On the other hand, it functions as a barrier that normally prevents invasion of potentially

harmful substances from the gut lumen. The permeability status of the mucosal barrier is pivotal in controlling translocation of such harmful substances and, therefore, critical in maintaining human health. Increased epithelial permeability in the small intestine facilitates penetration of luminal aggressors, which attract and activate leukocytes that evoke a local inflammatory reaction causing further damage to the epithelial barrier [70]. This can be illustrated by a vicious circle in which increased permeability of the small intestinal epithelium is a central factor (Fig. 1). An intact mucosal barrier is thus imperative in preserving normal physiology, which emphasizes its significance as an investigative tool in treatment evaluation or development.

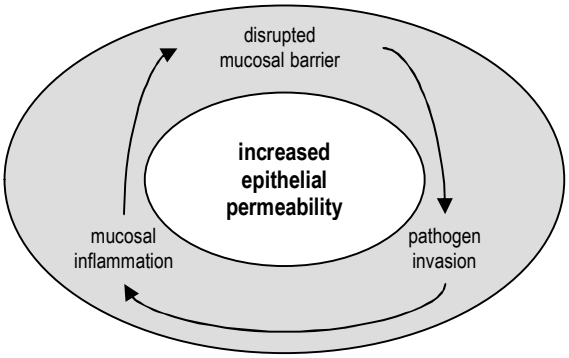


Figure 1. *Schematic model of the self-amplifying cascade of disrupted mucosal barrier function, increased pathogen invasion and mucosal inflammation.*

2.1.1 Mucosal barrier function and intestinal disease

The relevance of an intact mucosal barrier is also emphasized by the growing evidence suggesting that mucosal barrier function is compromised in intestinal diseases, including Crohn's disease [834]. The etiology of Crohn's disease is nowadays only partly understood. The current leading hypothesis is that it is a multifactorial disease with both genetic and environmental factors contributing to its pathogenesis. Altered epithelial integrity in the small intestine is thought to be one of those factors [896]. By assessing gastrointestinal barrier function, it has been demonstrated that mucosal permeability in patients with active Crohn's disease is increased relative to healthy controls, particularly in patients with small bowel involvement [839, 840, 845-847, 849, 897, 898]. Increased epithelial permeability in the small intestine was shown to correlate with disease activity and to predict clinical relapse in patients with Crohn's disease [839-841, 847, 849, 897-899]. Moreover, intriguing data suggest that a genetic mucosal barrier defect may be one of the predisposing factors for Crohn's disease. Several studies have shown that, in addition to patients with Crohn's disease, a subset of their first-degree relatives without clinical evidence of disease had raised epithelial permeability in the small intestine relative to healthy non-related controls [848, 900-902]. One case report actually describes a patient with Crohn's disease in whom an asymptomatic increase in intestinal permeability had already been observed 8 years prior to diagnosis, suggesting that a permeability defect may precede the onset of Crohn's disease [903]. A proportion of first-degree relatives of Crohn's disease patients were also shown to be more reactive to a provoked permeability increase by acetylsalicyclic acid and ibuprofen [904-906]. These findings might implicate an

inherited defect in mucosal barrier function, which may be of importance for the etiology of Crohn's disease. Genetic variation in the CARD15/NOD2 (caspase associated recruitment domain family, member 15/nucleotide binding oligomerisation domain 2) gene is presumably associated with susceptibility to Crohn's disease. Recent genetic studies have identified an association between mutations in the CARD15/NOD2 gene and increased intestinal permeability in patients with Crohn's disease as well as in their unaffected first-degree relatives [907, 908], suggesting that there may indeed be a genetic basis for impaired mucosal barrier function.

Since it is thought that patients with Crohn's disease mount abnormal mucosal immune responses to luminal antigens whose penetration into the gut wall is facilitated by compromised epithelial integrity, mucosal barrier dysfunction appears to be involved in the perpetuation of destructive inflammatory processes in the intestine. In this way, a dysfunctional mucosal barrier may prove to be a significant factor contributing to the pathogenesis of mucosal lesions in Crohn's disease. Studying such lesions could thus provide valuable insight into disease mechanisms, involving defects in immunoregulation as well as in mucosal barrier function. Assessing intestinal permeability is therefore an attractive approach for a better understanding of the role of defective mucosal barrier function in pathological conditions such as Crohn's disease.

2.1.2 Mucosal barrier function and extraintestinal disease

Further evidence indicating that the concept of intestinal permeability is an attractive concept for investigation derives from the putative pathological link between altered mucosal permeability with intestinal inflammation, as seen in Crohn's disease, and extraintestinal immune manifestations, including rheumatological conditions such as rheumatoid arthritis and spondyloarthropathy (the gut-joint axis) [909-913]. Many patients with intestinal disease develop associated arthropathy, especially in IBD. It has been reported that IBD is associated with episodes of peripheral arthritis that often coincide with disease exacerbations, parallelling the activity of intestinal inflammation. In some cases, such episodes may even precede clinical manifestations of IBD [914].

Recently, a new etiological mechanism has been proposed adding loss of mucosal barrier function as a key element in the development of autoimmunity [74, 75, 915]. This theory proposes that three main elements may be involved in the pathogenesis of autoimmune diseases: (i) a dysfunctional (mucosal) immune system allowing genetically susceptible hosts to initiate a disbalanced immune reaction to environmental antigens, (ii) a continuous exposure to environmental antigens perpetuating the abnormal inflammatory processes, and (iii) a compromised mucosal barrier facilitating interaction between the (mucosal) immune system and the environmental antigen [74, 75, 915]. The theory further proposes that mucosal barrier dysfunction may trigger inflammatory reactions not only in the intestine but also at sites distant from the intestine, therefore being an important determinant of human health (Fig. 2).

2.2 Human model of altered mucosal barrier function

In accordance with the above data which emphasize the importance of the permeability status of the small intestine in health and disease, we initiated two proof-of-principle experiments focusing on the

permeability status of the small intestine as a reflection of mucosal barrier function (chapter 3 and 4). For this purpose, we utilized a previously established human model of altered epithelial integrity, which enabled us to evaluate effects of ATP and adenosine on changes in small intestinal permeability *in vivo*. Small intestinal permeability changes were induced in healthy volunteers by short-term application of the nonsteroidal anti-inflammatory drug (NSAID) indomethacin. The model thus exploits a well-known side effect occurring with the use of NSAIDs.

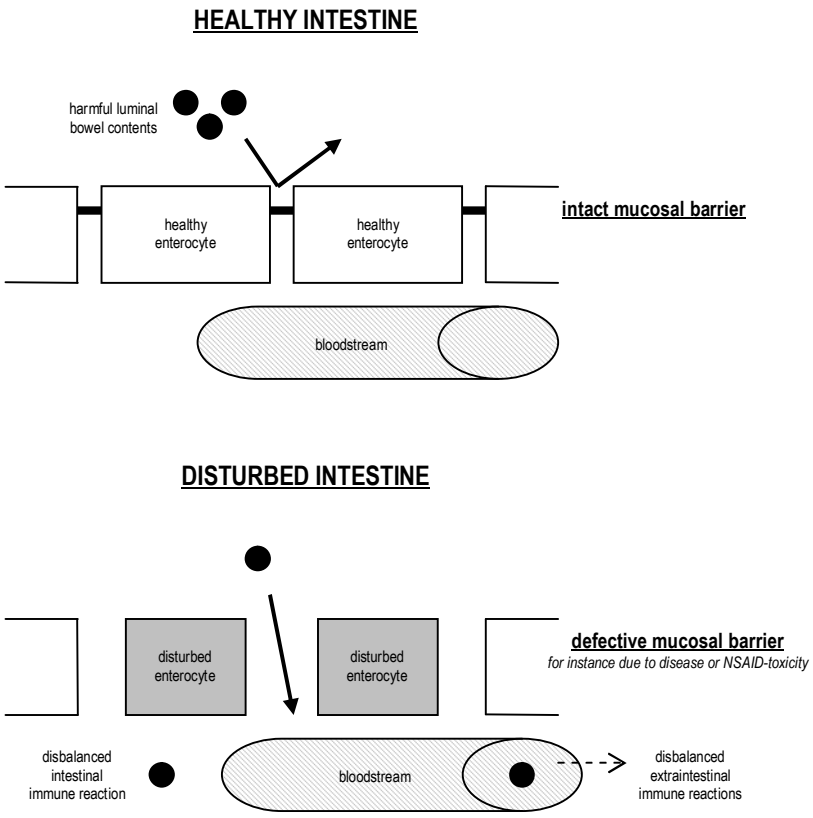


Figure 2. *Role of defective mucosal barrier function with increased epithelial permeability in the development of intestinal and extraintestinal (e.g. articular) immune reactions to environmental antigens, leading to uncontrolled inflammatory events at intestinal sites as well as sites distant from the intestine.*

2.2.1 NSAIDs and intestinal permeability

NSAIDs are effective anti-inflammatory and analgesic drugs that form the main pharmacological approach to treating various conditions associated with inflammation and pain. It is widely appreciated that frequent use of NSAIDs can cause considerable damage to the gastrointestinal tract. Gastrointestinal harm attributable to NSAIDs is well-described in the upper gastrointestinal tract, that is, the stomach and proximal duodenum (NSAID gastropathy). Gastric side effects of NSAIDs have

previously attracted considerable attention, which has led to the development of different gastroprotection strategies by selective cyclooxygenase (COX)-2 inhibition (coxibs). Detrimental NSAID effects in the lower gastrointestinal tract, especially the small bowel (NSAID enteropathy), have long been underappreciated. The pathogenesis of NSAID enteropathy is now assumed to be a multistage process resulting from a combination of COX-1/COX-2 inhibition and, more importantly, topical effects of NSAIDs on mucosal surfaces. One of the earliest events in the development of NSAID enteropathy is believed to be uncoupling of oxidative phosphorylation within enterocytes by direct topical NSAID irritation. This irritant effect induces deficiency of mitochondrial ATP synthesis, which leads to loss of control over intercellular tight junctions with an increase in paracellular permeability of the small intestinal epithelium [799, 817, 916]. Consequently, epithelial integrity is compromised, which causes a leaky mucosal barrier that amplifies exposure to aggressive luminal constituents and intensifies antigen absorption. In turn, this leads to over-accumulation and over-activation of immune cells in mucosal tissues, ultimately resulting in exaggerated immune responses with local intestinal inflammation. In this way, NSAIDs can cause IBD-like injury to the small bowel and may in fact induce exacerbations of underlying disease in NSAID-treated IBD patients. Clinical experience suggests the existence of pathological similarities between NSAID enteropathy and IBD, making these conditions sometimes difficult to distinguish clinically. Established (serious) complications of NSAID toxicity to the small intestine are ulcers, blood and protein loss, diaphragm-like strictures and perforations [72, 797].

The prevalence and severity of NSAID enteropathy has previously been underestimated, which was most likely due to two reasons. First, NSAID enteropathy is much more poorly characterized than NSAID gastropathy because it is mostly asymptomatic. Particularly early-stage changes in epithelial integrity are clinically silent and difficult to visualize. Second, diagnostic tools are not widely available, non-specific, inconvenient and/or expensive, rendering NSAID enteropathy difficult to diagnose. Nevertheless, it is now without doubt that NSAID-inflicted small bowel damage is common, with estimated prevalence rates of NSAID enteropathy maybe the same if not slightly higher than that of NSAID gastropathy [916-918]. Recent development of wireless video capsule endoscopy has substantially contributed to improved imaging of lesions in areas beyond the proximal small intestine. Using capsule endoscopy to assess macroscopic damage in the small bowel (*i.e.* erosions and ulcers), three recent studies reported high prevelance rates of 71% associated with long-term (> 3 months) [919] and of 55-68% associated with short-term (2 weeks) NSAID treatment [920, 921].

A drawback of imaging techniques, including capsule endoscopy, is the inability to visualize microscopic mucosal events in the lower regions of the gastrointestinal tract, including the early-stage epithelial permeability changes accompanying NSAID enteropathy. In these early stages of NSAID-inflicted damage to the intestinal wall, functional non-invasive assessment of the permeability status of the small intestinal epithelium is a valuable investigative measure.

2.2.2 Functional assessment of intestinal permeability

The permeability status of the small intestine can be assessed functionally by means of orally administrable permeability markers. These marker molecules generally include three groups: (i) sugars such as lactulose and rhamnose, (ii) polyethylene glycols, and (iii) radiolabeled molecules such as ^{51}Cr-

EDTA [922]. The underlying idea of using marker molecules as a functional measure of intestinal permeability refers to the passive movement of low-molecular weight molecules across the intestinal mucosa. Upon crossing the mucosal barrier, the molecules are transported to the bloodstream and excreted in the urine, where they can be quantified as an indication of non-specific intestinal wall injury. Increased excretion implies increased intestinal permeation. Although single permeability molecules such as ^{51}Cr-EDTA have previously been used frequently, assessment of the differential excretion ratio of two different-sized molecules following different routes of intestinal permeation is preferred (*e.g.* lactulose/rhamnose, lactulose/mannitol, ^{51}Cr-EDTA/rhamnose).

Because of individual variation in pre- and post-absorption processes, calculation of a ratio of the urinary recoveries of two marker molecules is considered to be a better measure of intestinal permeability than the urinary recovery of only a single molecule. Therefore, in our permeability experiments, we applied a sugar absorption test using a solution of two different-sized sugars (5 g lactulose and 0.5 g L-rhamnose) to assess changes in small intestinal permeability. A solution with lactulose and rhamnose administered in equal proportions at these dosages has previously been shown to discriminate significantly between normal and moderately increased permeability in healthy humans with sufficient sensitivity [812].

Thus, measuring intestinal permeability by determining the urinary lactulose/rhamnose (L/R) excretion ratio is thought to be a valid index for evaluation of early small intestinal permeability changes accompanying short-term NSAID administration.

2.2.3 Effect of ATP and other compounds on intestinal permeability

Previous experiments have demonstrated that intestinal permeability is increased within 8-10 hours after ingestion of two subsequent doses of the NSAID indomethacin (75 and 50 mg). The permeability increase is rapidly reverted, being no longer evident 48 hours after indomethacin ingestion [800, 815]. In our first human experiment (**chapter 3**), we used a short-term NSAID challenge to explore the effect of ATP on compromised mucosal barrier function in the small intestine. It was demonstrated that administration of ATP directly into the upper small intestine (*i.e.* duodenum, proximal jejunum) via a naso-intestinal tube attenuated an indomethacin-induced increase in small intestinal permeability in healthy human volunteers. Indomethacin increased the urinary L/R excretion ratio by 83% relative to the basal L/R ratio in the control experiment. ATP completely prevented this approximately twofold increase in urinary L/R ratio. Individual curves of median urinary L/R ratios in the control, indomethacin-plus-placebo and indomethacin-plus-ATP condition are shown in Fig. 3.

Thus, by attenuating early-stage indomethacin-induced small intestinal permeability changes, which are a prerequisite for the development of NSAID enteropathy, the finding presented in chapter 3 of this thesis would suggest that ATP may be beneficial in reducing small bowel side effects of NSAIDs by preserving mucosal barrier function. As mentioned above, preservation of mucosal barrier function may also be of relevance to the treatment of other intestinal disorders in which the mucosal barrier is compromised, such as in Crohn's disease.

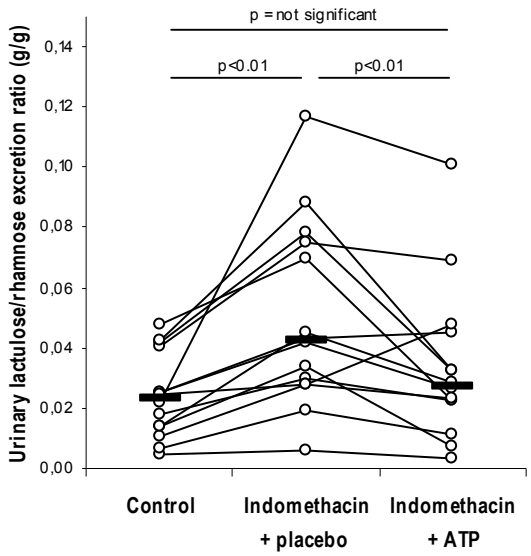


Figure 3. *Individual curves of urinary lactulose/rhamnose excretion ratios (g/g) in three experiments performed in randomized order with one week wash-out in between. Median L/R ratios (control: 0.023, indomethacin + placebo: 0.042, indomethacin + ATP: 0.027;* ***thick lines****) and between-group p-values are shown also (Wilcoxon signed ranks test, n=14).*

In addition to the beneficial effect of ATP demonstrated in our human model, the potential of other compounds in affecting indomethacin-induced permeability changes has also been evaluated in similar human experiments, of which several were carried out by Bjarnason and co-workers in the late-1980s and early-1990s. These authors first reported that administration of prostaglandin E_2, a derivative of the COX pathway, did not attenuate a rise in intestinal permeability as reflected by increased five-hour urinary excretion of ^{51}Cr-EDTA after two subsequent dosages of indomethacin (75 and 50 mg) [800]. Therefore, they next evaluated the effect of a more stable prostaglandin analogue, misoprostol. This agent was shown to attenuate the increase in urinary ^{51}Cr-EDTA excretion induced by indomethacin (75 and 50 mg), but the effect was incomplete because ^{51}Cr-EDTA excretion as well as the urinary ^{51}Cr-EDTA/L-rhamnose excretion ratio after misoprostol still remained significantly increased relative to baseline (no indomethacin) [814]. The inefficiency of targeting COX-dependent prostaglandin pathways is probably due to the fact that prostaglandins are thought not to be involved in the early-stage permeability changes induced by NSAIDs. Subsequently, Bjarnason and co-workers applied two other strategies to attenuate indomethacin-inflicted damage to the small intestine.

The first strategy comprised administration of the narrow-spectrum antibiotic metronidazole to patients who had NSAID enteropathy due to long-term use of several NSAIDs, including indomethacin (100-150 mg/day for more than six months). Metronidazole treatment reduced neutrophil-mediated intestinal inflammation, but proved to have no effect on the urinary ^{51}Cr-EDTA/L-rhamnose excretion ratio [923]. This would suggest that antibiotic treatment does not affect the altered intestinal permeability status accompanying NSAID enteropathy, but may be advantageous in attenuating the inflammatory processes that result from increased bacterial invasion of the mucosa.

The second strategy comprised normalisation of the indomethacin-induced mitochondrial ATP deficiency by co-administration of glucose and citrate, which are substrates for intracellular metabolic pathways (*i.e.*

glycolysis and Krebs' cycle). It was shown that the increased urinary ^{51}Cr-EDTA/L-rhamnose excretion ratio induced by two subsequent dosages of indomethacin (50 and 75 mg) was reduced by administering a formulation of indomethacin containing 15 mg glucose and 15 mg citrate per mg indomethacin [815]. Five-hour urinary excretion of ^{51}Cr-EDTA however still remained significantly increased relative to the baseline condition (no indomethacin) [815], suggesting incomplete treatment efficacy with a residual rise in intestinal permeability presumably still present.

The efficacy of another metabolic substrate, glutamine, was also tested in a more recent human study by den Hond et al. (1999). These authors showed that glutamine reduced the increase in six-hour urinary excretion of ^{51}Cr-EDTA induced by indomethacin (75 and 50 mg), but only when glutamine was administered at multiple doses in close timing with indomethacin-dosing [924]. Additionally, the effect of misoprostol was re-evaluated in this study. Although it was confirmed that misoprostol as a single agent did not affect elevated excretion of ^{51}Cr-EDTA after indomethacin ingestion, the inhibitory effect of glutamine was somewhat stronger when it was administered in combination with misoprostol [924]. However, again, the effect of glutamine in this study was incomplete as urinary ^{51}Cr-EDTA excretion after multiple-dose co-administration of glutamine with indomethacin still remained significantly elevated relative to baseline (no indomethacin), even when glutamine was administered in combination with misoprostol [924]. Recently, Troost et al. (2003) showed that an increase in urinary L/R ratio after ingestion of indomethacin (75 and 50 mg) was attenuated by concomitant administration of the nutritional compound lactoferrin [809], but once again the effect of lactoferrin on the indomethacin-induced rise in intestinal permeability was incomplete.

The above results show that several strategies have been applied previously in an attempt to counteract the detrimental effects of indomethacin on epithelial integrity in the small intestine. Although it appears that none of the applied strategies is fully effective in attenuating the permeability changes induced by indomethacin, the results do indicate that replenishing the energy supply of enterocytes may be a promising strategy to counteract early-phase metabolic changes in these cells due to topical NSAID toxicity. Our finding described in chapter 3 of this thesis suggests that topically administered ATP may hold promise as a strategy in the treatment of NSAID enteropathy. As ATP is the principal form of chemical energy directly available to cells, its favourable effect may have been mediated by replenishing of intracellular ATP pools, after being salvaged by the enterocytes whose energy metabolism had been disturbed by indomethacin. Another potential mechanism of action responsible for the observed effect may have been activation of purinergic receptors by ATP. Kreienberg et al. (1996) previously showed that intravenous injections of ATP-$MgCl_2$ prevented an ischemia-induced increase in mucosal permeability in the rat ileum [925]. Compared to non-ischemic controls, an 80% increase in permeability to ^{51}Cr-EDTA was observed during 90 minutes of ischemia. In contrast, rats pre-treated with ATP-$MgCl_2$ showed no change in intestinal permeation of ^{51}Cr-EDTA during ischemia [925]. It was also noted that tissue ATP levels in ileal segments declined during the ischemic period, a process which was not affected by prior administration of ATP-$MgCl_2$ [925]. Therefore, the authors hypothesized that ischemia-induced alterations in mucosal permeability in the rat ileum were attenuated by ATP-$MgCl_2$ through a mechanism other than providing enterocytes with an alternative energy source, possibly through binding of ATP to P2 receptors. Intestinal epithelial cells have been shown to express the majority of currently

identified P2 receptor subtypes on the apical (luminal) membrane [825, 826, 926, 927], which would suggest that the favourable effect of ATP in our experiment may indeed have involved P2 receptor signaling.

2.2.4 Limitations of the human model

At this point, potential limitations of the validity of the utilized human model of early-stage indomethacin-induced permeability changes need further discussion. The potential limitations are related to two applied methods in our experiments: the non-invasive method of assessing the indomethacin-induced permeability changes, and the method of monitoring correct placement of the naso-intestinal tube through which ATP was administered topically in our first experiment (chapter 3).

As already mentioned, we assessed intestinal permeability by the lactulose/rhamnose sugar absorption test. This type of permeability test may have two limitations. The first potential limitation refers to a possible lack of site-specific information regarding the exact intestinal region of the indomethacin-induced mucosal defect by assessing the differential urinary excretion ratio of marker molecules as a functional measure of intestinal permeability. However, it has been reported that site-specific discrimination between different regions of the gastrointestinal tract (*i.e.* stomach, small intestine, large intestine) can be achieved by using carefully chosen permeability marker molecules [75, 813]. Sugar probes such as lactulose and rhamnose undergo bacterial fermentation in the large intestine and, therefore, yield only information about permeability of the small intestine, but not about its subregions (*i.e.* duodenum, jejunum, ileum) [813]. Since indomethacin-induced mitochondrial damage to the small intestinal mucosa by topical irritation is most pronounced at the site of indomethacin absorption in the proximal to mid small intestinal subregions [819, 862], we administered ATP by naso-intestinal tube at the supposed site of mucosal damage inflicted by indomethacin. Therefore, the sugar probes lactulose and rhamnose were chosen to evaluate the effect of topically administered ATP on indomethacin-induced permeability changes in this small intestinal subregion. A second potential limitation of the sugar absorption test is the assumption that, when administering two sugars of different size, both molecules will be equally affected by pre-absorptive factors such as gastric emptying and intestinal transit, as well as by post-absorptive factors such as systemic distribution and renal clearance. On that assumption, the urinary excretion ratio of two different-sized sugars will directly reflect their size-dependent transit through different permeation pathways (paracellular vs. transcellular) [816, 844]. Because of the cross-over design of our experiments, between-subject variation in pre- and post-absorptive factors is irrelevant. Furthermore, conditions in the consecutive experiments were identical, except that we allowed for fluid intake ad libitum since, as demonstrated by Parviainen et al. (2005) [856], lactulose/rhamnose excretion ratios are independent of fluid intake and renal clearance.

A final potential limitation relates to the Bengmark-type naso-intestinal tube through which ATP (and placebo) was administered topically in the upper small intestine. This type of tube, commonly used as a small-bore enteric feeding tube, is a self-propelling nasoenteric silicone rubber catheter with a number of loops at its distal end that are straightened with a guidewire during insertion into the stomach [928, 929]. After removal of the guidewire, the coiled end of the tube facilitates spontaneous transpyloric passage, which is expected to occur within 4 hours after placement in the stomach [928, 929]. Once placed, the

coils of the tube tip also slightly anchor the tube safely into the duodenum or proximal jejunum, thereby minimizing the risk of regurgitation into the stomach [928]. In our experiment, tubes were inserted into subjects 4 hours before ingestion of the first indomethacin dose (75 mg) and the first administration of ATP or placebo through the tube. The tube then remained in place overnight and was removed approximately 15-30 minutes after ingestion of the second indomethacin dose (50 mg) and the second ATP or placebo administration. Although regular radiographic assessment (X-ray) is considered to be the most reliable method to confirm correct placement of blindly inserted tubes, it is cost prohibitive as well as unsafe to perform radiographs numerous times daily for the sole purpose of monitoring correct tube placement. Therefore, several nonradiographic so-called bedside methods exist to monitor correct tube position [930, 931]. In our experiments, tube position was monitored after insertion by aspiration of fluid from the tube at regular intervals. To differentiate between gastric and intestinal placement of a newly inserted tube, we observed characteristics of the aspirate and determined the pH value of the aspirated gastrointestinal juices by means of litmus paper. In the fasting state, gastric fluid is mostly clear and colorless or off-white with pH values generally being in the range of 1-5, whereas intestinal fluid generally has a bile-stained appearance, ranging in color from light to dark golden-yellow or brownish-green, with pH values being ≥ 6. Combined consideration of these two bedside methods has been reported to provide a valid and reliable indication of tube position, especially in fasting subjects [932-935], and would therefore appear to have provided sufficient assurance of correct tube position at the time of ATP or placebo administration during our experiments. Only during one out of 28 experiments, incorrect position of the tube at the time of ATP/placebo administration was suspected because of a clear and collorless aspirate with a low pH value of ~2, indicating that the tube was positioned in the stomach. The concerned experiment was discontinued and repeated on a different day, at which it was completed succesfully.

2.2.5 Hypothesized role of adenosine in the effect of ATP on intestinal permeability

Taken together, the above data support validity of the applied methods for assessment of both small intestinal permeability status and accuracy of tube placement (*i.e.* topical ATP delivery) in our first human experiment described in chapter 3. Based on this, we concluded that the observed effect of topically administered ATP on the indomethacin-induced increase in small intestinal permeability was genuine warranting further investigation. Thus, except for the method of administration, we utilized an identical experimental set-up in our second human experiment, which is described in **chapter 4** of this thesis. This experiment was primarily aimed at further exploration of the favourable effect of ATP on indomethacin-induced permeability changes in the small intestine via a more practically feasible mode of administration, that is, enteric-coated capsules. In addition to ATP, we also evaluated the effect of encapsulated adenosine on altered permeability of the small intestine in our second experiment, since we hypothesized that the observed favourable effect of topical ATP might have been partly mediated through its breakdown to adenosine. This hypothesis was based on three lines of evidence.

The first line of evidence refers to the notion that enterocytes have a poor capacity for *de novo* purine nucleotide biosynthesis. Therefore, these cells are forced to rely heavily on salvage pathways for their nucleotide needs, particularly under stressed conditions. At present no transporters are known which could mediate direct translocation of extracellular ATP across biological membranes to be salvaged into

the intracellular compartment for metabolic recycling. In contrast, a number of nucleoside transporters are known to be present at the plasma membrane of most cells, including absorptive epithelia [936-938]. These transporters mediate membrane translocation of physiologic nucleosides, including adenosine, for incorporation into intracellular nucleotides and nucleic acids. Two mammalian nucleoside transporter families with broad selectivities for purine and pyrimidine nucleosides have been categorized: the equilibrative nucleoside transporters (ENT1-4) and the concentrative nucleoside transporters (CNT1-3) [936-938]. The ENT family mediates bidirectional transport of nucleosides by facilitated diffusion along their concentration gradient, whereas CNT-mediated transport of nucleosides occurs against their concentration gradient through active cotransport across the membrane coupled to inwardly directed sodium gradients. In the human small intestinal brush border membrane, Patil et al. (1997) demonstrated the presence of CNT1 and CNT2, which exhibited highest activity in the jejunal region [939]. These concentrative nucleoside transporters could mediate uptake of adenosine into epithelial cells of the small intestinal mucosa, where adenosine serves as a precursor to intracellular nucleotides, including ATP. It has been shown in the rat intestine that topical administration of adenosine after mesenteric ischemia, with a > 80% reduction in tissue ATP concentrations, restored ATP levels in jejunal tissues to near normal [940]. Thus, luminal adenosine may be taken up by intestinal epithelial cells via CNT-type transport processes, and may thereby replenish deficient intracellular ATP pools during the early-phase metabolic changes induced by indomethacin.

The second line of evidence refers to data reported already over 25 years ago, which proposed that regulation of tight junctional permeability might be mediated by intracellular cAMP signaling [941]. Intracellular cAMP levels are known to be influenced by many physiological signaling mechanisms, including purinergic receptor signaling. The $P2Y_{11}$ receptor is the only receptor subtype that mediates an elevation of intracellular cAMP levels in response to ATP. In addition to this P2 receptor subtype, A_{2A} and A_{2B} subtypes of the P1 receptor family mediate an elevation of intracellular cAMP levels in response to adenosine. In this way, adenosine may be involved in the regulation of tight junctional permeability by affecting intracellular cAMP pathways through P1 receptor signaling. Friedman et al. (1998) demonstrated that exposure of T84 intestinal epithelial cells to hypoxia diminished intracellular cAMP signaling, which resulted in increased paracellular permeability and retarded recovery of the epithelial barrier following transmigration of neutrophils [942]. Furthermore, these authors showed that when the T84 intestinal epithelial cells were rendered unresponsive to adenosine by pre-exposure to the adenosine analogue NECA, these cells exhibited an enhanced permeability response during neutrophil transmigration [942]. It was suggested by the authors that adenosine-mediated P1 receptor signaling may be involved in the maintenance of epithelial permeability during neutrophil transmigration through elevation of intracellular cAMP levels.

Finally, our hypothesis that adenosine may have contributed to the favourable effect of ATP was based on a third line of evidence referring to the well-known anti-inflammatory and tissue-protective properties of adenosine in the intestine, as demonstrated in a number of animal models of intestinal inflammation and ischemia-reperfusion injury [57-61, 940, 943, 944]. Many of the processes in the inflamed and/or damaged intestine that are ameliorated by adenosine in these animal models resemble the detrimental processes accompanying NSAID enteropathy. This would suggest that adenosine might also be

beneficial in NSAID-inflicted damage to mucosal tissues in the small bowel, including the disruption of epithelial integrity. Indeed, in a pig model of hemorrhagic shock, prehemorrhage treatment with adenosine was shown to decrease the hemorrhage-induced rise in the passage of a macromolecular tracer from the ileal lumen to the portal blood, which was used as a measure of intestinal permeability [945]. Adenosine might thus preserve mucosal barrier function of the small intestine. Furthermore, inosine, a metabolite of adenosine previously considered to be inactive, has been shown to attenuate intestinal inflammatory processes and to improve gut barrier dysfunction in animal models of reperfusion injury, endotoxemic shock and colitis, possibly through P1 receptor signaling [56, 591, 946].

2.2.6 Effect of encapsulated ATP and adenosine on intestinal permeability

The three lines of evidence outlined above supported the hypothesis that adenosine, in addition to ATP, might be an effective compound in attenuating the indomethacin-induced early-stage alteration in the permeability status of the small intestinal mucosa, or that the favourable effect of ATP shown in chapter 3 was (partly) mediated via its breakdown to adenosine. Therefore, effects of both ATP and adenosine were evaluated in our second experiment described in **chapter 4** of this thesis. However, to our surprise, the early-stage indomethacin-induced permeability changes were not affected by oral administration of either encapsulated ATP or adenosine, contrasting with the favourable effect of topical ATP found earlier. In the discussion section of chapter 4, four possible explanations for the lack of effect of encapsulated ATP and adenosine were put forward.

The most plausible explanation is presumed to be twofold, related on the one hand to the site-specificity of ATP/adenosine delivery, and on the other hand to the timing of ATP/adenosine delivery. In our first experiment (chapter 3), ATP delivery was targeted into the upper small intestinal region (duodenum, proximal jejunum) and was timed to precede the permeability defects induced by indomethacin. This was accomplished by administering ATP by naso-intestinal tube immediately after oral intake of the indomethacin capsules. It has been shown that mitochondrial ATP deficiency by uncoupling of oxidative phosphorylation is induced as early as one hour after an oral dose of indomethacin [819, 947]. In our second experiment (chapter 4), ATP and adenosine were administered via enteric-coated capsules designed for targeted intestinal delivery in a pH-dependent fashion. This approach to deliver ATP/adenosine timely into the limited intestinal region of indomethacin-induced mucosal permeability changes is not an easy task. We used enteric-coated hydroxypropyl methylcellulose (HPMC) capsules that had been coated by means of an alternative coating method (*i.e.* separate coating of HPMC bodies and caps) developed by Huyghebaert and co-workers from the University of Ghent, Belgium [857]. The Eudragit© L30D-55 coating polymer of these capsules was shown to dissolve from pH 6.0-6.5 within one hour after transpyloric passage [857]. Luminal pH values in the healthy intestine are reasonably well-characterized, and are known to rise slowly along the length of the small intestine. Values range from pH 5.9 to 6.8 in the proximal small intestine (first third), pH 6.6 to 7.9 in the mid small intestine (second third) and pH 6.6 to 8.1 in the distal small intestine (final third) [948-950]. These data suggest that the enteric-coated capsules would release ATP/adenosine along the proximal to mid small intestinal region. It is of importance to realize at this point that the duodenum is approximately 20 to 30 cm in length, whereas the jejunum has an approximate length of 2.5 metres and the ileum of 3.5 metres [951].

In addition to depending on intestinal pH, site-specific and timed delivery of encapsulated ATP/adenosine would also depend on intestinal transit time. Transit of single units through the small intestine was previously assumed to be relatively constant with an average transit time of 3 to 4 hours, generally being independent of dosage form (capsule vs. pellets) as well as nutritional state (fasted vs. fed state) [952]. However, individual transit times of single units may be subject to considerable variation as shown more recently in a study in healthy fasting subjects (median transit time: 8 hr, range: 4.5-10.1 hr) [950].

In order to obtain post-hoc evidence of the timing of opening of HPMC capsules with the Eudragit© L30D-55 coating, we performed an additional experiment in four healthy subjects between 19 and 22 years of age. After an overnight faste, these subjects ingested five capsules which were identical to those used in the experiment described in chapter 4, but in addition contained 12 mg lithium per capsule, *i.e.*, 60 mg lithium in total. Blood samples were taken at regular intervals. Plasma lithium concentrations, which were assessed as a marker for opening of the capsules [953], were determined by flame atomic absorption spectroscopy.

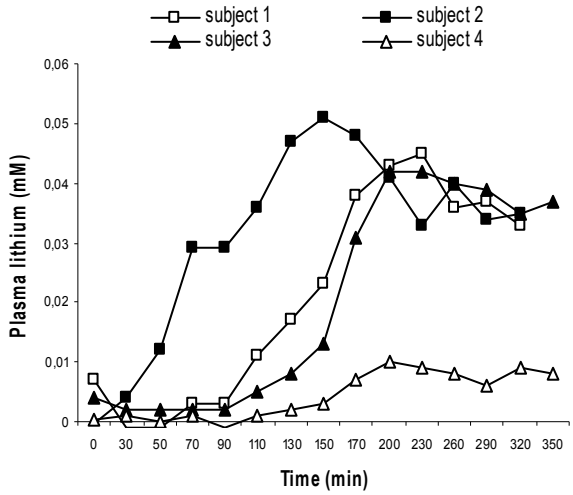


Figure 4. *Change in plasma concentrations of lithium over time. Subjects ingested five HPMC capsules with an Eudragit© L30D-55 coating, containing 60 mg lithium in total.*

As shown in Fig. 4, the timing and degree of elevation in plasma lithium levels showed considerable variation between subjects. In one subject, plasma lithium levels started to rise within 30 to 50 minutes after ingestion of the capsules (Fig. 4, subject 2). This was probably due to increased water intake because of nausea after taking baseline blood samples prior to capsule ingestion, which could have resulted in early dissolution of the coating in the stomach due to a buffering effect on stomach pH. In two subjects, we observed rising of lithium levels starting at ~90 minutes and reaching plateau values at ~200 minutes after capsule ingestion (Fig. 4, subjects 1 and 3), which would be suggestive of opening of the capsules in the proximal or mid region of the small intestine. In the fourth subject, only a marginal rise of plasma lithium levels was observed between 150 and 200 minutes (Fig. 4, subject 4). The results of this experiment would suggest that the timing or site of opening of the enteric-coated capsules, and therefore the intestinal delivery of ATP/adenosine, may have occurred at a point too late or too distant to

interfere with the NSAID-induced permeability changes in the small intestine.

The implications of the findings in **chapter 3 and 4** of this thesis will be discussed in the final section of this chapter, in which directions for future research will be suggested.

3. Enterocyte-driven inflammatory response

The experiments described in this thesis were aimed to explore the role of ATP and adenosine in mucosal defence in the small intestine. We focused primarily on epithelial defence mechanisms, which are accomplished by the enterocytes that reside in the small intestinal mucosa. On the one hand, these cells form an epithelial barrier as more or less passive mucosal defence against harmful particles in the gut lumen. In chapter 3, we presented results suggesting that ATP may preserve the integrity of the epithelial barrier. On the other hand, enterocytes can be considered as sentinels of the mucosal immune system and are therefore part of an immunological barrier. It is thought that the enterocytes are capable of initiating mucosal inflammatory responses through interaction with local immune cells, thereby establishing active mucosal defence against harmful luminal particles.

3.1 Cell experiment with human enterocytes

In **chapter 5**, results are described of an *in vitro* experiment, which was aimed at exploring effects of ATP and adenosine on an enterocyte-driven inflammatory response. For that purpose, we used the Caco-2 cell line. Under standard culture conditions, these human-derived colon adenocarcinoma cells undergo spontaneous differentiation, develop a polarized monolayer and lose their tumerogenic phenotype, expressing several morphological and functional characteristics of mature enterocytes of the small intestinal mucosa, including brush borders with microvilli [954]. Thus, differentiated Caco-2 cells constitute an appropriate culture model of highly enterocyte-like cells of the small intestine. Effects of extracellular ATP and adenosine on inflammatory responses mediated by Caco-2 cells have not been investigated to date.

3.1.1 Hypothesis

The evidence compiled in chapter 2 suggests that extracellular ATP and adenosine may be key regulators of leukocyte function, and thereby may modulate the outcome of inflammation and immune responses. ATP and adenosine were therefore expected to display comparable immunomodulatory properties in the small intestine. Since leukocyte function is affected by ATP and adenosine in a concentration-dependent fashion, we presumed that enterocyte function would be affected in a similar fashion. Specifically, we hypothesized that high extracellullar concentrations of ATP would enhance the generation of inflammatory mediators by Caco-2 cells, and that extracellular adenosine at a wide range of concentrations would suppress the generation of inflammatory mediators. As purinergic modulation of cell function requires the presence of purine-metabolizing ecto-enzymes that control extracellular concentrations of ATP and adenosine as well as the presence of receptors for ATP and adenosine, we also needed to establish the presence of ecto-enzymes and mRNA for P2 and P1 receptors. We

therefore performed two experiments with the Caco-2 cells, as described in chapter 5.

3.1.2 Findings

In the experiments with the Caco-2 cells, we found that:

i. Enterocyte-like Caco-2 cells exhibit potent ecto-enzyme activity and express mRNA for both P2 and P1 receptor subtypes;
ii. The generation of inflammatory mediators by Caco-2 cells, especially the production of cytokines, is affected by extracellular ATP. Effects of adenosine on cytokine production were not straightforward.

ATP at high extracellular concentrations (500-2500 μM) enhanced basal levels of IL-8. At similar levels, ATP also increased the production of IL-8 and TNFα by Caco-2 cells that had been exposed to the pro-inflammatory cytokines IFNγ and IL-1β. In contrast, ATP inhibited the production of IL-6 by IFNγ/IL-1β-stimulated Caco-2 cells. The P2 receptor agonist ATPγS had similar effects on IL-8 and IL-6 production by Caco-2 cells, thus suggesting P2 receptor involvement, possibly of a P2Y subtype, in the effects of ATP. Production of IL-8 and TNFα by enterocytes induces the recruitment and activation of leukocytes, particularly neutrophils, evoking local inflammation and immune responses towards invading pathogens [863]. These findings therefore seem to confirm our hypothesis that ATP mostly exerts immune-enhancing effects at high extracellular levels.

In common with ATP, adenosine at concentrations of 100 to 2500 μM enhanced basal levels of IL-8. This finding contrasts with our hypothesis that adenosine would display mostly anti-inflammatory and immunosuppressive properties. Unexpectedly, effects of adenosine on cytokine production by Caco-2 cells that had been IFNγ/IL-1β-stimulated in the presence of the ADA inhibitor EHNA were not straightforward. This was due to interference of EHNA with adenosine effects as already discussed in chapter 5. Nevertheless, the results do suggest that extracellular adenosine affects cytokine production by Caco-2 cells, even though its precise effects remain unclear at present.

Additional evidence suggesting that adenosine indeed affects immune function of enterocytes in the context of inflammatory conditions in the intestine derives from previous studies dealing with effects of adenosine on cytokine production in enterocyte-like cell lines.

3.2 Effects of adenosine on immune function of intestinal epithelial cell cultures

Besides the Caco-2 cell line, other human-derived colon carcinoma cell lines include HT-29 and T84 cells. These cell lines are frequently used to investigate the function of colonic epithelial cells. Jijon et al. (2005) demonstrated the presence of A_{2A} and A_{2B} receptors on HT-29 cells and showed that pre-treatment of these cells with adenosine at concentrations of 250 to 1000 μM reduced the production of IL-8 induced by TNFα, IL-1 or LPS [955]. The inhibitory effect of adenosine on IL-8 production was mediated by suppression of TNFα-induced activation of NFκB in these cells. Neither ATP (25-500 μM) nor ADP (100-1000 μM) mimicked the ability of adenosine to inhibit the production of IL-8 by HT-29 cells [955].

In contrast to expression of both A_{2A} and A_{2B} receptors by HT-29 cells, the A_{2B} receptor subtype has been shown to be the predominant adenosine receptor present in T84 cells [877, 956]. Sitaraman and co-workers (2001) showed that incubation of T84 cells with adenosine at concentrations of 1 to 100 μM elicited apically directed secretion of IL-6 [957]. More recently, these authors suggested that treatment of T84 cells with adenosine may also promote secretion of IL-8 [958]. Interestingly, A_{2B} receptor expression by T84 cells appears to be modulated under inflammatory conditions, since A_{2B} receptor-mediated signaling in these cells was recently shown to be down-regulated by IFNγ [959], but up-regulated by TNFα and hypoxia [55, 960]. Besides depending on a specific cell-culture model, the above data suggest that the effects of adenosine on cytokine production by enterocyte-like cells may also depend on its extracellular concentrations and the P1 receptor subtype activated at these concentrations. It could be speculated that immune function of intestinal epithelial cells may be enhanced via A_{2B} receptor activation by adenosine at low micromolar levels, whereas adenosine at high micromolar to millimolar levels may have inhibitory effects on mucosal inflammation via A_{2A} receptor activation.

Taken together, combined with results from previous studies, our findings indicate that the immune function of mucosal enterocytes is affected by extracellular ATP and adenosine, but further experiments are warranted to investigate the nature of the effects of these compounds on enterocyte-driven mucosal inflammation and the mechanisms involved.

Implications of the results described in **chapter 5** of this thesis will be discussed in the next section.

4. Overall conclusions and directions for future research

Based on the evidence deriving from the imposing amount of previous research as reviewed in **chapter 2**, we conclude that ATP and adenosine are ubiquitous molecules in the extracellular compartment playing a versatile role in the regulation of immunity and inflammation. Moreover, we conclude that ATP may have a role in intestinal defence, possibly fulfilling a dual function: (i) preservation of epithelial integrity which facilitates protection against invading pathogens (**chapter 3**) and (ii) stimulation of enterocyte-driven inflammatory responses towards luminal pathogens (**chapter 5**). Based on the data presented in this thesis, the precise role of adenosine in these epithelial defence mechanisms yet remains unclear (**chapters 4 and 5**). Overall, to corroborate and expand on the findings of this thesis, we suggest several directions for further research.

4.1 Mucosal barrier function

The observed favourable effect of ATP on mucosal barrier function merits further exploration. The first priority would be re-evaluation of the ATP effect on acute mucosal permeability changes by means of a more practically feasible and subject-friendly method of targeted intestinal delivery of ATP (and adenosine), using new encapsulation techniques that allow targeted release. Second, the presumed working mechanisms involved in the effect of ATP need more detailed clarification, that is, salvage-mediated and/or receptor-mediated effects. To this end, it might be relevant to assess the influence of

nucleoside transport inhibitors or P1 receptor antagonists in the human model of indomethacin-induced permeability changes.

4.2 Enterocyte-driven mucosal inflammation

The effects of ATP and adenosine on immune function of enterocyte-like Caco-2 cells also merit further investigation. Future experiments should be aimed at (i) detailed profiling of the expression of P2 and P1 receptors as well as ecto-enzymes on the Caco-2 cell surface, and (ii) assessment of the effects of specific P2 and P1 receptor ligands on cytokine production and ICAM-1 expression by the Caco-2 cells. In addition, similar experiments in other culture models of intestinal epithelial cells, such as the above mentioned T84 and HT-29 cell lines, would be relevant for a better insight into the role of ATP and adenosine in entrocyte-driven mucosal inflammation as a whole.

It is also imperative to provide an explanation for the observed interfering effect of the ADA inhibitor EHNA. Since this may have been due to release of ATP and/or adenosine in response to pro-inflammatory stimulation of Caco-2 cells, acute release of ATP and/or adenosine in the Caco-2 cell culture should be re-assessed using more sensitive methods to determine local ATP and adenosine concentrations than the HPLC assay in culture medium, which was applied in our experiments. Additional information about endogenous release of ATP and/or adenosine by the Caco-2 cells may also be provided by evaluation of the influence of ecto-enzymes or nucleoside transport inhibitors on the effects of ATP and adenosine.

4.3 Dysregulated mucosal defence in the small intestine

Based on the proposed dual role of ATP in mucosal defence in the small intestine, investigation of *in vivo* effects of ATP (and adenosine) on combined dysregulation of mucosal barrier function and mucosal inflammation in the intestine would be relevant. Two types of experiments may be suggested:

i. Animal studies with models of disrupted mucosal barrier function and intestinal inflammation. For instance, colitis models in which intestinal inflammation is induced by indomethacin, acetic acid or carrageenan are considered as appropriate models for studying impaired mucosal barrier function and early inflammatory events after mucosal injury [961]. In such models, the assessment of effects of ATP and adenosine as well as specific P2 and P1 receptor ligands on intestinal permeability and inflammatory markers in intestinal mucosal tissues would be valuable.
ii. Human studies assessing the effects of ATP (and adenosine) on alterations in gastrointestinal permeability and markers of mucosal inflammation in frequent NSAID-users and/or IBD patients would be worth while.

SUMMARY

The nucleotide adenosine 5'-triphosphate (ATP) is attracting increased attention as an extracellular signaling molecule playing a role in human biology. ATP is thought to be involved in the fine-tuning of various biological events via ligation of purinergic receptors, which have a widespread tissue distribution.

It was found previously in lung cancer patients that low-dose ATP infusions mediated immunomodulatory effects in vivo by preventing the increase in plasma C-reactive protein (CRP) levels that was observed in control patients who received no ATP. We therefore hypothesized that ATP (and its metabolite adenosine) may be beneficial in the treatment of chronic inflammatory disorders, which are characterized by dysregulated immunity, such as inflammatory bowel disease (IBD). In view of this hypothesis, our main objective was to further explore the role of ATP and adenosine in immunity and inflammation.

In **chapter 2**, we reviewed the existing literature on the role of ATP and adenosine in the immune system. Overwhelming evidence suggests that both ATP and adenosine are involved in the regulation of immunity and inflammation by modulating the function of a variety of immune cells, including neutrophils, monocytes, macrophages, dendritic cells and lymphocytes. A theory of immune regulation, which has gained growing interest over the last decade, poses that the immune system is primarily occupied with detecting dangers to the host instead of solely discriminating self from nonself. Besides stimulation of immunity by molecules originating from invading micro-organisms (pathogen-associated molecular patterns (PAMPs) or exogenous danger signals), this so-called Danger theory proposes that in the event of tissue damage or distress tissue-derived molecules (damage-associated molecular patterns (DAMPs) or endogenous danger signals) are also able to trigger immunity and to co-operate with the exogenous signals to direct the effector class of immune responses. Both ATP and adenosine fit well within this framework. They can be released into the extracellular compartment by activated or distressed cells where they mediate autocrine and paracrine effects on bystander immune cells via activation of P2 receptors for ATP and P1 receptors for adenosine. Via activation of these receptors, which are widely expressed in the immune system, ATP and adenosine exert diverse effects on processes such as cell recruitment, cell-mediated cytotoxicity, activation of transcription factors, production of inflammatory mediators and cell death. ATP at high extracellular concentrations is mostly immunostimulatory and pro-inflammatory, contributing in this way to acute inflammation and the initiation of primary immune responses. In contrast, ATP at lower extracellular levels appears to have immunomodulatory and anti-inflammatory properties, thereby being involved in the orchestration of ongoing inflammatory processes and immune responses. Adenosine mediates predominantly anti-inflammatory and immunosuppressive effects in the extracellular compartment, contributing to tissue protection and resolution of inflammation. Taken together, ATP and adenosine seem to be involved in the complex regulation of immunity and inflammation in a multifaceted and interdependent fashion.

Furthermore, we performed two types of experiments related to IBD aimed specifically at evaluating effects of ATP and adenosine on mechanisms of mucosal defence in the small intestine, including mucosal barrier function and enterocyte-driven inflammatory response.

In **chapter 3**, a human experiment is described that was aimed at evaluating the local effect of ATP on disturbed barrier function of the small intestinal mucosa, which is common in IBD. The nonsteroidal anti-inflammatory drug (NSAID) indomethacin was used to induce an increase in epithelial permeability in the upper small intestine. Frequent use of NSAIDs is associated with an elevated risk of damage to the mucosal epithelium which lines the gastrointestinal tract lumen. One of the earliest events in NSAID toxicity is mitochondrial dysfunction with intracellular ATP deficiency, leading to an increase in epithelial permeability in the upper small intestine. We performed a randomized cross-over experiment with 14 healthy human volunteers. Each subject participated in three experiments with one week wash-out in between (control (= no intervention), placebo, ATP). Basal permeability of the small intestine was assessed as a control condition. As a model of increased intestinal permeability, indomethacin was administered to the subjects at 10 hours (75 mg) and 1 hour (50 mg) before permeability assessment. Concomitant with indomethacin ingestion, placebo or ATP (30 mg/kg) was administered into the upper small intestine through a Bengmark-type naso-intestinal tube. Intestinal permeability was assessed by the sugar absorption test, that is, ingestion of a test drink containing lactulose (5 g) and rhamnose (0.5 g) followed by 5-hour collection of total urine; urinary excretion of lactulose and rhamnose was determined by high-pressure liquid chromatography (HPLC). The urinary lactulose/rhamnose (L/R) excretion ratio is a sensitive measure of intestinal permeability changes. An increased L/R ratio implies increased intestinal permeability. Results showed that the urinary L/R excretion ratio was significantly increased relative to control following administration of indomethacin. The indomethacin-induced increase in L/R ratio was significantly attenuated by topical administration of ATP. The L/R ratio following administration of ATP was similar to the basal L/R ratio in the control condition. It was concluded that topical administration of ATP into the upper small intestine counteracts an increase in small intestinal permeability induced by a short-term indomethacin challenge in healthy humans. This finding implicated that ATP might also be beneficial in the treatment of intestinal disorders in which epithelial integrity is compromised, such as IBD.

As described in **chapter 4**, we performed a follow-up experiment to evaluate a more practically feasible mode of ATP administration. Utilizing the same human model of indomethacin-induced intestinal permeability changes, we administered ATP via enteric-coated hydroxypropyl methylcellulose (HPMC) capsules with an Eudragit© L30D-55 coating. In an attempt to provide better insight into the mechanism of action of ATP, we also administered adenosine via the enteric-coated HPMC capsules. ATP may be salvaged by mucosal epithelial cells subsequent to its breakdown to adenosine. A total of 33 subjects participated in four experiments that were carried out in randomized order with one week wash-out in between (control, placebo, ATP, adenosine). Intestinal permeability was assessed by the L/R sugar absorption test. Similar to our previous experiment, it was shown that indomethacin ingestion significantly increased the urinary L/R excretion ratio relative to control. In contrast to our previous experiment, however, neither ATP nor adenosine administration affected the indomethacin-induced increase in L/R ratio. It was concluded that the observed lack of effect of encapsulated ATP and adenosine was likely due to opening of the enteric-coated supplement at a site distal from the intestinal site damaged by indomethacin. Further experiments on effectiveness and site-specific delivery of ATP (and adenosine) are warranted.

Besides constituting a cellular barrier, the enterocytes residing in the intestinal mucosa also actively contribute to intestinal defence by initiating mucosal immune responses in co-operation with local immune cells. In **chapter 5**, an experiment with enterocyte-like Caco-2 cells is described in which we evaluated effects of ATP and adenosine on an inflammatory response mediated by these human-derived intestinal cells. It is known that Caco-2 cells in long-term culture differentiate into enterocyte-like small intestinal epithelial cells exhibiting a variety of immunological properties. In accordance with the concentration-dependent effects of ATP and adenosine on immune cells, we hypothesized that ATP at high extracellular concentrations would enhance the enterocyte-driven inflammatory response, whereas adenosine would have mostly anti-inflammatory effects at a wider range of concentrations.

We performed two experiments with the Caco-2 cell culture. In the first cell experiment, time-dependent metabolism of ATP and adenosine under inflammatory conditions was assessed as an indication of ecto-enzyme activity. To induce an inflammatory reaction, 20-day differentiated Caco-2 cells were stimulated with the pro-inflammatory cytokines interferon (IFN)-γ and interleukin (IL)-1β. Cells were co-incubated with ATP or adenosine for 24 hours and culture medium was collected at different time points for HPLC analysis of ATP, adenosine and their metabolites. Results showed that ATP and adenosine were completely metabolized in the Caco-2 cell culture within approximately 2 to 6 hours with sequential formation of their metabolites. The adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA) minimized breakdown of adenosine, whereas the ecto-ATPase inhibitor 6-N,N-Diethyl-D-β,γ-dibromomethylene ATP trisodium salt (ARL-67156) had no effect on ATP breakdown in Caco-2 cell culture medium. We also determined mRNA expression of P2 receptors for ATP and P1 receptors for adenosine in the first cell experiment. Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that Caco-2 cells express mRNA for $P2Y_1$, $P2Y_2$, $P2Y_{11}$, $P2Y_{12}$, A_{2A}, A_{2B} and A_3 receptors. Following 24-hour stimulation of Caco-2 cells with IFNγ/IL-1β, a ~64% decrease in $P2Y_1$ mRNA expression was noted; expression of other subtypes was not changed.

In the second cell experiment, effects of ATP and adenosine on intercellular adhesion molecule (ICAM)-1 expression and cytokine production were assessed. We incubated 18-day differentiated Caco-2 cells with IFNγ/IL-1β and different concentrations of ATP or adenosine. In an attempt to minimize their breakdown during incubation, ARL-67156 was added 30 minutes before ATP and EHNA was co-incubated with adenosine. Following 16 hours of incubation, expression of ICAM-1 protein was determined and levels of IL-6, IL-8 and tumor necrosis factor (TNF)-α were measured in culture medium. Results showed that ATP and adenosine did not affect ICAM-1 expression by IFNγ/IL-1β-stimulated Caco-2 cells. In contrast to its lack of effect on ICAM-1 expression, ATP did influence the production of cytokines by the Caco-2 cells. ATP increased basal levels of IL-8 in cell culture medium. Also, the IFNγ/IL-1β-induced production of IL-8 and TNFα was increased by ATP, but the production of IL-6 was decreased by ATP. Basal levels of IL-8 and IFNγ/IL-1β-induced production of IL-6 were affected in a similar fashion by the ATP analogue and P2 receptor agonist adenosine 5'-O-(3-thiotriphosphate) (ATPγS), suggesting a P2 receptor-mediated mechanism. Unexpectedly, the adenosine deaminase inhibitor EHNA interfered with effects of adenosine on cytokine production by Caco-2 cells. EHNA as a single agent inhibited the IFNγ/IL-1β-induced production of IL-6 and enhanced the production of IL-8 and

TNFα in response to IFNγ/IL-1β. Because of this interference by EHNA, effects of adenosine on cytokine production by the Caco-2 cells remained unclear. When cells were incubated with adenosine in the absence of EHNA, increased levels of IL-8 and decreased levels of IL-6 were observed.

Thus, results of the two cell experiments showed that (i) enterocyte-like Caco-2 cells exhibit ecto-enzyme activity allowing them to control extracellular levels of ATP and adenosine, (ii) Caco-2 cells co-express mRNA for both ATP and adenosine receptors, (iii) ICAM-1 expression by Caco-2 cells during an inflammatory reaction is not affected by either ATP or adenosine, and (iv) cytokine production by Caco-2 cells appears to be affected by ATP and perhaps by adenosine. It was concluded that extracellular ATP and adenosine seem to be involved in the modulation of an enterocyte-driven inflammatory response.

Overall conclusions of this thesis are:

i. Substantial literature evidence indicates that ATP and adenosine are extracellular signaling molecules which play a role in the regulation of immunity and inflammation by modulating various immune cell functions;
ii. ATP is likely to play a role in intestinal defence by affecting intestinal permeability on the one hand and enterocyte-driven cytokine production on the other hand;
iii. Adenosine seems to exert immunomodulatory effects on human enterocytes, but its precise role remains unclear.

SAMENVATTING

Hoewel al lang bekend is dat adenosine 5'-trifosfaat (ATP) binnenin de cel fungeert als een energieleverancier, krijgt deze nucleotide steeds meer aandacht als een signaalmolecuul buiten de cel (extracellulair). Tegenwoordig wordt aangenomen dat ATP betrokken is bij de regulering van verscheidene biologische processen in het menselijk lichaam door te binden aan zogenaamde purinerge receptoren, welke op de buitenzijde van vrijwel alle lichaamcellen voorkomen.

In eerder onderzoek bij longkankerpatiënten die gedurende een half jaar regelmatig werden behandeld met een lage dosering ATP per infuus, werden immunologische effecten van ATP waargenomen. Behandeling van deze patiënten met ATP leidde tot een stabilisering van de concentratie van het ontstekingseiwit CRP (C-reactive protein) in het bloedplasma, terwijl bij onbehandelde patiënten in de controlegroep een toename in de tijd van de CRP-concentratie werd waargenomen. Op grond van dit immunomodulerende effect werd de hypothese gevormd dat toediening van ATP gunstig zou kunnen zijn bij de behandeling van chronische ontstekingsziekten die gekenmerkt worden door een verstoorde immuunfunctie, zoals inflammatoire darmziekten (Inflammatory Bowel Disease, afgekort IBD). Het project dat is beschreven in dit proefschrift was gebaseerd op deze hypothese en had als doel de rol van ATP in het afweersysteem nader te bestuderen.

In **hoofdstuk 2** wordt een overzicht gegeven van de bestaande literatuur over de rol van ATP en zijn afbraakproduct adenosine in het immuunsysteem. In de literatuur bestaan sterke aanwijzingen dat zowel ATP als adenosine betrokken zijn bij de regulering van immuniteit en inflammatie (ontsteking) door de functie van verschillende immuuncellen te beïnvloeden, o.a. neutrofielen, monocyten, macrofagen, dendritische cellen en lymfocyten. Het afgelopen decennium heeft een nieuwe theorie over immuunregulering in toenemende mate aandacht gekregen. Deze theorie stelt dat het immuunsysteem primair gericht is op het herkennen van gevaar ('danger') voor de gastheer in plaats van uitsluitend, zoals eerder werd aangenomen, lichaamsvreemde van lichaamseigen stoffen te onderscheiden. Naast activering van het immuunsysteem door lichaamsvreemde moleculen die afkomstig zijn van binnendringende micro-organismen (zogenaamde 'Pathogen-Associated Molecular Patterns' (PAMP's) of ook wel exogene 'danger'-signalen), suggereert deze zogeheten 'Danger'-theorie dat er bij weefselschade lichaamseigen moleculen vrijkomen uit de beschadigde weefsels (zogenaamde 'Damage-Associated Molecular Patterns' (DAMP's) of ook wel endogene 'danger'-signalen), die eveneens in staat zijn het immuunsyteem te activeren en die in samenspel met de exogene signalen tot een efficiënte immuunrespons leiden. Zowel ATP als adenosine passen als DAMP's uitstekend binnen deze theorie. Ze worden beide ten gevolge van celactivering of -beschadiging uitgescheiden in het extracellulaire compartiment, waar ze via binding aan P2 receptoren voor ATP en P1 receptoren voor adenosine verschillende effecten hebben op nabij gelegen immuuncellen. Door activering van deze purinerge receptoren, die in het gehele immuunsysteem voorkomen, beïnvloeden ATP en adenosine diverse immunologische processen. Een acuut verhoogde extracellulaire ATP concentratie, bijvoorbeeld ten gevolge van celschade, heeft voornamelijk immunostimulerende en ontstekingsbevorderende effecten, waardoor ATP bijdraagt aan acute ontstekingsreacties en primaire immuunresponsen. Daarentegen lijkt een chronische, lagere ATP concentratie meer immunomodulerende en ontstekingsremmende effecten te hebben, waardoor ATP een bijdrage kan leveren aan de regulering van aanhoudende ontstekingsprocessen en immuunresponsen. Adenosine heeft vooral

ontstekingsremmende en immunosuppressieve effecten, en is als gevolg daarvan betrokken bij het uitdoven van ontstekingshaarden en bij weefselherstel. Samenvattend kan gezegd worden dat ATP en adenosine een veelzijdige en onderling afhankelijke rol spelen bij de complexe regulering van immuniteit en inflammatie.

Behalve het bovengenoemde literatuuroverzicht is een aantal proeven uitgevoerd met als doel het onderzoeken van de effecten van ATP en adenosine op twee afweermechanismen in de dunne darm, namelijk de barrièrefunctie van de darmwand en de immuunfunctie van darmcellen.

In **hoofdstuk 3** wordt een proef beschreven die gericht was op het onderzoeken van het effect van ATP op een verstoorde barrièrefunctie van de dunne darm, een veel voorkomend symptoom bij patiënten met IBD. Met behulp van de ontstekingsremmer indomethacine (een zgn. Non-Steroidal Anti-Inflammatory Drug, afgekort NSAID) werd een verhoging geïnduceerd van de doorlaatbaarheid van de darmwand (darmpermeabiliteit). Veelvuldig gebruik van dergelijke ontstekingsremmers blijkt samen te hangen met een verhoogd risico op schade aan het slijmvlies van de darmwand (de darmmucosa). Een van de eerste schadelijke effecten van indomethacine is het verstoren van de productie van ATP als energievoorraad in de darmcellen. Door een tekort aan ATP kan de darm zijn barrièrefunctie niet meer goed vervullen, hetgeen leidt tot een verhoging van de darmpermeabiliteit in het eerste deel van de dunne darm. Om het effect van ATP op de darmpermeabiliteit te onderzoeken werd een experiment uitgevoerd met 14 gezonde vrijwilligers. Elke deelnemer deed mee aan drie verschillende experimenten (controle (= geen interventie), placebo, ATP) met daartussen steeds een periode van één week. De volgorde van de experimenten werd door loting bepaald. In het controle experiment werd de basale darmpermeabiliteit gemeten, d.w.z. de darmpermeabiliteit onder normale omstandigheden. In de andere twee experimenten werd de darmpermeabiliteit kunstmatig verhoogd doordat de deelnemers tweemaal de ontstekingsremmer indomethacine innamen. Tegelijkertijd met het innemen van de indomethacine werd placebo (water) of ATP rechtstreeks in het eerste deel van de dunne darm toegediend via een darmsonde. De darmpermeabiliteit van de deelnemers werd bepaald door middel van een meting van de suikerabsorptie: nadat de deelnemers een oplossing van de twee suikers lactulose en rhamnose hadden gedronken, verzamelden ze gedurende 5 uur al hun urine waarin vervolgens de uitscheiding van lactulose en rhamnose werd gemeten. Door de verhouding te bepalen tussen lactulose en rhamnose in de urine, de L/R ratio, kunnen permeabiliteitsveranderingen gemeten worden (een verhoogde L/R ratio wijst op een verhoogde darmpermeabiliteit). Na het innemen van de ontstekingsremmer indomethacine was de L/R ratio in de urine verhoogd ten opzichte van de controle conditie. Deze verhoging van de L/R ratio trad niet of nauwelijks op wanneer ATP tegelijkertijd met indomethacine werd toegediend. Hieruit kan worden geconcludeerd dat de door indomethacine veroorzaakte verhoging van de darmpermeabiliteit bij gezonde vrijwilligers kan worden voorkomen door gelijktijdige toediening van ATP in de dunne darm. Mogelijk heeft ATP dus ook gunstige effecten bij de behandeling van ziekten met een verstoorde barrièrefunctie van de darm, zoals IBD.

In **hoofdstuk 4** wordt een vervolgproef beschreven die gericht was op het testen van een meer praktische en patiëntvriendelijke toedieningsvorm van ATP. Hiervoor werd wederom gebruik gemaakt van een verhoging van de darmpermeabiliteit van gezonde vrijwilligers door de ontstekingsremmer

indomethacine. In deze proef werd ATP echter toegediend in de vorm van gecoate capsules. Behalve een experiment waarin ATP per capsule werd gegeven, gaven we in een extra experiment ook adenosine in de vorm van deze capsules om zo meer inzicht te verkrijgen in het mogelijke werkingsmechanisme van ATP. Het kan namelijk zijn dat ATP in de darm wordt afgebroken tot adenosine, en als zodanig wordt opgenomen door de darmcellen. Aangezien binnenin de darmcellen adenosine vervolgens weer kan worden omgezet tot ATP, zou door het geven van adenosine per capsule de ATP voorraad in de darmcellen dus eveneens kunnen worden aangevuld. In totaal deden 33 vrijwilligers mee aan vier experimenten (controle, placebo, ATP, adenosine) waarvan de volgorde door loting werd bepaald, opnieuw met steeds een tussenperiode van één week. De darmpermeabiliteit werd weer bepaald met behulp van een meting van de suikerabsorptie. Net als in ons vorige experiment veroorzaakte het innemen van indomethacine een verhoging van de L/R ratio in de urine ten opzichte van de controle conditie. In tegenstelling tot ons vorige experiment bleken echter zowel ATP als adenosine in capsulevorm geen invloed te hebben op deze door indomethacine veroorzaakte verhoging van de L/R ratio. De vermoedelijke verklaring voor het ontbreken van een effect van de ATP en adenosine capsules was dat de capsules te laat waren opengegaan, waarschijnlijk pas op een plek na het gedeelte van de dunne darm dat beschadigd was door indomethacine. Verder onderzoek zal nodig zijn om een duidelijker beeld te krijgen van de werking van ATP (en adenosine) alsmede om een effectieve en tevens patiëntvriendelijke toedieningsvorm te ontwikkelen.

Naast het vormen van een celbarrière voor schadelijke stoffen en bacteriën, zijn de darmcellen (enterocyten) ook actief betrokken bij het opwekken van een zogenaamde mucosale immuunrespons in samenspel met de in de darm aanwezige immuuncellen. In **hoofdstuk 5** wordt een experiment beschreven met de zogeheten Caco-2 cellijn, waarin de effecten van ATP en adenosine zijn onderzocht op een ontstekingsreactie geïnitieerd door deze van mensen afkomstige cellen. Het is bekend dat gekweekte Caco-2 cellen te vergelijken zijn met enterocyten uit de dunne darm. De Caco-2 cellen vertonen dan verscheidene immunologische eigenschappen. Op basis van voorkennis over de effecten van ATP en adenosine op immuuncellen namen we aan dat een hoge ATP concentratie een kunstmatig opgewekte onstekingsreactie in deze Caco-2 cellen zou bevorderen, terwijl we van adenosine vooral ontstekingsremmende effecten verwachtten.

Er werden twee experimenten met de Caco-2 cellen uitgevoerd. In het eerste celexperiment werd de afbraak van ATP en adenosine tijdens een kunstmatige ontstekingsreactie in de tijd gevolgd als aanwijzing voor de activiteit van zogenaamde ecto-enzymen. Deze enzymen zijn aanwezig aan de buitenzijde van de celwand en reguleren daar de afbraak van ATP en adenosine. Om een kunstmatige ontstekingsreactie in de celkweek op te wekken werden Caco-2 cellen gedurende 24 uur gestimuleerd met twee ontstekingsbevorderende stoffen: interferon-gamma (IFNγ) en interleukine-1-bèta (IL-1β). Tegelijkertijd werden de cellen blootgesteld aan ATP of adenosine. Op verschillende momenten werden de concentraties van ATP, adenosine en hun afbraakproducten in de celkweek gemeten. Het bleek dat ATP en adenosine binnen ca. 2 tot 6 uur volledig werden omgezet in hun afbraakproducten. Om de afbraak van ATP en adenosine te remmen, zodat ze gedurende een langere periode zouden kunnen binden aan hun receptoren en zo de functie van de Caco-2 cellen langdurig zouden kunnen beïnvloeden, voegden we in een aantal deelexperimenten tevens afbraakremmers toe: ARL-67156 voor

remming van de ATP afbraak en EHNA voor remming van de adenosine afbraak. Terwijl EHNA de afbraak van adenosine minimaliseerde, had ARL-67156 geen invloed op de afbraak van ATP. In het eerste celexperiment toonden we in de Caco-2 cellijn tevens de aanwezigheid aan van een aantal receptoren voor ATP (zgn. $P2Y_1$, $P2Y_2$, $P2Y_{11}$ en $P2Y_{12}$ receptoren) en adenosine (zgn. A_{2A}, A_{2B} en A_3 receptoren).

In het tweede celexperiment werden de effecten van ATP en adenosine op een aantal parameters voor ontstekingsactiviteit in de Caco-2 cellijn bepaald, namelijk effecten op de hoeveelheid van het zogeheten adhesiemolecuul ICAM-1 (intercellular adhesion molecule-1) en de productie van een aantal ontstekingseiwitten (cytokinen). Er werd weer een kunstmatige ontstekingsreactie opgewekt in de Caco-2 celkweek en de cellen werden tegelijkertijd blootgesteld aan verschillende concentraties ATP of adenosine. Ook werden in een aantal deelexperimenten weer de afbraakremmers ARL-67156 voor ATP en EHNA voor adenosine toegevoegd. Vervolgens werden de hoeveelheid ICAM-1 en de productie van de cytokinen interleukine-6 (IL-6), interleukine-8 (IL-8) en tumor necrosis factor-alpha (TNFα) gemeten. ATP en adenosine hadden geen invloed op de hoeveelheid ICAM-1 in de Caco-2 cellijn. In tegenstelling tot het ontbreken van een effect van ATP op ICAM-1, had ATP wel invloed op de productie van cytokinen door de Caco-2 cellen. De productie van IL-8 en TNFα tijdens een kunstmatige ontstekingsreactie werd bevorderd door ATP, terwijl de productie van IL-6 geremd werd door ATP. De effecten van adenosine op de cytokinenproductie door de Caco-2 cellen werden ook onderzocht, maar vanwege een onverwachte verstorende invloed van de afbraakremmer EHNA in een aantal deelexperimenten, waren de resultaten voor adenosine niet goed te interpreteren.

De resultaten van deze twee experimenten met de Caco-2 cellijn laten zien dat (i) Caco-2 cellen over afbraakenzymen aan de buitenzijde van de celwand beschikken waardoor ze de concentraties van ATP en adenosine buiten de cel kunnen beïnvloeden, (ii) Caco-2 cellen zowel receptoren voor ATP als adenosine bevatten, (iii) de hoeveelheid van het adhesiemolecuul ICAM-1 tijdens een ontstekingsreactie niet beïnvloed wordt door ATP en adenosine, en (iv) de productie van cytokinen door Caco-2 cellen waarschijnlijk beïnvloed wordt door ATP en mogelijk ook door adenosine. Geconcludeerd wordt dat ATP en adenosine betrokken lijken te zijn bij de modulering van de immuunfunctie van enterocyten.

De algemene conclusies van dit proefschrift zijn:

i. Er zijn sterke aanwijzingen uit de literatuur dat ATP en adenosine als extracellulaire signaalmoleculen een belangrijke rol spelen bij de regulering van immuniteit en inflammatie door het beïnvloeden van diverse functies van immuuncellen;
ii. ATP speelt waarschijnlijk een rol in de darmafweer via het beïnvloeden van enerzijds de darmpermeabiliteit en anderzijds de immuunfunctie van enterocyten;
iii. Hoewel ook adenosine invloed lijkt te hebben op de immuunfunctie van enterocyten, is de precieze rol van adenosine nog onduidelijk.

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ABBREVIATIONS

2-MeS-ATP	2-methylthio-ATP
5-LO	5-lipoxygenase
AA	arachidonic acid
ABC	ATP-binding cassette
ADA	adenosine deaminase
Ado	adenosine
ADPβS	adenosine 5'-*O*-(2-thiodiphosphate)
AP-1	activator protein-1
ARL-67156	6-N,N-Diethyl-D-β,γ-dibromomethylene ATP trisodium salt
ATL-146e	4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester
ATPDase	ATP-diphosphohydrolase
ATPγS	adenosine 5'-*O*-(3-thiotriphosphate)
ATTC	American Tissue Type Collection
BSA	bovine serum albumin
BzATP	benzoyl ATP
CARD15/NOD2	caspase associated recruitment domain family, member 15/nucleotide binding oligomerisation domain 2
CCL2	monocyte chemoattractant protein (MCP)-1
CCL3	macrophage inflammatory protein (MIP)-1α
CCL4	macrophage inflammatory protein (MIP)-1β
CCL5	regulated upon activation normal T-cell expressed and secreted (RANTES)
CCL17	thymus- and activation-regulated chemokine (TARC)
CCL20	macrophage inflammatory protein (MIP)-3α
CD23	immunoglobulin (Ig)-E receptor
CD73	ecto-5'-nucleotidase
CGS-21680	2-[p-(2-carboxyethyl)phenylethylamino]-5'-N-ethylcarboxamidoadenosine
Cl-IB-MECA	2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-methylcarboxamide
CLL	chronic lymphocytic leukaemia
CNT	concentrative nucleoside transporter
COX	cyclooxygenase
$cPLA_2$	cytosolic PLA_2
CTL	cytotoxic T cell
CXCL2	macrophage inflammatory protein (MIP)-2α
CXCL8	interleukin-8
CXCL10	IFNγ-inducing protein [355]-10
DAMP	damage-associated molecular pattern
DC	dendritic cell
DMEM	Dulbecco's minimum essential medium
EDTA	ethylenediaminetetraacetate
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride
ELISA	enzyme-linked immunosorbent assay

E-NPP	ectonucleotide pyrophosphatase/phosphodiesterase
ENT	equilibrative nucleoside transporter
E-NTPDase	ectonucleoside triphosphate diphosphohydrolase, CD39
FCS	fetal calf serum
fMLP	formyl-methionyl-leucyl-phenylalanine
FSDCs	fetal skin-derived dendritic cells
G3PDH	glyceroaldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage colony-stimulating factor
H. pylori	helicobacter pylori
HPLC	high-pressure liquid chromatography
HPMC	hydroxypropyl methylcellulose
IBD	inflammatory bowel disease
ICAM-1	intercellular adhesion molecule-1
IFNγ	interferon-γ
IL	interleukin
$iPLA_2$	Ca^{2+}-independent PLA_2
LAK cell	lymphokine-activated killer cell
LC	Langerhans cell
LDL	low-density lipoprotein
LFA-1	lymphocyte function-associated antigen-1, CD11a/CD18
LIF	leukemia inhibitory factor
LPAM-1	lymphocyte Peyer's patch adhesion molecule-1
LPS	lipopolysaccharide
LT	leukotriene
L/R	lactulose/rhamnose
Mac-1	macrophage antigen-1, CD11b/CD18
MHC	major histocompatibility complex
MoDC	monocyte-derived dendritic cell
Mo/Mø	monocytes/macrophages
NEAA	non-essential amino acids
NECA	5'-N-ethylcarboxamidoadenosine
NFκB	nuclear factor-κB
NK cell	natural killer cell
NOS	nitric oxide synthase
NSAID	nonsteroidal anti-inflammatory drug
oATP	oxidized ATP
PAF	platelet activating factor
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PDC	plasmacytoid dendritic cell

PECAM-1	platelet endothelial cell adhesion molecule-1
PEG	polyethylene glycol
PG	prostaglandin
PHA	phytohemagglutinin
PKA	protein kinase A
PLA_2	phospholipase A_2
PLD	phospholipase D
PMA	phorbol myristate acetate
PMN	polymorphonuclear neutrophil
PRR	pattern-recognition receptor
PS	penicillin streptomycin
RA	rheumatoid arthritis
RNS	reactive nitrogen species
ROS	reactive oxygen species
R-PE	recombinant-phycoerythrin
RT-PCR	real-time transcription-polymerase chain reaction
SCID	severe combined immunodeficiency
SP	sodium pyruvate
TCR	T cell receptor
TGFβ	transforming growth factor-β
Th cell	T helper cell
TLR	Toll-like receptor
TNFα	tumor necrosis factor-α
TSP-1	thrombospondin-1
Tub	tubercin
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VLA4	very late antigen-4

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Martijn Jan Leo Bours was born on July 31 1975 in Weert, The Netherlands. After obtaining his secondary school diploma (VWO) in 1994 at the Philips van Horne Scholengemeenschap in Weert, he studied Physiotherapy at the Hogeschool Zuyd in Heerlen. After his graduation, he worked for a short period of time as a physiotherapist at the Division of Rehabilitation in the Atrium Medical Center in Heerlen. In 1999, he went to Maastricht to study Health Sciences at Maastricht University, from which he graduated in 2001.

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