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Pro-inflammatory role of P2Y$_6$ receptor signalling
during vascular inflammation

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To my parents Helga and Dr. Rainer Stenz,
to my sisters Carolin Franziska and Kerstin Julia Stenz
to Jens-Jochen Riegel and
Margit, Adam, Max, Christel, Dagmar and Martin,
to my grandparents and to my family-in-law :-)

Abbreviations

1321 N1 astrocytoma cell line	Human glial cell line from brain astrocytoma, naturally lacking any known P2 receptor
A1	Adenosine receptor type 1
A2a	Adenosine receptor type 2a
A2b	Adenosine receptor type 2b
A3	Adenosine receptor type 3
AC	Adenylate cyclase
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumine
Ca^{2+}	Calcium
cAMP	Cyclic Adenosine Monophosphate
CD39	Ecto-Apyrase
CD73	Ecto-5'-nucleotidase
CDP	Cytidine diphosphate
COX-2	Cyclooxygenase 2
CTP	Cytidine triphosphate
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl sulfoxide
ERK 1/2	Extracellular signal-regulated kinases 1 and 2 = specific subset of MAPK
GDP	Guanosine diphosphate
G-protein	Guanine-nucleotide binding protein
$G_{\alpha q}$	α-subunit of heterotrimeric G_q-proteins
G_i	Heterotrimeric G-protein that inhibits the production of cAMP from ATP.
$G_{q/11}$	Family of heterotrimeric G-proteins that stimulate the membrane-bound PLC-ß and increase the intracellular

	concentration of IP_3 and cAMP.
G_s	Heterotrimeric G-protein that stimulates the production of cAMP from ATP and activates the AC.
GRK	G-protein coupled receptor protein kinase
GTP	Guanosine triphosphate
$[H^3]$	Tritium, a radioactive form of heavy hydrogen
HPLC	High performance liquid chromatography
IDP	Inosine diphosphate
IL	Interleukin
IP_3	Inositol-Triphosphate
ITP	Inosine Triphosphate
MAPK	Mitogen-activated protein kinase
mRNA	Messenger Ribonucleic Acid
NDP	Nucleoside diphosphate
NF-κB	Nuclear factor kappa B
NTP	Nucleoside triphosphate
P2	Purinergic nucleotide receptor
P2X	Purinergic ionchannel receptor
P2Y	Purinergic / pyrimidinergic G-protein coupled receptor
PBS	Phosphat Buffered Saline
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A
PKC	Phosphokinase C
PLC-ß	Phospholipase C beta
PMN	Polymorphonuclear leukocytes
P_i	Inorganic phosphate
PP_i	Pyrophosphate
RIPA	Radio-Immuno Precipitation Assay
RNA	Ribonuclein Acid
SDS	Sodium dodecyl sulfate
RT-PCR	Real-Time Polymerase Chain Reaction
TBS	Tris Buffered Saline

TRIS	Tris(hydroxymethyl)aminomethane
UDP	Uridine Diphosphate
UMP	Uridine Monophosphate
UTP	Uridine Triphosphate

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1. Introduction

During adverse conditions such as inflammation or ischemia, multiple cell types release nucleotides and activation of extracellular nucleotide receptors has been implicated in vascular inflammation.

1.1. Extracellular nucleoside and nucleotide receptors

Signalling by extracellular nucleosides and nucleotides was first described by Drury and Szent-Györgyi in 1929, when they discovered that intravenous administered adenosine and adenylic acid slowed down the heart rate, circumvented signal transduction from atrium to the ventricles and led to a general arterial dilatation including the coronary vessels [1]. In 1944 Buchthal and Folkow discovered, that not only extracellular adenosine and adenylic acid, but also extracellular ATP functions as a signalling molecule by causing tetanic contractions in bundles of muscle fibres [2]. After several studies of different authors [3], which established the role of ATP as an extracellular signalling molecule, Geoffrey Burnstock introduced the still today used classification for extracellular nucleoside and nucleotide receptors in 1978 [4]. Because of the purinergic ligands adenosine, ADP and ATP, the receptors were classified as P-receptors. Furthermore, Burnstock subdivided the receptors into

- P1-receptors with the ligand adenosine and

- P2-receptors with the ligands ADP and ATP.
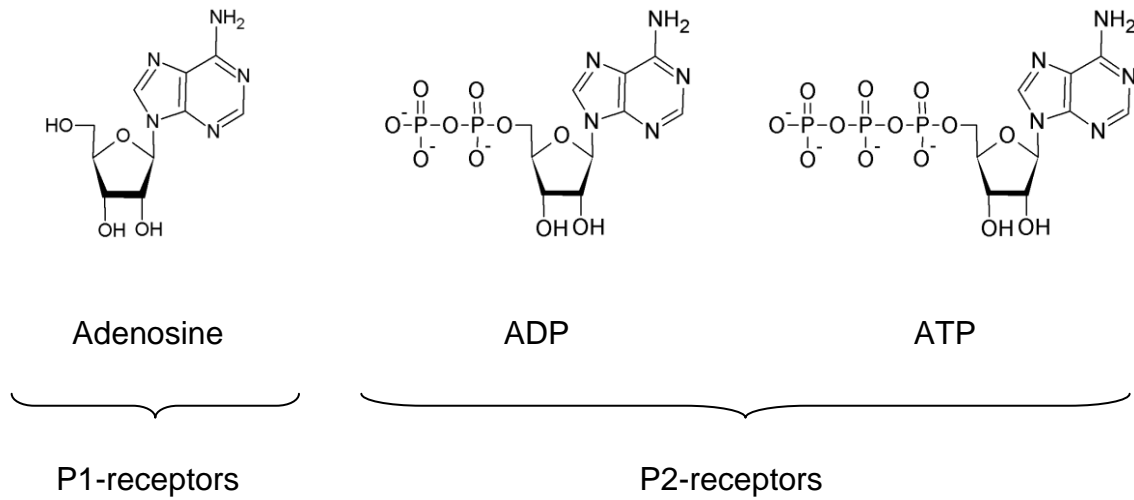
Illustration 1: *Classification of P1- and P2-receptors [4]*

In 1985, Burnstock and Kennedy suggested, that the ATP sensitive P2 receptors consist of two groups which could be further subdivided [5]. They discovered, that there are two groups of receptors with two different rank orders of agonist potencies, which led to the classification of P2X and P2Y receptors [5].

Additional studies revealed, that the P2 receptors not only differed in agonist rank orders, but also in protein structure and function. Abracchio and Burnstock specified in 1994, that P2X-receptors are ion-channels and P2Y-receptors are G-protein coupled receptors [6].

ionchannels   G-protein coupled receptors

ionotropic P2X-receptors   metabotropic P2Y-receptors
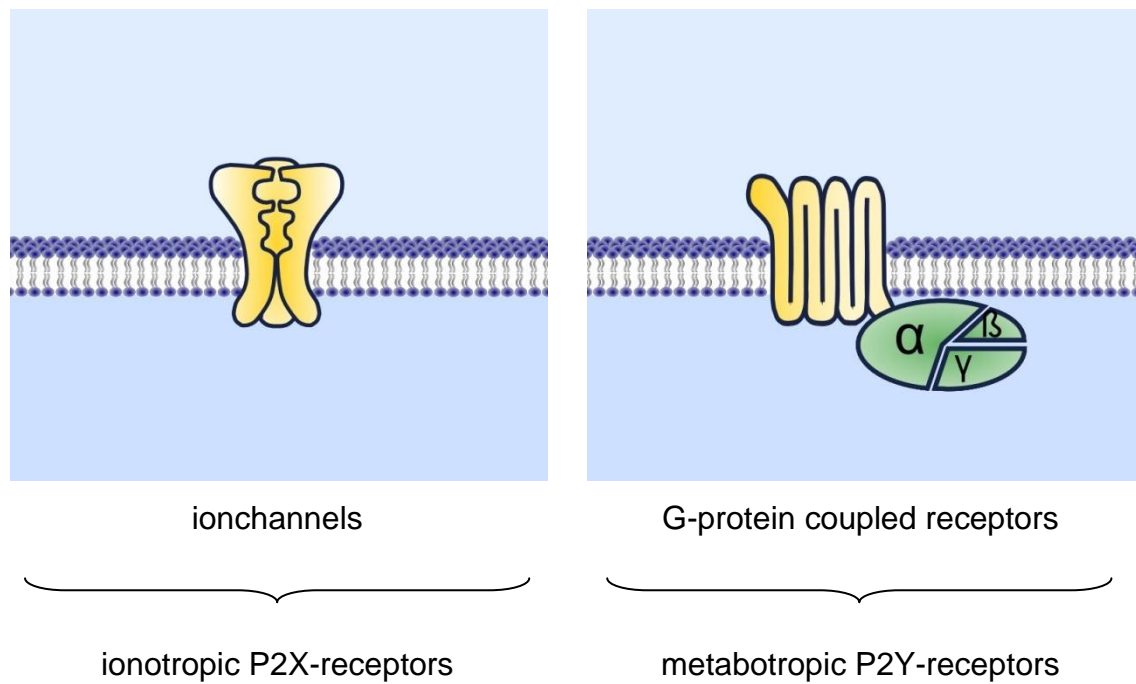
Illustration 2: *Classification of P2X- and P2Y-receptors* [6]

Today, four human P1-receptors, seven human P2X- and eight human P2Y-receptors are known: $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$. The missing numbers of the P2Y-receptors represent either non-mammalian orthologues or receptors which are under investigation, so far without convincing results for a responsiveness to nucleotides [7].

Table 1: *Nucleoside and nucleotide receptors*

P2-receptors		
P1-receptors (adenosine)	**P2X-receptors (ATP (>ADP))**	**P2Y-receptors (NTP, NDP)**
A1	$P2X_1$	$P2Y_1$
A2a	$P2X_2$	$P2Y_2$
A2b	$P2X_3$	$P2Y_4$
A3	$P2X_4$	$P2Y_6$
	$P2X_5$	$P2Y_{11}$
	$P2X_6$	$P2Y_{12}$
	$P2X_7$	$P2Y_{13}$
		$P2Y_{14}$
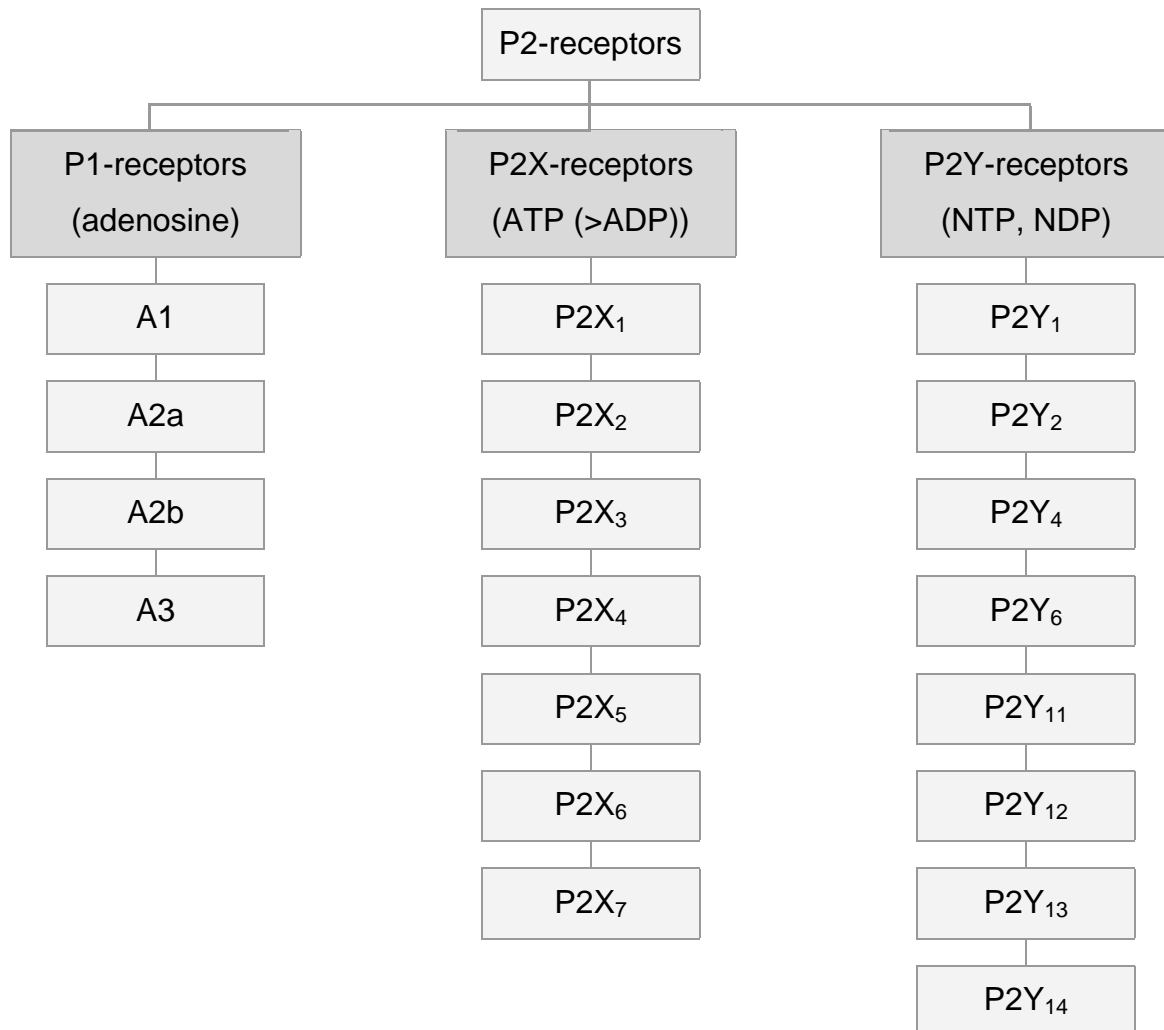
Further research revealed, that not only adenosine and adenosine phosphates, but also uridine, and to a lesser extent guanine, thymine and cytosine molecules participate in nucleotide signalling [8-19]. However, other nucleotides than ATP act solely on P2Y-receptors.

1.2.   P1 receptors

In 1989, the first two P1-receptors were identified [20]. Until today, four P1 receptors are known: the A1-, A2a-, A2b- and A3-receptor. All four receptors are seven-transmembran domain and G-protein coupled receptors. The A1- and A3-receptor couple to inhibitory G-proteins, which diminish the intracellular cAMP concentration after receptor stimulation [21], whereas the A2a- and A2b-receptor couple to stimulatory G-proteins, which induce an intracellular rise in cAMP concentration [21].

Table 2: *Natural ligands of P2X receptors and mechanisms of action*

Name	Natural occurring ligand	Mechanism of action
A1	Adenosine	G_i / Adenylate cyclase → cAMP ↓ [22]
A2a	Adenosine	G_s / Adenylate cyclase → cAMP ↑ [22]
A2b	Adenosine	G_s / Adenylate cyclase → cAMP ↑ [22]
A3	Adenosine	G_i / Adenylate cyclase → cAMP ↓ [22]

P1-receptors are known today for mediating the negative chronotropic and dromotropic effects of adenosine to the heart (A1) [23] influencing brain function (A1) [24-27], myocardial perfusion (A2a), drug addiction (A2a) [28-29] , supporting inflammatory responses of diverse cell types (A2b) [30-32], preventing ischemic cell damage (A1-3) [33-34] and much else.

1.3.   P2X receptors

Extracellular P2X-receptors are ligand gated cation permeable ion-channels which allow the passage of cations into and out of the cell. Therefore, they are also called ionotropic receptors. The naturally occurring agonist of all of the P2X-receptors is ATP [22] and to a lesser extent for some of the P2X-receptors also ADP.

The first cDNA of a P2X receptor was isolated in 1994. Today, seven types of P2X-receptors are known (P2X$_1$ - P2X$_7$).

Table 3: *Natural ligands of P2X receptors and mechanisms of action*

Name	Natural occurring ligand	Mechanism of action
P2X$_1$	ATP >> ADP	Na$^+$, K$^+$, Ca^{2+} [22]
P2X$_2$	ATP	Na$^+$, K$^+$ [22]
P2X$_3$	ATP	Na$^+$, K$^+$, Ca^{2+} [22]
P2X$_4$	ATP	Na$^+$, K$^+$ [22]
P2X$_5$	ATP > ADP	Na$^+$, K$^+$, Ca^{2+} [22]
P2X$_6$	ATP > ADP	Does not function as homomultimer [22]
P2X$_7$	ATP	Na$^+$, K$^+$ [22]

The P2X-receptors are thought to be composed of three subunits [22,35], which make them different from other tetrameric and pentameric ion-channel families [35]. A trimeric ion pore can either be formed of one type of P2X-receptor (homomeric), or can consist of different P2X-receptors (heteromeric) [22]. Heteromeric receptor trimers were found for P2X$_{2/3}$, P2X$_{1/2}$, P2X$_{1/5}$, P2X$_{2/6}$,

$P2X_{4/6}$ and $P2X_{1/4}$. $P2X_7$-receptors only occur as homomeric receptors. In contrast, for the $P2X_6$-receptor no homomeric receptor was identified.
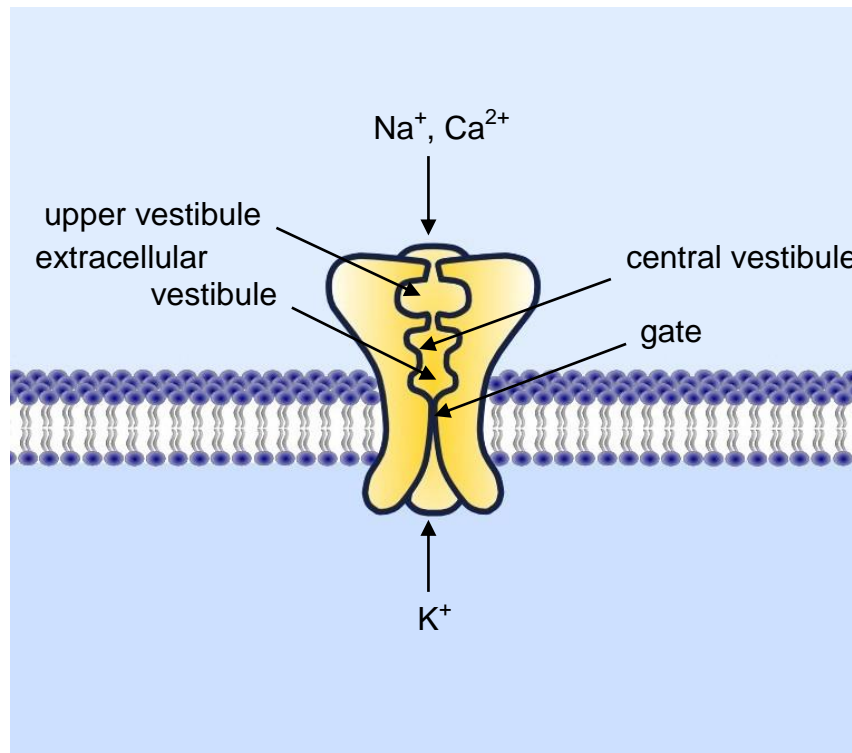
Illustration 3: *Simplified structure of a trimeric homomeric $P2X_4$-receptor Adapted from Silberberg et al.[35] and Kawate et al.[36]*

Activation of P2X-receptors leads to membrane depolarization and an increase of intracellular Ca^{2+} because of a fast Na^+ and Ca^{2+} influx and a K^+ efflux [37]. The increased intracellular Ca^{2+} in turn activates mitogen-activated protein kinases [38].

Diverse functions of P2X-receptors like increase of blood pressure [39] and nociceptive sensory processing in chronic inflammatory and neuropathic pain [40-41] are known. In addition different functions of heteromeric P2X-receptors can be suggested because of different pharmacological properties like the EC_{50}-value [42].

1.4.   P2Y receptors

P2Y-receptors are G-protein coupled receptors with 7 transmembrane regions [6,8]. Because of their coupling to G-proteins, they are also called metabotropic receptors in contrast to the ionotropic P2X receptors (ionchannels). Unlike P2X-receptors, the P2Y-receptors have more diverse ligands than ATP. They are not only activated by purines like ATP, but also by pyrimidines.

__Table 4:__ Natural ligands of P2Y receptors

Name	Natural occurring ligand
$P2Y_1$	ADP > ATP [8,18]
$P2Y_2$	ATP = UTP [8,13,15]
$P2Y_4$	UTP > UDP > ITP > ATP [10]
$P2Y_6$	UDP > UTP > ADP >> ATP [11]
	UDP > TDP > IDP > GDP > ADP >> CDP [16]
$P2Y_{11}$	ATP [43]
$P2Y_{12}$	ADP [14,44]
$P2Y_{13}$	ADP >ATP > IDP [19]
$P2Y_{14}$	UDP glucose > UDP galactose [9]

The first P2Y receptor was cloned in 1993 [45-46]. After this breakthrough, several other P2Y receptors soon were identified and cloned.

Cloned recombinant P2Y-receptors mainly couple to G_q-proteins, which activate PLC-ß and increase intracellular Ca^{2+}. In contrast to this narrow range of action, endogenous P2Y-receptors show a much expanded range of actions (PLD, AC,

MEK/MAP kinase system, Rho-dependent kinase). The reason for these differences might possibly be related to the limited effector targets in the used expression systems.

Table 5: *P2Y-receptor signalling [21]*

Name	Main ligand	Signalling
$P2Y_1$	ADP	$G_q/G_{11} \rightarrow$ PLC-ß; $IP_3\uparrow$; $Ca^{2+}\uparrow$ and PKC [21]
$P2Y_2$	ATP = UTP [47]	$G_q/G_{11} \rightarrow$ PLC-ß; $IP_3\uparrow$; $Ca^{2+}\uparrow$ and PKC [48-49]
$P2Y_4$	UTP [47]	$G_q/G_{11} \rightarrow$ PLC-ß; $IP_3\uparrow$; $Ca^{2+}\uparrow$ and PKC [50]
$P2Y_6$	UDP [47]	$G_q/G_{11} \rightarrow$ PLC-ß; $IP_3\uparrow$; $Ca^{2+}\uparrow$ and PKC [21]
		G12/13 $\rightarrow$ AC, cAMP$\uparrow$ [51]
$P2Y_{11}$	ATP	$G_q/G_{11} \rightarrow$ PLC-ß; $IP_3\uparrow$; $Ca^{2+}\uparrow$ and PKC [52]
		$G_s \rightarrow$ cAMP $\uparrow$ [52]
$P2Y_{12}$	ADP	$G_{i/o} \rightarrow$ cAMP$\downarrow$ [53-55]
$P2Y_{13}$	ADP	$G_{i/o} \rightarrow$ cAMP$\downarrow$ [56]
$P2Y_{14}$	UDP glucose	G_q/G_{11} [22]; $G_{i/o} \rightarrow$ cAMP$\downarrow$ [9,57]

In the last years, the interest in compounds acting at P2Y-receptors increased because of their implication in a wide range of diseases [58].

The **$P2Y_1$-receptor** is known to be important in platelet aggregation and glucose homeostasis. A lack of $P2Y_1$-receptor expression leads to a slightly prolonged bleeding time, whereas an overexpression leads to a shortened bleeding time and a rise in sensitivity to platelet aggregation [59-60]. Moreover, elevated blood glucose levels and body weight are observed in mice lacking the $P2Y_1$-receptor [61].

The **P2Y$_2$-receptor** is responsible for Cl$^-$ secretion and inhibition of Na$^+$ uptake in epithelial cells. Therefore, P2Y$_2$-receptor activation by an agonist was suspected to be helpful in the treatment of cystic fibrosis, which is caused by an defect of the Cl$^-$-channel CFTR and a subsequent trapping of Cl$^-$ in the cells. Phase III studies could show, that the P2Y2-receptor agonist Denufosol® (dCp$_4$U; INS37217) improves lung function in CF patients [62-63]. An additional Phase III study is planned [64]. Moreover, P2Y$_2$-receptor agonists are frequently used in the treatment of dry eye disease because of their mediated increase in ion fluxes.

The **P2Y$_4$-receptor** is implicated in the function of the inner ear. It is suggested, that nucleotides are important in noise adaptation of the cochlea [65-67]. In addition, the P2Y4-receptor is important in intestinal Cl$^-$-secretion [68].

The **P2Y$_6$-receptor** is - like the P2Y$_4$-receptor - also important in Cl$^-$-secretion in diverse epithelial cells [69-70]. Furthermore the P2Y$_6$-receptor is implicated in multiple functions in the innate immune system [71-72]. There might also exist a function in chronic inflammatory diseases like inflammatory bowel disease [73].

The **P2Y$_{11}$-receptor** also plays a role in immune function [74-75], as well as it exhibits secretory functions in pancreas [76].

As the P2Y$_1$-receptor, the **P2Y$_{12}$-receptor** is important in platelet function. It plays a role in fibrinogen receptor activation, dense granule secretion and thrombus formation [61,77]. The most famous drug acting at P2-receptor is the P2Y$_{12}$-receptor antagonist Clopidogrel (Plavix®), which is used in the prevention and treatment of thrombosis and stroke [78]. According to the the IMS (Institute for Healthcare in Informatics, information as current as april 7, 2011), Clopidogrel is the third best selling drug in the US since several years. From the follow-up drugs Prasugrel [79], Cangrelor and Ticagrelor (Brilinta®), the latter showed the most promising study results. In the New England Journal of Medicine a significant decrease in mortality and myocardial infarction without an

increase of overall major bleedings was reported recently [80]. Also because of gene polymorphisms, which circumvent the action of Clopidogrel®, alternative drugs have long been awaited.

With a number of search results of 34 in pubmed in april 2011, it can be stated, that the **P2Y$_{13}$-receptor** is barely examined. One study revealed a role of P2Y$_{13}$-receptor in lipoprotein metabolism, more precisely HDL endocytosis [81].

Also barely examined, the **P2Y$_{14}$-receptor** is assumed to play a role in neuroimmune functions [57] and chemotaxis [82].

1.5.   P2Y$_6$-receptor

The P2Y$_6$-receptor is a G-protein coupled receptor with the major ligand UDP [11,47]. The existence of a receptor specifically recognizing UDP was primarily hypothesized in 1994 [83]. One year later, Chang et al. succeeded in cloning this receptor [84].

1.5.1.   Tissue distribution

Evidence for P2Y$_6$-receptor expression can be found in various tissues and cell lines. Already in 1997 was assumed, that the widespread distribution might indicate physiological importance of the receptor [69].

Table 6: *P2Y$_6$-receptor expression*

Technique	Tissue / Cell line
Nothern Blot	Human tissue: Spleen [11], placenta [11,85], thymus [11], intestine [11], blood leukocytes [11] Human cell lines: A549 [85], 16HBE14o- [85], 1HAEo- [85]
Southern Blot	Human isolated cells: Polymorphonuclear cells [86], monocytes [86], lymphocytes [86], human umbilical vein endothelial cells [86]

Human cell culture cell lines:

promyelocytic HL-60 [86], megakaryocytic Dami cells [86]

RT-PCR   Human tissue:

Spleen [87], placenta [87], kidney [87], lung [87], intestine [87], adipose [87], bone [87], heart, some brain regions [87], cardiomyocytes [88], pancreas [89]

Human cell lines:

Retinal pigment epithelium (ARPE-19) [90], nasal epithelial cells (HNEC) [62], monocytic THP-1 cells [71], human pancreatic duct cell line PANC-1 [91], human pancreatic duct cell line CFPAC-1 [91], human pancreatic duct cell line Capan-1 [92], bronchial surface epithelial cell line 16HBE14o- [93]

Murine tissue / cell lines:

Colonic tissue [73], J774 macrophages [94-95], peritoneal macrophages of BALB/c mice [95], cardiomyocytes [88], whole pancreatic islets [96], pancreatic ß-cells [96]

Rat tissue / cell lines:

Chondrocytes [97], aortic vascular smooth muscle cell (VSMC) [98], microglia [72], C6-2B glioma [47]

Rabbit tissue / cell lines:

Osteoclasts [99]

Western   Human tissue / cell lines:

Cardiomyocytes [88]

Murine tissue / cell lines:

Skeletal myoblast cell line C2C12 [58],

Rat tissue / cell lines:

Microglia [72]

Immunohisto-chemistry Human tissue / cell lines:

Cardiomyocytes [88]

Keratinocytes, (peripheral) neurons and U937 cells exhibit mRNA of $P2Y_6$ receptor, but have been shown to lack functional $P2Y_6$-receptor responses [100-105]. Also in the two different human airway epithelial cell lines CF/T43 and IB3-1, UDP do not lead to an increase of IP_3 after stimulation [106-107]. Bronchi of cotswold pigs also didn't show an increase in bronchial ion transport after stimulation with UDP [108].

1.5.2. Agonists and antagonists

Further research on the $P2Y_6$-receptor was longtime impaired by the lack of specific and stable $P2Y_6$-receptor agonists and antagonists. In the last years, some substances have been developed. Unfortunately they still exhibit problems in solubility and stability.

Table 7: *$P2Y_6$-agonists*

Ligand	Action	pEC$_{50}$	EC$_{50}$	Selectivity	Reference
MRS2782	Full agonist	6,18 =	0,794µM		[109]
UDP	Full agonist	6,50 =	0,316µM (species not	No	[11]

				known)		
				0,013µM		[110]
				0,190µM (rat receptor)		[47]
				0,015µM		[111]
				0,047µM		[112]
				0,086µM		[113]
Up3U	Full agonist	6,69	=	0,204µM		[114]
				0,200µM		[15]
INS48823	Full agonist	6,90	=	0,126µM		[114]
3-phenacyl-UDP	Full agonist	7,20	=	0,063µM	Yes	[115]
UDP-ß-S	Full agonist	7,55	=	0,028µM	Yes	[110,114]
MRS2693	Full agonist	7,83	=	0,015µM	Yes	[58,110]
2MeSATP	Partial agonist	4,00	=	100,0µM	No	[11,47]
CDP	Partial agonist			88,00µM	No	[110]
ADP	Partial agonist	4,50	=	31,62µM	No	[11]
				65,00µM		[110]
GDP	Partial agonist			45,00µM	No	[110]
IDP	Partial agonist			34,00µM	No	[110]
UTP	Partial agonist	5,20	=	6,310µM	No	[11]
5BrUTP	Partial agonist	6,10	=	0,794µM	No	[11]
				0,130µM		[47]

Table 8: *P2Y$_6$-antagonists*

Ligand	Action	pIC$_{50}$	IC$_{50}$	Selectivity	Reference
Reactive blue 2	Antagonist	4,5-0,0	31,62µM – 1M	No	[114]
MRS2567	Antagonist	6,9-7,0	0,100µM – 0,126µM		[116]
MRS2578	Insurmountable, non-competitive antagonist	7,0-7,4	0,040µM – 0,100µM	Yes	[116]
cysLT1 antagonists	Antagonist	<6,0	< 1,0µM	No	[117]

1.5.3.   Cell signalling

By definition, the natural agonist with the lowest EC$_{50}$-value is suspected to be the main ligand of the receptor. As the only P2Y-receptor, whose EC$_{50}$-value for UDP is lower than for other nucleotides, the P2Y$_6$-receptor is assumed to be the only UDP receptor [113].

1.5.3.1. G$_{q/11}$, PLC, IP$_3$, DAG, Ca^{2+}, PKC and ERK 1/2

Because of the complete resistance of the P2Y$_6$-receptor signalling to inhibition by pertussis toxin, a coupling to G$_{q/11}$-proteins was suggested early in 1997 [16,118]. However, not until 11 years later, the coupling to G$_{q/11}$-proteins could be confirmed through G$_{\alpha q}$-deficient mice by Roach et al. [119].

In these mice, lacking the α-subunit of G_q-proteins, the downstream receptor response of intracellular calcium increase was completely lost after $P2Y_6$-receptor stimulation [119].

$G_{q/11}$-protein coupled receptors are known to activate the phospholipase C beta (PLC-ß) [119]. Of the four identified PLC isoforms [120], activation of PLC-ß3 and PLC-ß4 after stimulation with UDP has been confirmed in a study by Roach et al., whereas PLC-ß2 activation was not verifiable [119]. PLC-ß1 was not expressed in the cell lines which were tested, so that no conclusion about a participation of PLC-ß1 can be made [119].

The PLC-ß cleaves phosphatidylinositol-4,5-bisphosphate (PIP$_2$) into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP$_3$). IP$_3$ acts at the endoplasmatic reticulum and leads to an increase of intracellular Ca^{2+}.
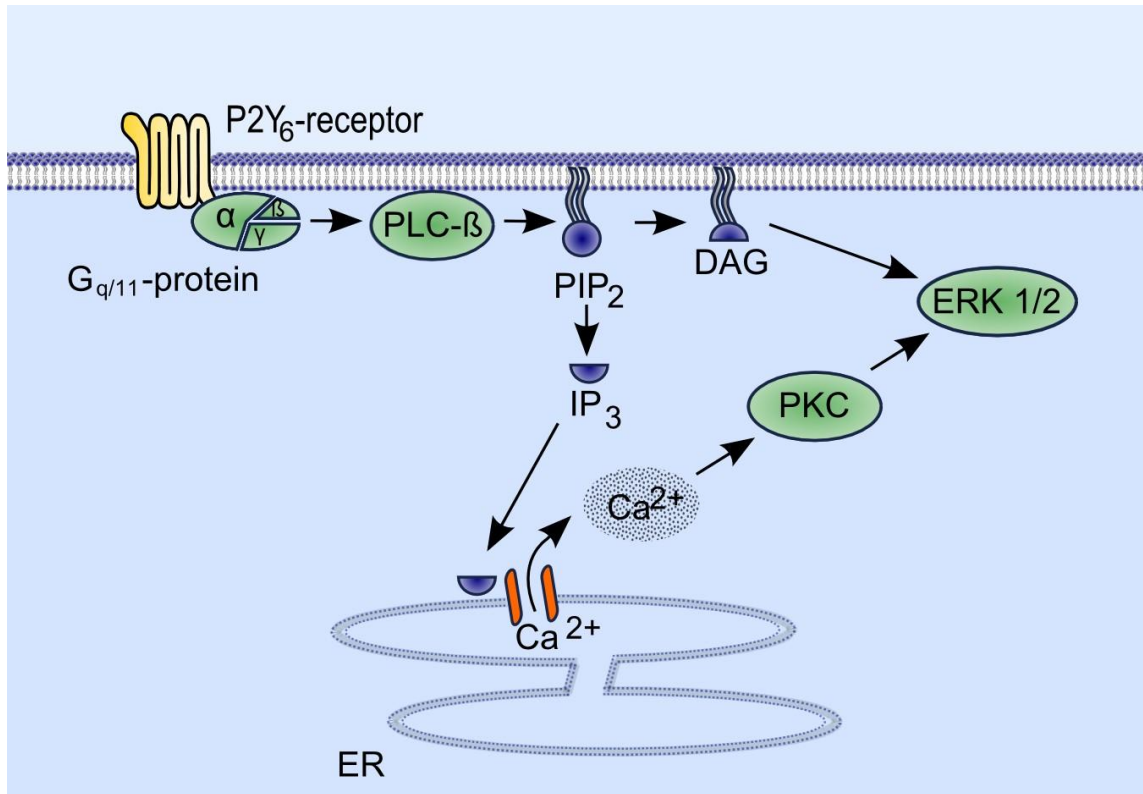
Illustration 4: *P2Y$_6$-receptor signal transduction through G$_q$-proteins.*

An increase of intracellular IP$_3$ after stimulation with UDP was observed in multiple studies [16,69,95]. After stimulation with UDP, the intracellular IP$_3$ concentration increases rapidly and reaches its maximal level only after 15-30 min. Thereafter, the level of intracellular IP$_3$ decreases slowly but remains elevated for at least 1h [16]. If the agonist UDP is removed, the level of intracellular IP$_3$ returns almost to control level, indicating that the continuous presence of the agonist is needed to maintain the IP$_3$ elevation.
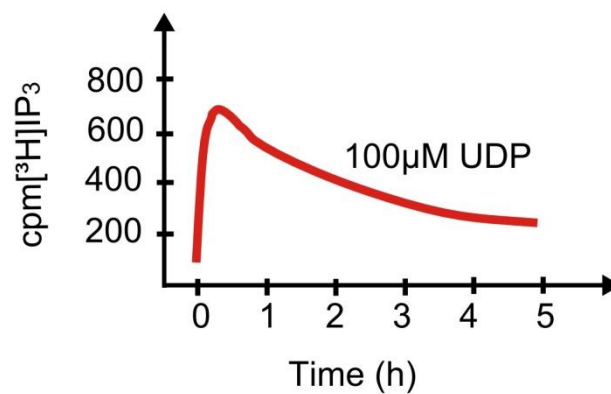
Illustration 5: _Increase of intracellular IP_3 following $P2Y_6$-receptor stimulation with UDP._
Adapted from Robaye et al. [16]

Intracellular levels of free <u>calcium</u> (Ca^{2+}) are responsible for the regulation of multiple intracellular processes. Levels of intracellular calcium are therefore accurately regulated [121-123]. Meanwhile it is known, that the duration and the level of calcium increase after stimulation of a G-protein coupled receptor influence important cellular responses like transcription, apoptosis, endocytosis and metabolism [95,123]. Both, the naturally occurring $P2Y_6$-receptor UDP and the synthetic $P2Y_6$-receptor agonist INS48823 are known to induce a transient elevation of intracellular Ca^{2+} [72,95,97,99,102,119].

Ca^{2+} and DAG induce the activation of protein kinase C (PKC). PKC is not a single enzyme, but consists of a family of at least 10 isoforms of serine/threonine kinases [124].

Inhibition of protein kinase C inhibits UDP-induced phosphorylation of ERK 1/2 (in rat chondrocytes [97]). Based on those studies and studies with the PKCδ-inhibitor rottlerin, Kudirka et al suggest, that the PKC isoform PKCδ is responsible for the UDP-induced phosphorylation of ERK 1/2 [97]. Mamedova et al. studied the isoforms PKC-α, PKC-β and PKC-θ after stimulation with the $P2Y_6$-receptor agonist MRS2693 and could not show a corresponding activation

of these three isoforms to the curve progression of ERK 1/2 phosphorylation [58]. Therefore it seems most likely, that the PKC isoform δ is responsible for P2Y$_6$-receptor stimulation induced ERK 1/2 phosphorylation.

The extracellular signal-regulated kinases 1 and 2 = <u>ERK 1/2</u> are activated in response to growth factors and phorbol esters and regulate the proliferation and differentiation of cells. In several studies it is demonstrated that stimulation of cells with UDP increases ERK 1/2 phosphorylation [71,73,97,119]. In a recent study, ERK 1/2 phosphorylation was confirmed also after stimulation with the specific P2Y$_6$-receptor agonist MRS2693 [58]. In addition, Grbic et al. could show, that the specific P2Y$_6$-receptor antagonist is able to inhibit UDP-induced ERK 1/2 phosphorylation [73].

1.5.3.2. G$_{12/13}$, AC, cAMP, PKA and Rho

Already in 1999, a coupling of the P2Y$_6$-receptor to more than one type of G-protein was suspected [125]. It was also noticed, that there was a difference in signalling in cloned and endogenous P2Y-receptors with the endogenous receptors showing a much expanded range of used signalling pathways. It was hypothesized, that this might be due to the limited effector targets in the used expression systems of cloned P2Y-receptors.

It was not until 2008, that the second type of G-protein was identified. Nishida et al. could show, that the P2Y$_6$-receptor also couples to <u>G$_{12/13}$-proteins</u> [51], which activate other downstream targets than G$_{q/11}$-proteins. With their α-subunit, the G$_{12/13}$-proteins activate the membrane bound enzyme <u>adenylate cyclase</u> (AC), which converts ATP into <u>cAMP</u>. The increased level of intracellular cAMP in turn activates the cAMP dependent <u>protein kinase A</u> (PKA).
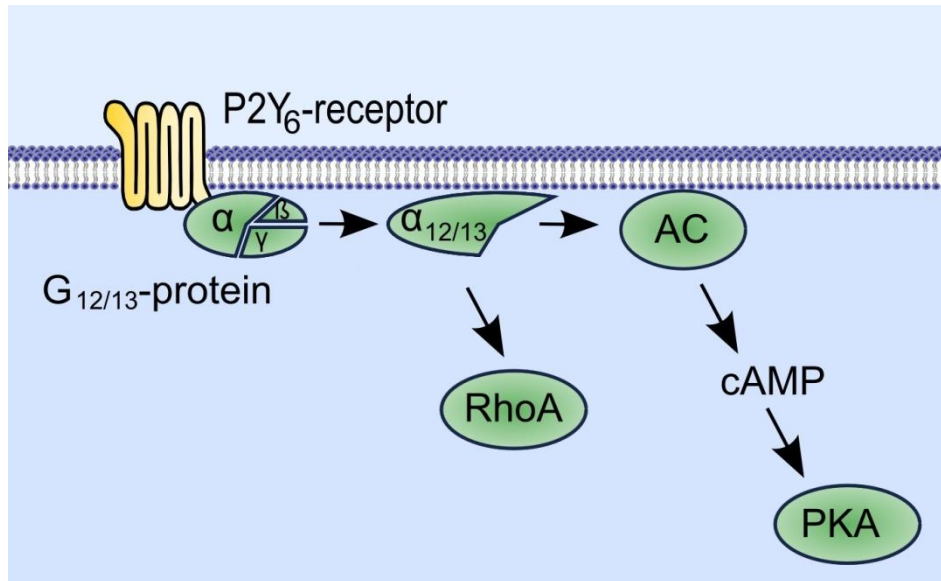
Illustration 6: *P2Y$_6$-receptor signal transduction through G$_{12/13}$-proteins*

To verify the activity of the G$_{\alpha 12/13}$ subunit, <u>Rho</u>-proteins are a very good indicator [126]. Rho-proteins are small GTP-binding proteins, which consist of a family of 23 members in mammals by now [127]. Treatment with extracellular UDP increased Rho-activation, whereas treatment with the specific P2Y$_6$-receptor antagonist MRS2578 suppressed stretch-induced Rho-activation [51].

1.5.3.3. PLD

Phospholipase D inhibition inhibits UDP-induced ERK 1/2 phosphorylation [97], therefore it can be concluded, that PLD is necessary for ERK 1/2 phosphorylation through UDP.

1.5.3.4. PI3K

The $G_{\beta\gamma}$-subunit has the ability to activate the PI3-Kinase [128], whereas the $G_{\alpha q}$-subunit is able to inhibit the PI3-Kinase. Although many G-protein coupled receptors activate the PI3-Kinase [129], UDP doesn't activate the PI3K [119], instead it inhibits the phosphorylation of Akt to phospho-Akt mediated by PI3K [119].
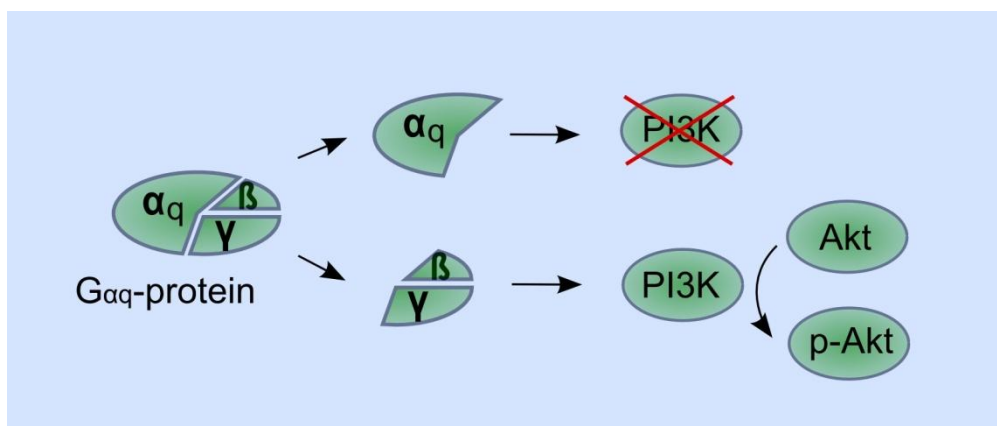

Illustration 7: *$G_{\alpha q}$-subunits inhibit the PI3-Kinase, whereas $G_{\beta\gamma}$-subunits activate the PI3K*

1.5.3.5. NF-κB

The human NF-κB family consists of a family of dimeric transcription factors: RelA/p65, RelB, c-Rel/Rel, p52 (precursor p100) and p50 (precursor P105), which are able to form fifteen different homo- or heterodimeric complexes [130-131].
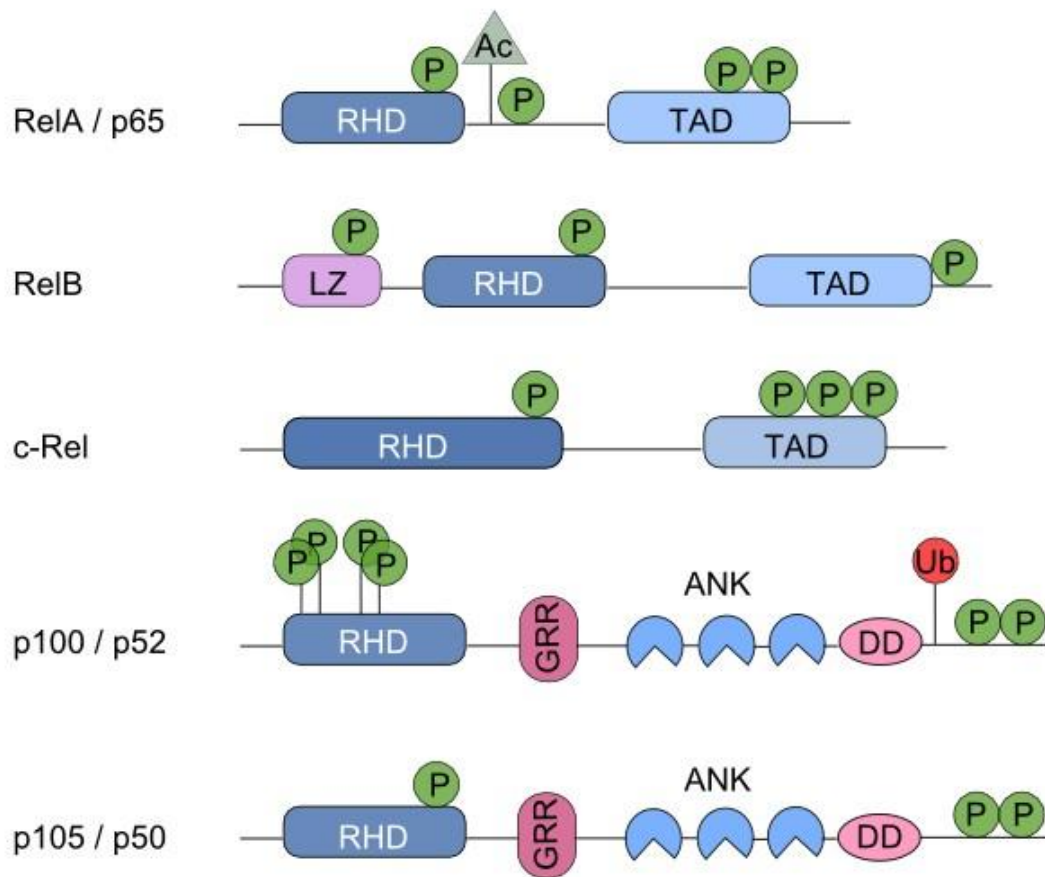
Illustration 8: *NF- κB family members*
Adapted from Vallabhapurapu et al. [131]
RHD = Rel homology domain, TAD = Trans activation domain, LZ = Leuzin zipper motif, GRR = glycine rich region = "hinge", ANK = ankyrin rich repeats. Posttranslational modification sites: Ub = Ubiquination, Ac = Acetylation and P = Phosphorylation. P105/p50 is ubiquitinated at multiple sites. For the benefit of clarity some ANK symbols in one queue were spared

NF-κB is a "rapid-acting" transcription factor, which does not need new protein synthesis to function. This enables NF-κB to act as a "first responder" to detrimental cellular stimuli. Bound to inhibitory proteins (IκBs), inactive NF-κB proteins reside in the cytoplasm. The inhibitory proteins hide the nuclear localization signals of NF-κB proteins and in this way prevent translocation of NF-κB proteins into the nucleus. Phosphorylation and ubiquitination of the inhibitory proteins lead to degradation of the inhibitory proteins in the

proteasome and enable nuclear translocation of NF-κB. In the nucleus, the transcription factor NF-κB regulates the transcription of a variety of genes.

The family of inhibitory kappa B proteins (IκB) consist of three classical IκBs (IκBα, IκBβ and IκBε) as well as some novel members like IκBζ, IκBNS and BCL-3. The C-terminal halves of p100 and p105 also funtion as IκBs because of their IκB-like structure. The p105 C-terminal ankyrin repeat is therefore called IκBγ. It also binds to RelA and c-Rel and keeps them in the cytoplasm [131]. The C-terminus of p100 is called IκBδ.
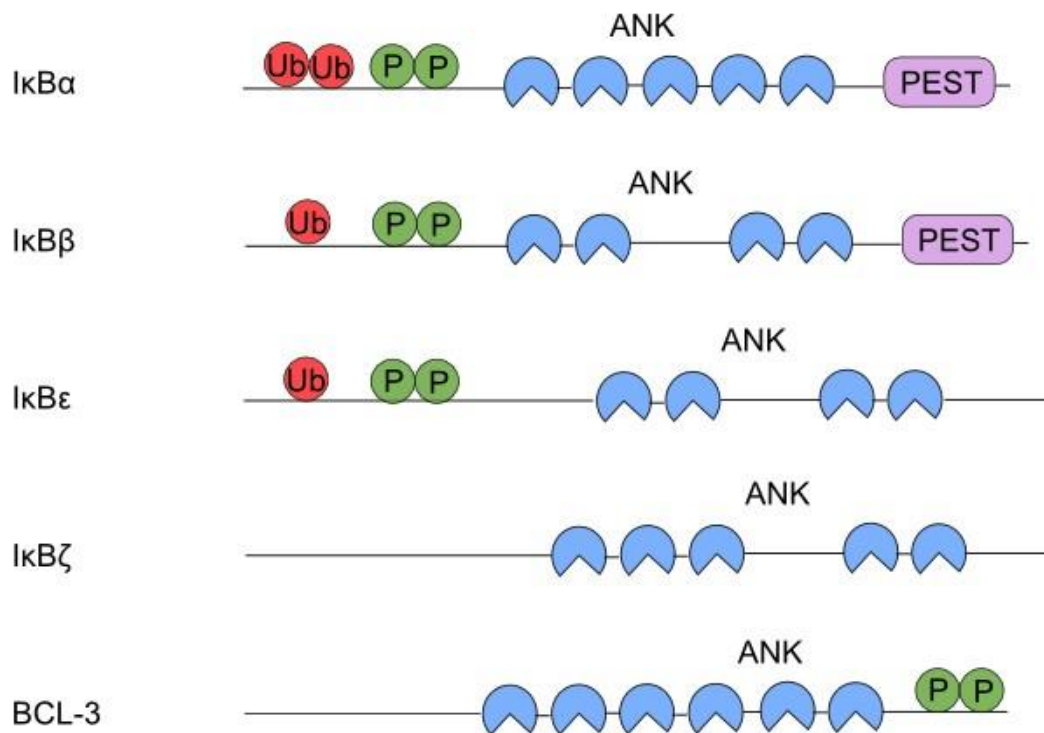

__Illustration 9:__ IκB family members
Adapted from Vallabhapurapu et al. [131]
ANK = ankyrin rich repeats, PEST = proline, glutamic acid (=e), serine and threonine domain. Posttranslational modification sites: Ub = Ubiquination and P = Phosphorylation. For the benefit of clarity some ANK symbols in one queue were spared.

The classical (or "canonical") NF-κB signalling pathway is the major NF-κB activation pathway utilized by most stimuli [131]. It mainly involves the activation of p65/p50 heterodimers and c-Rel/p50 heterodimers [131]. Heterodimers of p65/p50 are simultaneously the most prevalent appearance of NF-κB [132].

In a luciferase reporter assay with a 5'-GGGACTTTCC-3' NF-κB binding site, increased NF-κB activity after stimulation with UDP had been confirmed in a dose dependent manner (in rat vascular smooth muscle cells) [98]. Because the p65 monomer recognizes the 5'-TTCC-3' section, while the p50 binds to the 5'-GGGAC-3' section, this reporter assay indicates that NF-κB p50/p65 heterodimers are activated as downstream targets of UDP stimulation [132-133].

A following study could confirm by immunofluorescence, that UDP induces nuclear translocation of the NF-κB p65 subunit in a time and dose dependent manner [99]. A maximum was observed after 3h of stimulation with 10µM of UDP. The specific agonist INS48823 at the $P2Y_6$-receptor also induces nuclear translocation of NF-κB [99]. Both, UDP and INS48823 induced nuclear translocation of NF-κB were inhibited by SN50, a peptide which interferes with the NF-κB translocation through the nuclear pore [73,99].
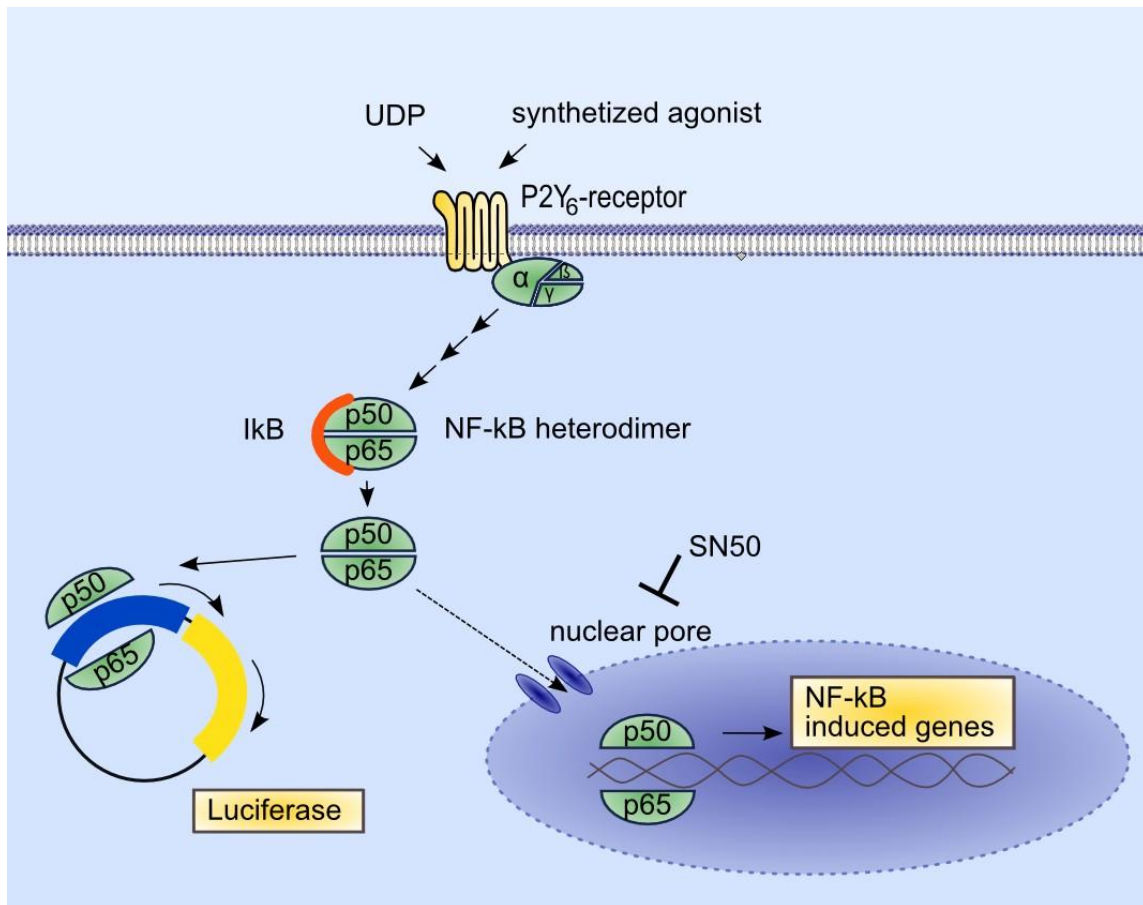
Illustration 10: *UDP induces an increase in NF-κB activity*
UDP induces an increase in NF-κB activity in rat vascular smooth muscle cells
[98] and induces the translocation of NF-κB in the nucleus in rabbit osteoclasts [99].

Although UTP also showed a slight induction of NF-κB reporter activity [98], no induction of nuclear translocation of NF-κB could be shown [98-99]. Only cRel appeared to be slightly enhanced in the nucleus after stimulation with UTP [98]. ATP and ADP revealed no induction of NF-κB reporter activity [98] and ADP also didn't induce a nuclear translocation of NF-κB [99]. This indicates that the response is mediated through a selective UDP receptor like the P2Y$_6$-receptor.

Because of the lack of a specific antagonist, antagonist data confirming the P2Y$_6$-receptor as a mediator of this action were longtime missing. In our studies

we could complement, that inhibition of the P2Y$_6$-receptor antagonist leads to a sustained repression of NF-κB activity.

The "canonical" (or classical) NF-κB signalling pathway comprises proteasome mediated degradation of IκB proteins [99]. If the proteasome degradation is inhibited by lactacystin, the UDP-induced NF-κB activation is omitted [99]. This indicates additionally, that the canonical pathway is involved in UDP-induced NF-κB activation.
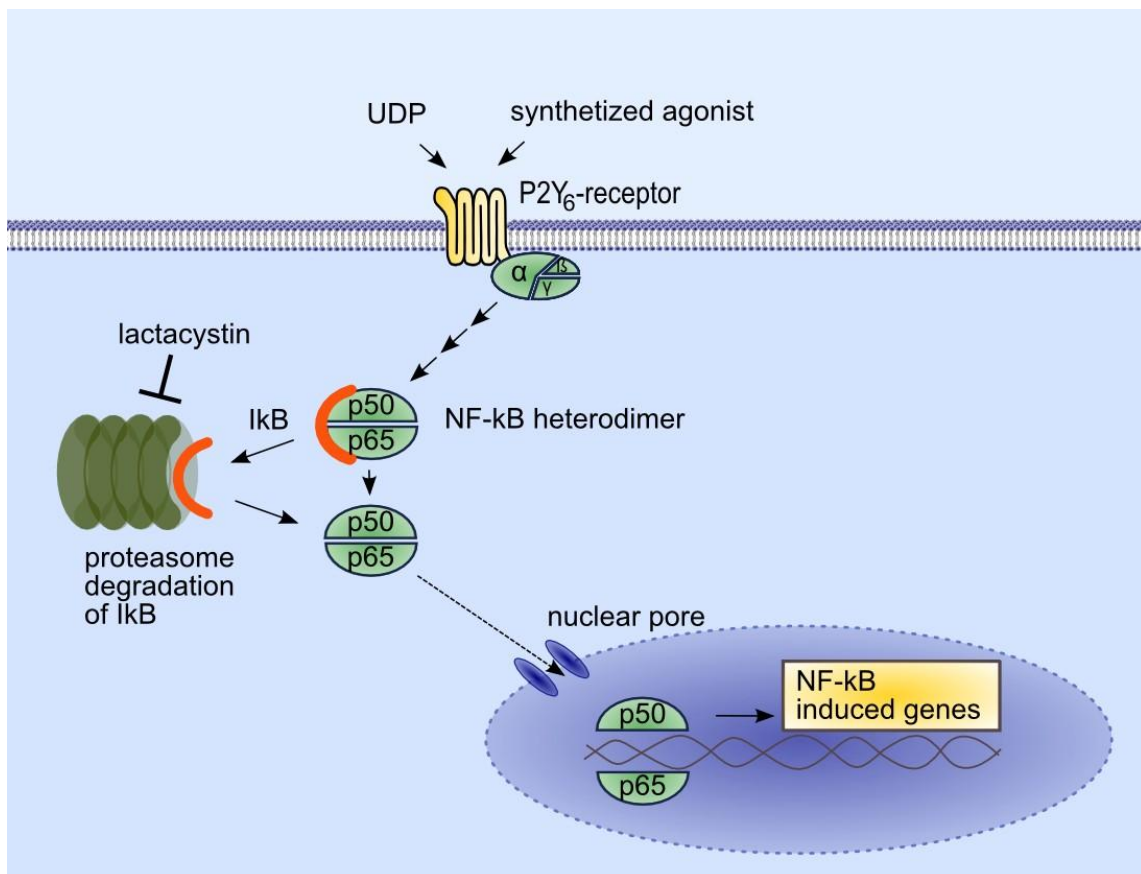

Illustration 11: *Proteasome degradation of IκB*
Proteasome degradation of IκB as well as nuclear translocation through the nuclear pores are necessary for UDP evoked NF-κB activation [99].

1.5.4. Induced genes after P2Y$_6$-receptor stimulation

The identification of genes induced by P2Y$_6$-receptor signalling is still a matter of research. Only for the release of IL-8 through P2Y$_6$-receptor signalling there exist several data from different work groups [71,73,90,102]. In addition, the release of CCL20 after stimulation with UDP or the specific P2Y$_6$-receptor agonist INS48823 has recently been shown to occur from human nasal epithelial cells and human dendritic cells [62].

In a cytokine antibody array, stimulation of Caco-2 cells with 100µM UDP revealed no induction of eotaxin, GM-CSF, IL-1α, IL-1β, IL-3, IL-6, IL-10, IL-17, IP-10, TNF-α, leptin, rantes, MIP-1α, MIP-1β and MIP-5 after 6h of stimulation [73]. The observed induction of IL-4 and the increase of IL-12 release was neither specific to UDP (also UTP evoked induction of IL-4 and the increase of IL-12 release), nor reversible through administration of specific P2Y$_6$-receptor antagonist MRS2578 [73].

In recombinant U973/hP2Y$_6$ cells however, release of TNF-α, IP-10 and MCP-1 was increased after stimulation with UDP [102]. Whereas no induction of IL-1β release was confirmed, although mRNA levels of IL-1β, IP-10 and MCP-1 were substantially increased [102]. In contrast to the increased release of TNF-α, there was no increase of mRNA levels of TNF-α in real-time RT-PCR [102]. More data are necessary to confirm these results and to arrange them in the context of P2Y$_6$-receptor signalling.

1.5.4.1. Induction of IL-8 after P2Y$_6$-receptor stimulation

UDP is known to induce release of IL-8 from monocytes [71,102,134], intestinal epithelial cells [73] and mature dendritic cells [135], as well as U937 stably transfected with P2Y$_6$-receptor [102], and 1321N1 astrocytoma cells stably

transfected with P2Y$_6$-receptor [71,102]. Thereby, the EC$_{50}$ of UDP-induced production of IL-8 ranks about 2-3µM in 1321N1 astrocytoma cells stably transfected with P2Y$_6$-receptor [71,102]. It was shown, that the increase in IL-8 release is regulated at the transcriptional level [73]. In addition, the causal involvement of the P2Y$_6$-receptor was confirmed by experiments with the specific P2Y$_6$-receptor antagonist MRS2578, which could specifically abolish the UDP-induced IL-8 release in human monocytes and in Caco-2 epithelial cells [73,134].

A few different studies analyzed the possible signalling molecules which might be important in IL-8 induction.

- ERK 1/2 seems to be an important mediator, because the ERK 1/2 inhibitor UO126 specifically inhibits UDP induced IL-8 regulation [73].

- Promoter studies of Grbic et al. included a minimal IL-8 promoter which consisted several binding sites for transcription factors: AP-1, NF-IL-6, NF-κB and a TATA box [73].

Grbic et al. could not confirm an influence of p38 MAPK, JNK 1/2, and NF-κB on UDP induced IL-8 induction with different pharmacological inhibitors [73].

1.5.4.2. Pathways shown not to be involved in P2Y$_6$-receptor signal transduction

UDP does not induce phosphorylation of the AMP-activated protein kinase (AMPK) [136]. Also no increase in p58 or JNK 1/2 phosphorylation was shown after UDP stimulation [73].

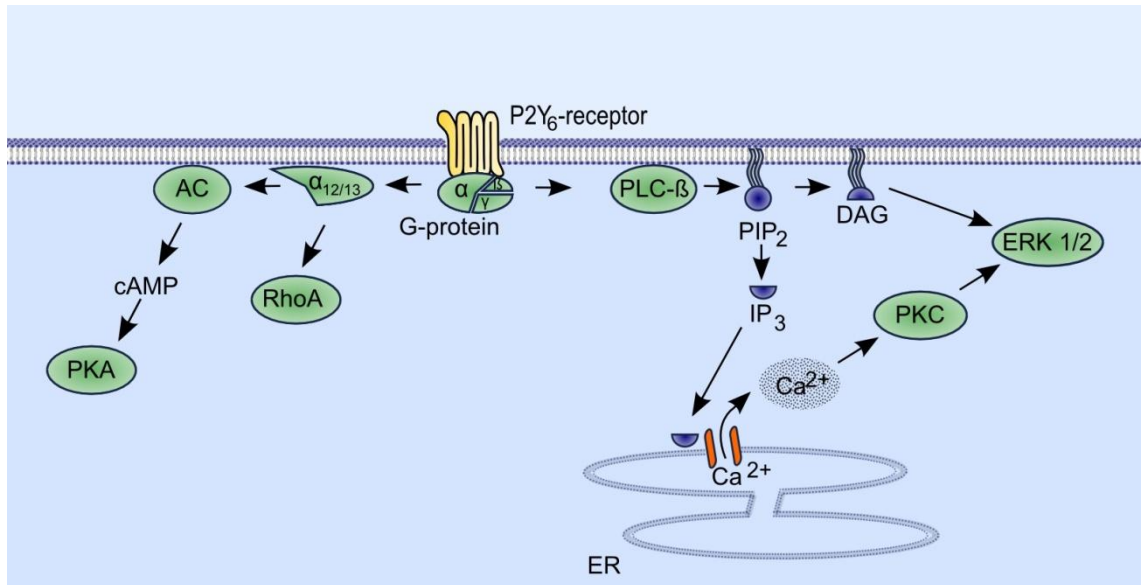## 1.5.4.3. Summarized illustration of P2Y$_6$-receptor cell signalling pathways

Illustration 12: *P2Y$_6$-receptor cell signalling pathways*

1.5.5.   P2Y$_6$-receptor desensitization and regulation

Receptor desensitization means weakening of signalling in response to persistent or intermittent application of an agonist. The weakening occurs as a consequence of alterations at the level of the receptor.

Common mechanisms are:

- Phosphorylation of the receptor through Proteinkinase A (PKA),
- Phosphorylation of the receptor through special G-protein coupled Receptor protein Kinases (GRK),
- or binding of arrestin, which leads to an internalization of the receptor.
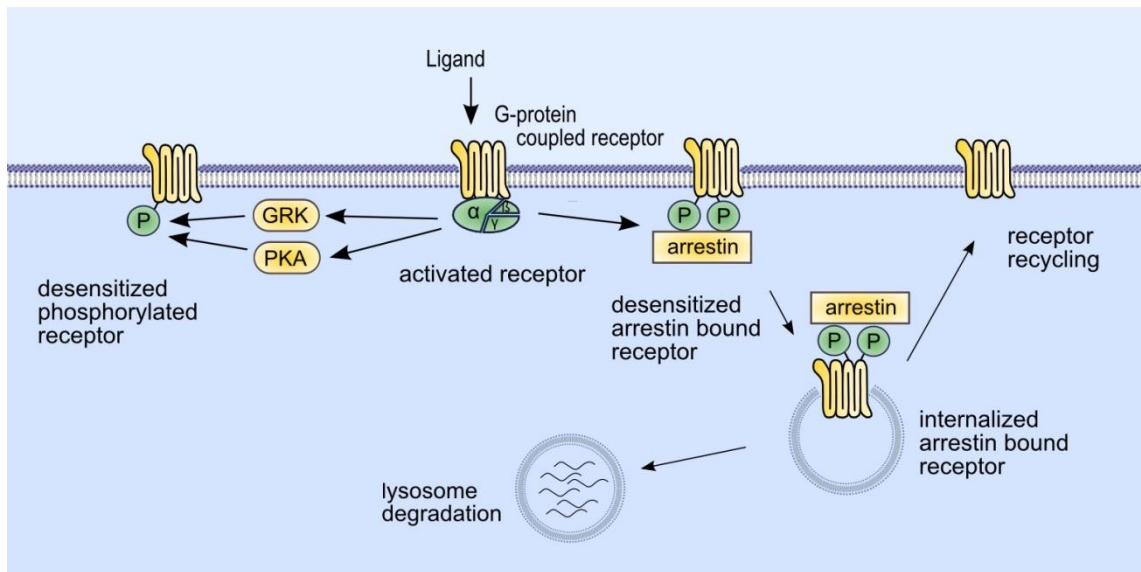
Illustration 13: _Receptor desensitization_
Adapted from R. Deutzmann

In contrast to the other P2Y-receptors, that show a quick desensitization, the P2Y$_6$-receptor shows as a unique characteristic a slow desensitization after stimulation with an agonist [16,137]. The current assumption is that the short C-terminal sequence (only 22 amino acids), which contains no serine residue (=S), no tyrosine (=Y) and only one threonine (=T) for possible phosphorylation sites for protein kinases, is responsible for this effect [137]. Also the third cytoplasmic loop, which has been shown to be important for phosphorylation of other G-protein coupled receptors, contains no one of these three amino acids, although this had been differently reported in a recently published minireview [138]. The first cytoplasmatic loop is supposed not to be involved in receptor regulation through phosphorylation [137].
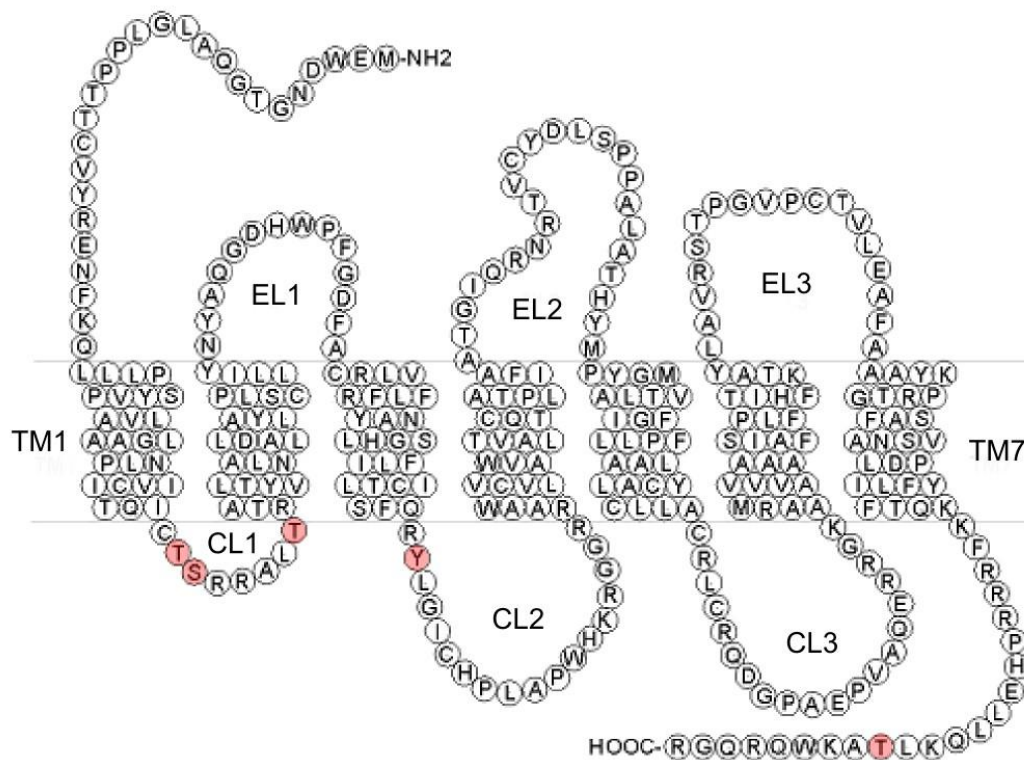
Illustration 14: *Structure of amino acids of the P2Y$_6$-receptor*
According to von Kügelgen et al.[139] and according to the protein sequence NP_789767.1 published on pubmed.net, last updated June 09.

Successive measurement of the intracellular IP$_3$ response during continuous UDP stimulation revealed a linear accumulation of IP$_3$ over at least 15-30min [137]. Thereafter, the level of intracellular IP$_3$ decreased slowly but remained elevated for at least 1h and a significant response was maintained for at least 3h [16]. When after a first stimulation with UDP, the P2Y$_6$-receptor expressing cells were washed and incubated for further 10min without the presence of the agonist, the level of intracellular IP$_3$ returned almost to control level, indicating that the continuous presence of the agonist is needed to maintain the IP$_3$ elevation [16]. The slow decrease of IP$_3$ level after the maximum at 15-30min could be due to the partial degradation of the agonist, since when fresh UDP was readded during the first hour, the maximal response was restored.

Surface $P2Y_6$-receptor levels remain constant for at least 10min of stimulation. After 1h of stimulation $P2Y_6$-receptor levels decline to ~75% and reach their baseline after 6h with ~30% surface receptor expression. Interestingly, the $P2Y_6$-receptor surface expression doesn't recover within 1h after privation of the agonist, which leads to the assumption, that a real down-regulation of $P2Y_6$-receptor occurs [137].

In summary it can be stated, that the $P2Y_6$-receptor is not susceptible to agonist-induced rapid desensitization, but does down-regulate during longer incubation time with the agonist UDP [137].

1.5.6. $P2Y_6$-receptor knockout mice

In 2008, knockout mice for the $P2Y_6$-receptor were first published [140]. Macroscopically, they appeared indistinguishable from wildtype mice. Also there is no difference in fertility reported [140].

Bar et al. examined $P2Y_6$-receptor wildtype and knockout macrophages and found out, that the 9 fold increase of IP_3 in wildtype macrophages after stimulation with 100μM of UDP or UTP is abolished in knockout macrophages [140]. Furthermore they could display, that UDP leads to a rapid phosphorylation of the MAP kinase ERK1/2, which could be detected already 2 min after stimulation with UDP in wildtype macrophages and did not occur in knockout macrophages. However this was a transient phosphorylation. 15 minutes after a stimulation with UDP, levels of phosphorylation of ERK1/2 were identical in both macrophage types [140]. A 15 min stimulation with 30ng/ml TNF-α induced ERK1/2 phosphorylation as well as in wildtype and knockout macrophages [140].

UDP and LPS stimulated macrophages of $P2Y_6$-receptor knockout mice produced less MIP-2 (which is the murine equivalent to the human IL-8), and

less IL-6 than wildtype macrophages compared to LPS stimulated macrophages without UDP [140].

1.6. Extracellular nucleotides

As powerful signalling molecules, extracellular nucleotides exert many different functions, depending on their extracellular concentration, the duration of concentration changes and the cell type they are acting on [141]. Due to their short half life and fast metabolism, extracellular nucleotides mostly act as autocrine and paracrine molecules and are dynamically regulated through release and nucleotide metabolism [8,142].

Nucleotides are released by all cells [142]. In excitable neuronal cells, ATP is stored in presynaptic vesicles and release occurs after stimulation of exocytosis, according to the role of a classical neurotransmitter [143].

Non-excitable cells like epithelial and endothelial cells were shown to release basal rates of nucleotides in steady state [144-145], as well as an increasing amount of nucleotides due to stressful events [8]. Different study results about the release mode in non-excitable cells indicate the involvement of vesicular and channel-mediated release of nucleotides [142,146]:

- Vesicular mediated release of nucleotides:
  Vesicular exocytosis as a mode of nucleotide release is one of the earliest assumptions about how nucleotides might be released in the extracellular space [147].
  In a model which minimized cell surface hydrolysis, Tatur et al. analyzed the composition of nucleotides in the extracellular fluid. It was shown, that not only ATP and UTP, but also ADP and UDP are released [148]. Because of the higher extracellular ADP:ATP and UDP:UTP ratios (1:3 vs. 1:2), compared with the intracellular nucleotide ratios, Tatur et al.

concluded, that nucleotides are probably released from a nucleotide diphosphate rich compartment like the endoplasmatic reticulum and golgi [148]. Moreover, Tatur et al. showed the increased vesicular exocytosis under conditions with increased nucleotide release and demonstrated, that this nucleotide release is calcium dependent [148]. Taken together, it can be assumed, that calcium dependent vesicular exocytosis is one mode of nucleotide release of adenine and uridine molecules.

- Channel mediated release of nucleotides:

Different channel forming molecules have been examined with various techniques and a lot of assumptions have been made about the structures offering well-directed nucleotide release.

Patch clamp experiments finally confirmed, that Pannexin 1 proteins, which form large conductance nonselective channels, are permeable to ATP [146]. In addition, it was shown, that channel activity increases under mechanical stress [146]. Following studies showed also the importance of Pannexin 1 in paracrine signalling. It is known for many tissues, that they are able to communicate over a long distance. Besides the possible passage of IP_3 through gap junctions, one of the mechanisms might be the ATP induced ATP release mediated through Pannexin 1 and P2Y receptors [149]. Lovocei et al. could show, that Pannexin 1 channels can be activated by extracellular ATP, which stimulates P2Y-receptors [149]. A consequent intracellular calcium increase after P2Y-receptor stimulation leads to a Pannexin 1 activation [149], which leads to increase in released ATP. This might underline the role of ATP as a "danger signal" to the cells.
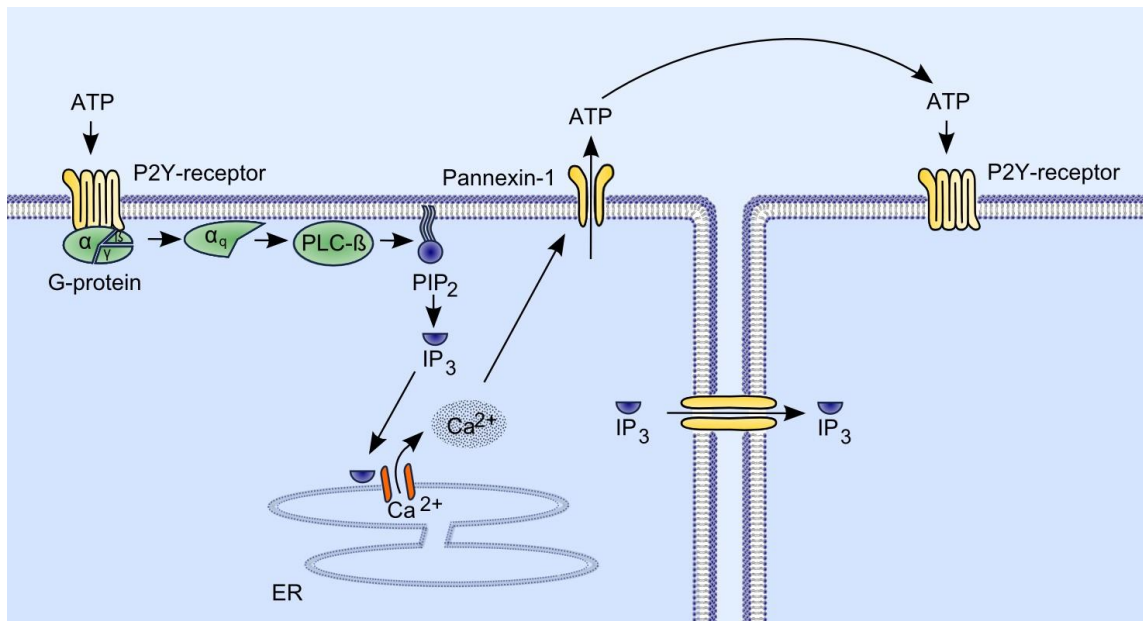
Illustration 15: *Pannexin-1*
Pannexin-1 mediated ATP release after stimuation of P2Y receptors and increase of intracellular calcium, adapted from Locovei et al.

Not only for Pannexin-1, but also for Connexin 43 hemichannels, there are now single channel recordings available documenting the direct ATP efflux through the Connexin 43 channels [150]. It also seems likely, that Connexin 26 as well as Connexin 32 mediate ATP efflux out of different cell lines [151].

Moreover, $P2X_7$-receptor gated channels are suspected to contribute to ATP release [152-153]. And also volume-sensitive channels seem to release ATP [154].

Besides the non-lytical release mechanisms described above, loss of membrane integrity in cell lysis leads to extensive uncontrolled release of the

intracellular nucleotide stores. However, this mechanism of nucleotide release accounts only for a few of the known actions of nucleotides [8].

Responses to extracellular nucleotides are terminated by diverse soluble and membrane-bound enzymes, which rapidly metabolize nucleotides [8,155-159]. Most, if not all cell types exhibit nucleotide metabolizing enzymes on their cell surfaces [8].

Beside the release of purine nucleotides like ATP and ADP, in the last years, evidence for the release also of pyrimidine nucleotides was generated. Under clinical aspects, the discovery of pyrimidine actions opened possibilities for new therapeutic approaches. Because pyrimidines are not degraded into adenosine, which is known to induce bronchoconstriction and possibly supports airway inflammation and pulmonary fibrosis, they seem to be preferable in some therapeutic treatment settings e.g. in cystic fibrosis [160].

Since the endogenous ligand of the $P2Y_6$-receptor is uridine diphosphate (UDP), the focus in the following subsections will lie on the release, metabolism and reuptake of uridine phosphates. Today, several different uridine phosphates are known to occur in the extracellular space: UTP, UDP and UMP as well as Up4A, a dinucleotide containing a uridine and an adenine nucleotide with four phosphates in between, and UDP coupled to various sugars.
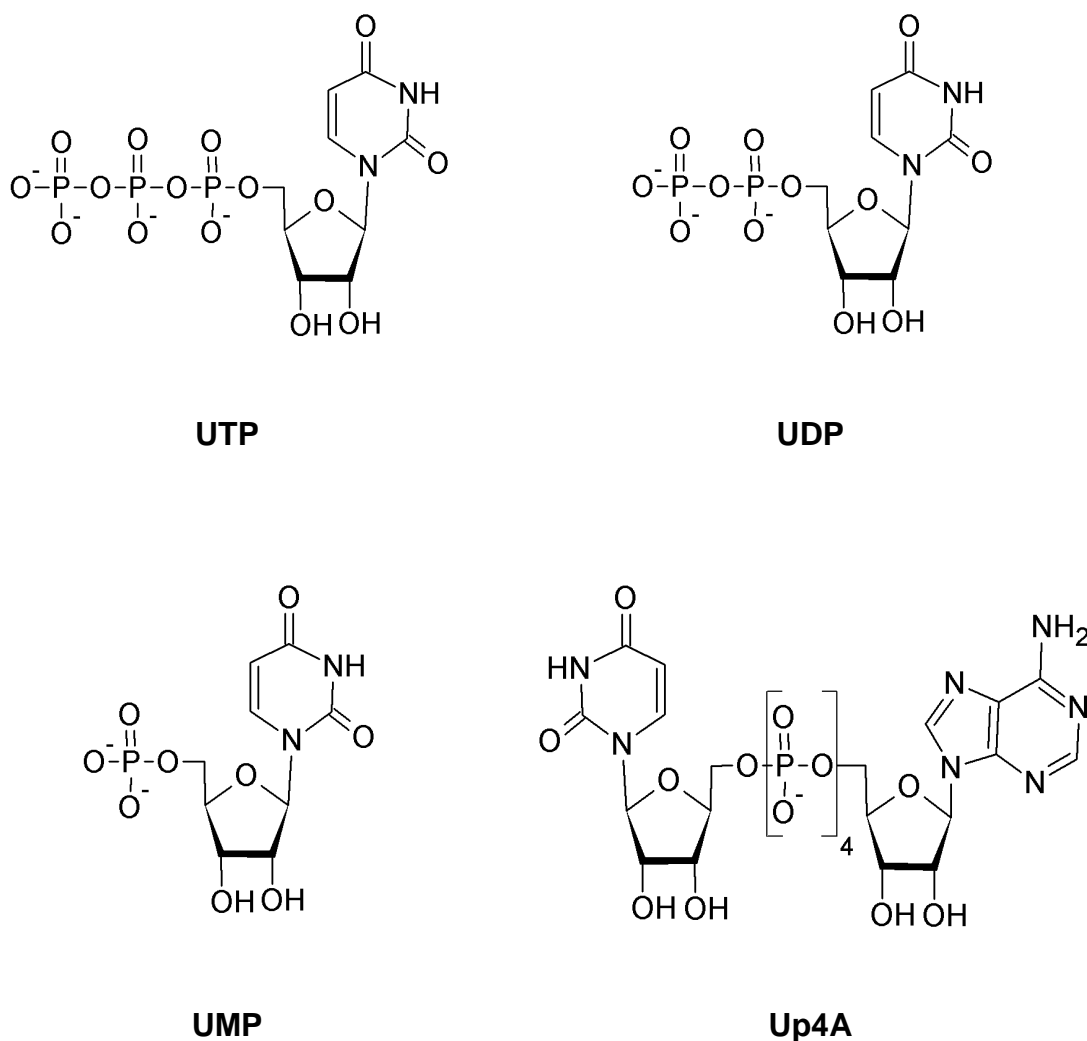
UTP

UDP

UMP

Up4A

Illustration 16: *Extracellular uridine nucleotides*

However, several complicating factors in the field of uridine phosphate release and metabolism long time delayed the exploration of extracellular uridine phosphates and their actions.

One of these factors has probably been the difficult measurement method of extracellular uridine phosphates. The radioactive HPLC method to detect uridine phosphates demands handling with radioactive substances, is complicated and costly [147,161].

A general problem with estimating nucleotide concentrations is the observation that measurements in the bulk medium underestimate the real concentration of nucleotides found in the close vicinity of the plasma membrane. Moreover, sampling the liquid layer directly upon cell surfaces remains difficult [147]. With a surface-attached luciferase assay it could be shown, that measurement in bulk media underestimate the local ATP concentration by at least one order of magnitude [162]. Respectively, experiments testing the local accumulation of UTP with subsequent $P2Y_4$-receptor activation in $hP2Y_4$-1231N1-cells indicated, that the pericellular concentration of UTP are several fold higher than the concentration in the bulk medium [163].

Due to the difficulties in the measurement of UDP, some earlier studies only evaluated extracellular UTP and assumed, that some effects might be due to $P2Y_6$-receptor stimulation because of the rapid degradation of UTP to UDP and possible extracellular accumulation of UDP [69,88]. These conclusions however are dangerous, because of oppositional effects of UDP and UTP [62] and metabolism of UTP through NDPK into ATP.

Because of the lack of stable and selective agonists and antagonists at the $P2Y_6$-receptor, most of the data of the last years were generated with UDP or even UTP as an agonist of $P2Y_6$-receptor. However UDP itself exhibits a low chemical and metabolic stability and therefore, specificity of UDP evoked responses were legitimately doubted [69].

In summary, these circumstances limit the available data, which enable to make firm conclusions about extracellular uridine phosphates, expecially UDP, and their actions at their P2Y-receptors. However, the developments of the last few years led to the introduction of some new effective research tools - like an UTP and UDP sensitive assay and diverse $P2Y_6$-receptor agonists and antagonists.

1.6.1.   Release of uridine phosphates

The realisation, that beside adenine nucleotides also uridine nucleotides and uridine nucleotide sugars are released from cells, either independent or in parallel to the release of adenine nucleotides, made the research in the field of extracellular nucleotides more complex [147,164-165].

One of the early hints for the release of uridine phosphates was the finding of measurable extracellular uridine phosphate concentrations in the supernatant of cell cultures. With the first measurement methods, extracellular UTP was confirmed in supernatant of diverse cell types like epithelial [147] and endothelial cells [166-167], astrocytoma cells [147,163], polymorphonuclear leukocytes [147] and platelets [147].

Because of the action of extracellular uridine phosphates only on the "slower" metabotropic g-protein coupled P2Y-receptors, it was suggested that extracellular uridine phosphates evoke responses, which do not need to happen in milliseconds of time [47].

Besides the release of mononucleotides, also the release of an uridine dinucleotide phosphate could be confirmed [168]. Uridine adenosine tetraphosphate (Up4A) was shown to be released from pharmacologically and mechanically stimulated endothelial cells [168].

1.6.1.1. Basal release of uridine phosphates

It is assumed, that many non-excitable cells like endothelial or epithelial cells release nucleotides continually at a basal rate under steady-state conditions [144-145]. This way, an equilibrium between nucleotide release and metabolisation is maintained [144-145]. The extracellular levels of nucleotides are thereby

determined in the low nanomolar range (0,5-10nM) in most cell cultures [144-145]. Despite the low levels of basal extracellular nucleotides, which are likely to be underestimated due to the limited measurement methods [147], alterations in these extracellular nucleotide concentrations are suspected to be harmful to the tissue and basal autocrine and/or paracrine activation of uridine nucleotide sensitive receptors, which is considered to be possibly important for maintaining physiological cell functions [103,163]. Detection of constant extracellular UTP levels in resting cell cultures of different cell types led to the assumption, that a constitutive release of UTP might be important for the regulation of basal cell functions [147,163].

Due to studies of extracellular nucleotide metabolizing enzymes, Lazarowski et al. hypothesized in 1997 the accumulation of extracellular UDP [163]. In 2000, Lazarowski et al. provided data for a constitutive release of nucleotides and a basal extracellular equilibrium between nucleotides [145]. A basal release of UTP could be confirmed in primary astrocytes and astrocytoma cell lines in 2006 [169].

Nucleoside diphosphates like ADP, UDP and GTP are usually detected in comparable or higher amounts than their corresponding triphosphates [145].
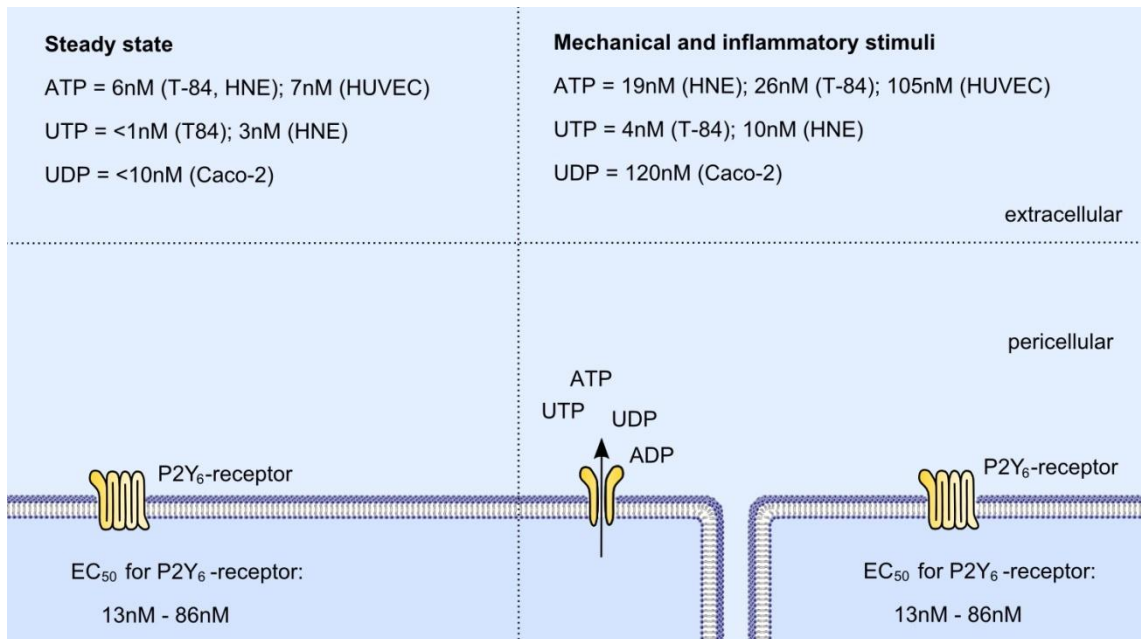
Illustration 17: *Extracellular nucleotide concentrations*
T-84 and HNE data are derived from Lazarowski et al. [147]*, HUVEC*
measurements were performed by Yegutkin et al. and Schwiebert et al. [159,170]
and UDP release of Caco-2 cells was determined by Grbic et al. [73]

1.6.1.2. Release of uridine phosphates because of stressful events or trigger molecules

In response to detrimental stimuli like diminished supply of oxygen (hypoxia, anoxia, ischemia), inflammation (bacterial invasion, inflammatory mediators) and mechanical stimulation (shear stress, osmotic swelling in hypotonic shock), the amount of nucleotide release increases. Therefore, it has been proposed, that the autocrine / paracrine signalling through nucleotides serves as a fast first alarming response, which might enable the cell to protect itself through autocrine signalling, counteract damage and lead to the release of helpful mediators as well as transfer neighboring cells in an activated state through paracrine signalling [103,171].

Increased release of uridine nucleotides (mainly UTP) has been widely demonstrated for miscellaneous stressful conditions like hypoxia, bacterial invasion and inflammation [73] and mechanical stimulation (shear stress [51,147,165,172] and osmotic swelling in hypotonic shock [148]).

Although it has been proved in the most, but not in all studies, a non-lytic mechanism of nucleotide release with preservation of the integrity of the outer cell membrane is assumed in moderate stress conditions [103]. Mechanically stimulated cells which are pretreated with an inhibitor of vesicle trafficking in vesicular exocytosis, exhibit decreased ATP concentrations in the extracellular medium [164], indicating that vesicular exocytosis plays a role in mechanically stimulated ATP release.

A release of UTP could be first demonstrated in 1995 by Saïag et al., who used [H³]uridine-labelled vascular endothelial cells. Increased flow, creating increased shear stress, induced an increase in the release of [H³]UTP [173]. Two years later, Lazarowski et al. succeded in measuring UTP in the extracellular fluid of cells and confirmed the hypothesis, that mechanical stimulation yields in nonlytic release of UTP [163]. Further investigation about the release mechanism was made in a study in 2008, where the hypothesis of uridine nucleotide release through vesicular exocytosis could be substantiated [148].

Moreover, considerations for the active release of uridine diphosphate were made [148]. Therefore, a model for hypotonic stress on pulmonary epithelial cells was used and time curves of nucleotide release were measured. To diminish the effect of extracelullar nucleotide metabolizing enzymes, the experiments were performed in a flow-through chamber. The measured curves of adenine nucleotides as well as uridine nucleotides peaked at the same time, which indicated, that a release of both groups of nucleotides occurred [148]. UTP and UDP were measured and interestingly, a 27-fold increase in UDP could be displayed (ATP 5,6-fold, ADP 3,7-fold, UTP > 7-fold) [148]. Ratios of nucleotide

diphosphates (ADP, UDP) to their corresponding nucleotide triphosphates were markedly elevated over their cytosolic ratios (UDP:UTP = 1:2; ADP:ATP = 1:3) [148]. In this model of diminished influence of extracellular nucleotide metabolizing enzymes, these results confirmed the hypothesis, that not only nucleotide triphosphates were actively released, but also nucleotide diphosphates. Because of the high nucleotide diphosphate to nucleotide triphosphate ratios, the source of nucleotide diphosphates didn't seem to be the cytosol with a much smaller ratio but rather vesicles derived from the endoplasmatic reticulum rich in nucleotide diphosphates [148].

The release of the vesicles seemed to be Ca^{2+} dependent, as vesicular exocytosis was completely abolished by the calcium chelator BAPTA [148].

Grbic et al. recently confirmed, that uridine diphosphate release is sufficient to stimulate $P2Y_6$-receptors under inflammatory conditions [73]. They determined a concentration of ~120nM in the supernatant of TNF- and IFN- stimulated cells [73]. Since the human $P2Y_6$-receptor exhibits an EC_{50}-value between 13 and 87nM, the amount of UDP is sufficient to evoke a receptor response.

Moreover it is suggested, that there is an autocrine/paracrine signalling loop, which provides elevated extracellular uridine nucleotide concentrations in response to stressful conditions [103].

1.6.1.3. Release of uridine phosphates because of cell lysis

Unlike the non-lytic release of nucleotides described in the previous chapter, lytic release occurs if the integrity of the cell is destroyed which happens during cell lysis. Bacterial adherence, diminished oxygen supply, apoptosis and necrosis are major causes of cell lysis. The nucleotide-rich cytoplasm and the content of the cell organelles pour out of the cells and lead to a pronounced increase in extracellular nucleotides in the vicinity of the damaged cells [103] or even in the plasma [88]. During cardiac ischemia in pigs and humans it could be

shown, that plasma levels of uridine nucleotides are significantly elevated [88,174].
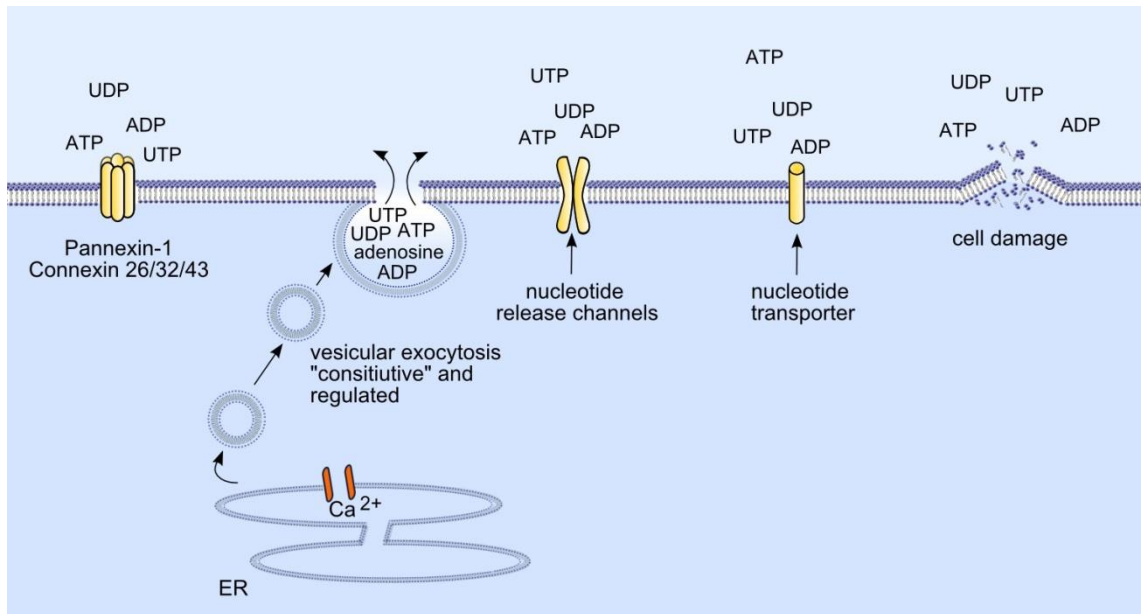Not only the release of mononucleotides, but also to the release of dinucleotides could be confirmed [175].

_Illustration 18__: Release of uridine nucleotides._
Vesicular exocytosis is a well studied possible release mechanism of uridine nucleotides [148]. Release of uridine nucleotides through nucleotide release channels or nucleotide transporters is still under investigation, whereas the release of uridine nucleotides through cell damage is widely accepted [88,174].

1.6.2.   Extracellular metabolism of uridine phosphates

The wide distribution and diversity of cell surface enzymes, which catalyze nucleotide degradation and interconversion underlines the importance of controlling extracellular nucleotide levels [164]. Through the action of these ectoenzymes, the concentration of extracellular nucleotides is precisely regulated [176].

A study in the rat/mouse neuroblastoma glioma cell line NG108-15 concerning the metabolism of uridine phosphates in cell cultures revealed, that exogenous UTP has a half life of approximately 14 - 30min [69,161], and is metabolized more than 90% after 4h and more [98,161]. In multiple studies a transient increase in the UDP-level could be demonstrated (three-fold [98] vs. ~40% of constituted UTP [161], a major breakdown product [69]), which might be an effect of the 2- to 3-fold faster metabolisation of UTP than of UDP [69,177]. Thus, released UTP might therefore also be able to provoke responses at the $P2Y_6$-receptor through its degradation product UDP [69,98].
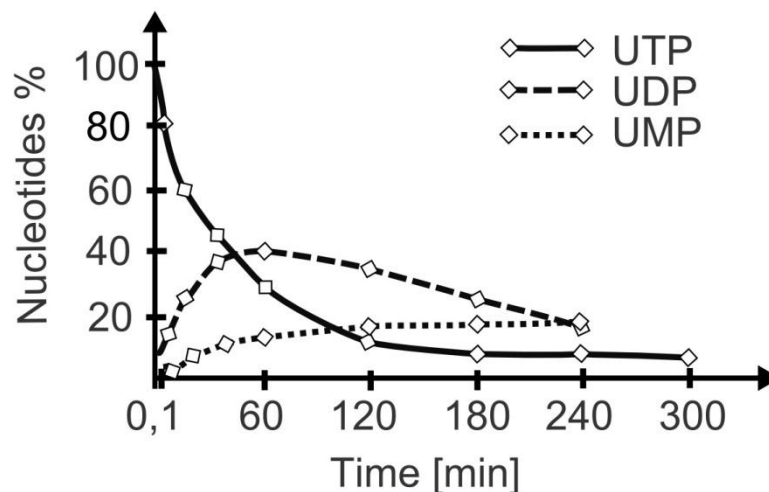

Illustration 19: *Extracellular metabolism of UTP*
Summarized time curves of two studies [98,161]. Metabolism of UTP lead to a transient increase on UDP-levels.

Exogenous UDP has a half life of approximately 27 - 50min [69,112,161]. A degradation of approximately 90% of the introduced amount of UDP was observed after 150min [112,161].
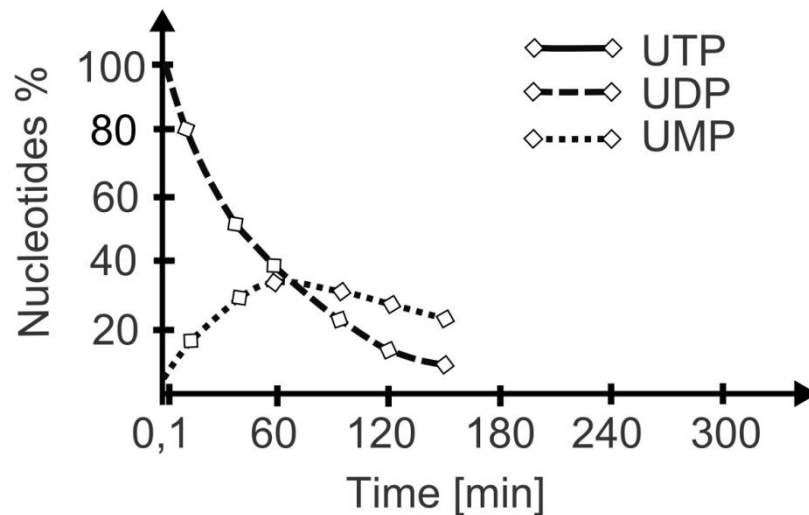

Illustration 20: _Extracellular metabolism of UDP_
Adapted from Kaulich et al. [161] _. Metabolism of UDP does not lead to a transient increase on UTP-levels._

Comparative measurements of adenine nucleotide metabolism revealed, that metabolism of uridine nucleotides was significantly slower than metabolism of adenine nucleotides [161,177]. The velocity of metabolism is suspected to be dependent on the expression pattern of multiple nucleotide metabolising enzymes. It has been speculated, that probably because of the lower extracellular concentrations, the metabolism does not have to be as fast as the one for purines [147].

1.6.2.1. Ecto-nucleoside 5'-triphosphate diphosphohydrolases

The most prominent members of extracellular nucleotide metabolizing enzymes are the ecto-nucleotidase 5'-triphosphate diphosphohydrolases (E-NTPDases) [176]. The E-NTPDases are able to hydrolyze nucleotide triphosphates and nucleotide diphosphates (NTP → NDP + P_i → NMP + 2 P_i). Extracellular UTP is converted into UDP as well as UDP into UMP by E-NTPDases.
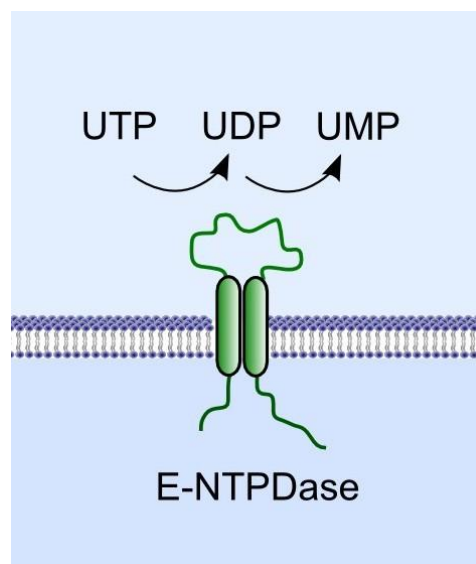

Illustration 21: *Ecto-nucleoside 5'-triphosphate diphosphohydrolase*

E-NTPDases hydrolyze β- and γ-phosphate residues of nucleotides, however they are not able to hydrolyze the α-phosphate to hydrolyze UMP to uridine and phosphate [178]. Of the family of eight known NTPDases, four NTPDases are anchored in the outer cell membrane, which are E-NTPDase 1, 2, 3 and 8 [178-179]. In addition, soluble NTPDases in the extracellular space are found. Some are supposed to be cleaved from the NTPDases 1, 2, 3, and 8 located at the outer cell membrane, whereas NTPDase 5 and 6 are secreted in the

extracellular space [179]. Entirely intracellularly located are only the NTPDases 4 and 7 [179].

Table 9: *NTPDases*

NTPDase	Preferred substrates (values of human enzymes unless other indicated)	Localisation
(E-) NTPDase 1 CD39	K_m ATP = 17µM [180] K_m ADP = 22µM [180] K_m UTP = 47µM [180] K_m UDP = 135µM [180]	Outer cell membrane
(E-) NTPDase 2	K_m ATP = 70µM [180] K_m ADP = ND [180] K_m UTP = 393µM [180] K_m UDP = ND [180]	Outer cell membrane
(E-) NTPDase 3	K_m ATP = 75µM [180] K_m ADP = 31µM [180] K_m UTP = 58µM [180] K_m UDP = 67µM [180]	Outer cell membrane
NTPDase 4	UDP > GDP = CDP = UTP > GTP >>>> ATP, ADP	Entirely intracellularly [141]
NTPDase 5 45kDa ER-UDPase CD39L4	UDP, GDP, IDP K_m UDP = 200-500µM [181] K_m GDP = 200-500µM [181] K_m ADP = 12,7mM [182]	ER and soluble
NTPDase 6 CD39L2	Nucleoside diphosphates GDP > IDP > UDP > CDP > ADP [183] K_m ADP = 10,6mM [184]	Golgi and soluble
NTPDase 7		Entirely

intracellularly [141]

(E-) NTPDase 8   Murine: Outer cell

K_m ATP = 13µM [180]   membrane

K_m ADP = 41µM [180]

K_m UTP = 47µM [180]

K_m UDP = 171µM [180]

NTPDase 5 is known to be secreted in the extracellular space and exhibits maximal specificity for UDP [185].

Soluble NTPDase 6 hydrolyzes triphosphates only barely [186].

Regarding the Michaelis constants K_m, the assumption rises, that extracellular UTP might lead to an accumulation of UDP [180]. Small K_m-values, which indicate that the enzyme needs only a small concentration to become saturated, are mainly found for UTP, not for UDP, indicating that maximum velocity of UTP metabolism is reached at lower concentrations and subsequently leads to an accumulation of UDP [180]. The larger K_m-values for UDP express, that higher substrate concentrations are necessary to reach the maximum reaction velocity.

Regarding the overall K_m-values, it becomes also clear, that extracellular ATP and ADP metabolism is likely to happen faster than that of UTP and UDP in most tissues, because of lower K_m-values of the E-NTPDases.

1.6.2.2. Extracellular nucleoside diphosphate kinase (NDPK)

In addition to phosphate hydrolyzing, nucleotides are substrates for trans-phosphorylation. The extracellular nucleoside diphosphokinase catalyzes the conversion of nucleoside diphosphates (e.g. UDP and ADP) to their respective nucleoside triphosphates (e.g. UTP and ATP) by transfer of the phosphate from another NTP to the NDP [145].
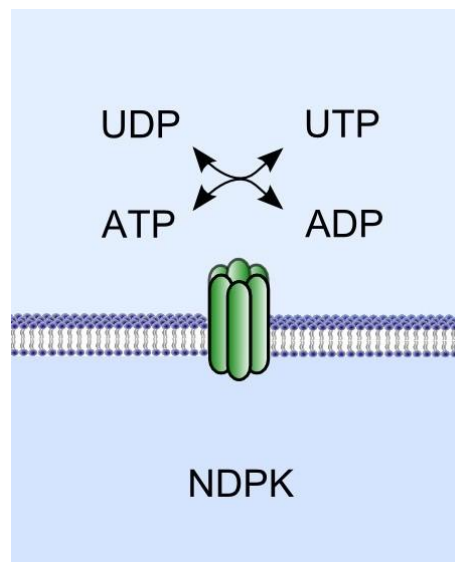

Illustration 22: *Extracellular-nucleoside diphosphate kinase*

Because the nucleoside diphosphokinase is a reversible enzyme, it is able to convert nucleotides in both directions. The direction, in which the enzyme catalyzes, depends on which side of the equilibrium the reaction is shifted.

$$ATP + UDP \rightleftharpoons ADP + UTP$$

Experiments with radioactive labelled ATP indicated, that γ-phosphates of extracellular ATP are transferred to UDP by an ecto-nucleoside diphosphokinase [145]. Similarly, γ-phosphates of added radioactive labelled UTP are transferred to ADP, indicating an extracellular equilibrium between the reaction components ATP, UDP, ADP and UTP [145]. That the metabolism of UDP to UTP depends on the availability of the second reaction partner ATP was shown in a study by Lazarowski et al. After the addition of solely 1µM UDP no conversion to UTP was demonstrated [69]. If ATP was also added and the balance between the reaction partners was shifted to the left, a prominent increase in UTP was noted [69].

$$\text{ATP + UDP} \rightarrow \text{ADP + UTP}$$

It is obvious, that these results complicate the attribution of UDP evoked effects only to stimulation of the UDP sensitive $P2Y_6$-receptor [69].

1.6.2.3. Extracellular nucleotide pyrophosphatase / phosphodiesterase

The ectonucleotide pyrophosphatase / phosphodiesterase (E-NPP) family consists of seven members, three of them have been implicated in hydrolysis of nucleotides (E-NPP 1-3) [187]. E-NPPs hydrolyze nucleotide triphosphates in their corresponding monophosphate and the reaction product pyrophosphate (NTP → NMP + PP_i). In nearly all tissues, NPP1-3 have been confirmed [188]. In 2000, Lazarowski et al. could confirm the activity of an extracellular nucleotide pyrophosphatase, which hydrolyzed UTP into UMP and PP_i [145]. Diadenosine polyphosphates and diguanine polyphosphates are hydrolyzed by E-NPP 1-3 [189].
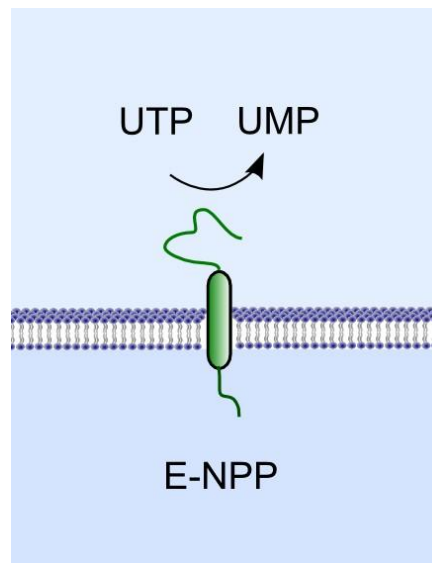
Illustration 23: *Extracellular nucleotide pyrophosphatase /phosphodiesterase*

1.6.2.4. Ecto-5'-nucleotidase

The ecto-5'-nucleotidase is the only member of the family of seven nucleotidases, which is expressed at the cell surface. The ecto-5'-nucleotidase catalyzes the hydrolysis of ribo- and deoxyribonucleoside 5'-monophosphates into nucleosides and orthophosphate [190]. Thereby ribonucleotides are better substrates than deoxyribonuleotides [191]. AMP is the most efficient substrate with a Km-value of 3 to 19µM [191-194]. UMP exhibits a Km-value of 110µM in guinea-pig skeletal muscle [193,195]. Also in zebrafish and in the C6 rat glioma cell line it was shown that the enzyme is able to hydrolyze UMP [190].

During inflammation, the ecto-5'-nucleotidase is inactivated in vascular endothelial cells by oxygen radicals [196]. Therefore, extracellular nucleotides accumulate in inflammation.

1.6.2.5. Alkaline phosphatase

The alkaline phosphatase hydrolyzes non-specifically inorganic phosphates from nucleotides [178].

NTP $\rightarrow$ NDP + Pi

NDP $\rightarrow$ NMP + Pi

NMP $\rightarrow$ nucleoside + Pi

The metabolization of UMP to uridine by alkaline phosphatase from calf intestine was shown early in 1987 [197]. In 2003, Kaulich et al. could show, that UMP and especially UDP accumulate after stimulation with UTP if alkaline phosphatase is blocked, which underscored the importance of the alkaline phosphatase in uridine nucleotide metabolism [161].

1.6.2.6. Summary metabolism of nucleotides

Even though the bigger part of earlier studies concentrated mainly on nucleotide metabolism of adenine nucleotides, meanwhile, several data also exist about uridine nucleotide metabolism.

It seems to be likely, that the different members of four families of nucleotide metabolizing enzymes, which also are responsible for adenine nucleotide metabolism, also take part in uridine nucleotide metabolism. These are E-NTPDases, E-NPPases, E-NDPK and alkaline phosphatase.
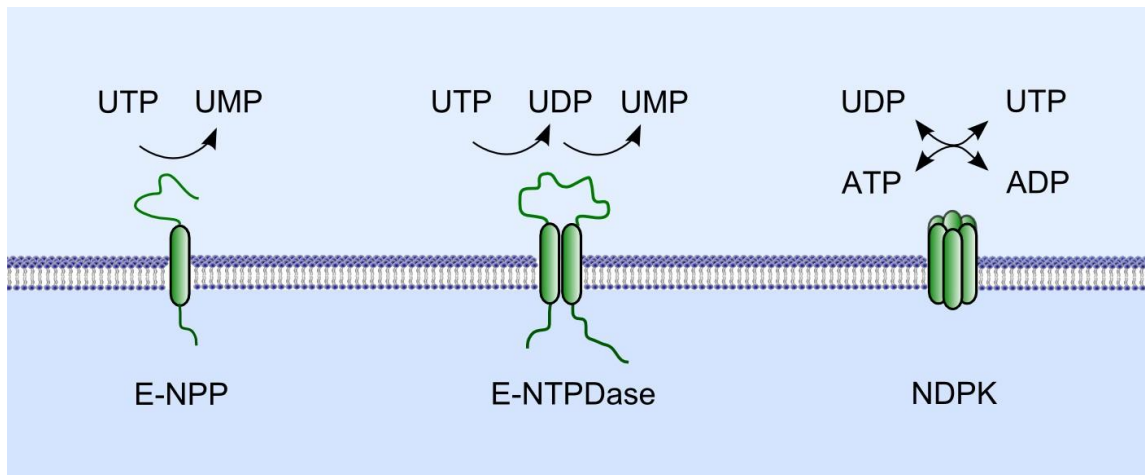
Illustration 24: *Extracellular metabolism of UTP and UDP*

Based on present knowledge, UMP is mainly metabolized to uridine by Ecto-5'-nucleotidase and alkaline phospatase, like AMP.

A recent theory suggests moreover, that not only receptors for UTP and UDP, but also receptors for uridine exist. Since metabolism of UTP and UDP leads to a massive accumulation of extracellular uridine [69,161], uridine might also possibly have signalling functions. Already in 2001, Kimura et al. hypothesized, that uridine might possibly act on uridine receptors, which might exist in brain [198].

1.6.3.   Reuptake of extracellular uridine

Uptake of extracellular uridine occurs through nucleoside transporters [199-200]. Seven types of nucleoside transporters are known by now [178]. Three of them are Na+ dependent ("concentrative"; CNT1 - CNT3) and two of them are Na+-independent nucleoside transporters ("equilibrative"; ENT1 – ENT4) [200]. The human nucleoside transporter CNT1 selectively transports pyrimidines, whereas

the human nucleoside transporter CNT2 selectively transports purines [200].The K_m value for human CNT1-mediated uridine transport is 59μM [201].

Uridine uptake was confirmed in rabbit aortic endothelial cells [166], rabbit ear artery endothelial cells [167], and erythrocytes [202]. In sympathetic nerve terminals however, an uptake of uridine nucleotides doesn't seem to exist [167].

Interestingly, after incubation with extracellular radioactive uridine, not only an uptake could be confirmed, but also increased levels of UMP, UDP and UTP were found in the extracellular fluid, which were suspected to have been released from the cells [166-167].

Table 10: *Nucleoside transporters*

Transporter	Mechanism	Na$^+$-dependency
ENT1	Facilitated diffusion	Na$^+$-independent
ENT2		
ENT3		
ENT4		
CNT1	Cation symporter	Na$^+$-dependent
CNT2		
CNT3		

Additionally, in inflammation, an increase of hCNT2, hENT1 and hENT2 mRNA expression levels was observed [203].

1.7.   P2Y$_6$-receptor in inflammation

Contact of organisms to invading bacteria, and in the case of gram-negative bacteria contact with the bacterial endotoxin LPS, induces a sequence of responses known as inflammatory reaction [95]. Thereby, LPS is a potent activator of the innate immunity [204]. LPS mediates its actions mainly through TLR-4 and induces a release of diverse soluble inflammatory molecules [205].

As a early defense mechanism, TNF-α is produced and released by various cell types [206]. In addition, release of non-protein mediators like purines and pyrimidines plays an important role in supporting the inflammatory reaction [38,207-209].

There is growing evidence, that P2-receptors are involved in cytokine release (e.g. IL-1β from microglial cells) induced by inflammatory mediators [208,210]. As a consequence, the family of P2-receptors is now increasingly accepted as participant in the inflammatory reaction [38].

Early studies already assumed, that an intracellular Ca^{2+} increase, as well as PKC and ERK activation are supportive for LPS-mediated release of inflammatory cytokines [95]. The inflammatory response evoked by LPS was suspected to be amplified by stimuli, which could induce Ca^{2+} increase, PKC and ERK activation [95]. The assumption, that P2-receptors might possibly play a major role in inflammation, arose, when it was discovered, that ERK activation could be inhibited by P2-receptor antagonists [211].

Treatment with Thapsigargin, a molecule which raises intracellular calcium levels, increases the production of LPS induced genes like TNF-α, PGE$_2$ and IL-6 [95]. In contrast, BAPTA/AM, which is an intracellular Ca^{2+} chelator, reduces the production of LPS induced genes after LPS stimulation [95].

More precisely, later studies of the connection between inflammatory stimuli and P2-receptor signalling implicated a special role of $P2Y_6$-receptors in mediating proinflammatory cytokine release. Inflammatory stimuli like TNF-α, LPS or IFN-γ were shown to induce IL-8 release via $P2Y_6$-receptor [73,138]. Moreover, also the release of the proinflammatory chemokine CCL20 seems to be mediated by the $P2Y_6$-receptor [62]. These findings indicate an important role of the $P2Y_6$-receptor in immune defense.

1.7.1. $P2Y_6$-receptor upregulation in inflammation

Nucleotide receptor upregulation in acute injury combined with increased release of nucleotides is assumed to be a first attempt to counteract stressful events [103]. Up to now, three different studies, which examined the $P2Y_6$-receptor expression in inflammation, confirmed an upregulation of the $P2Y_6$-receptor under inflammatory conditions.

First of all, Somers et al. discovered in 1998 a $P2Y_6$-receptor upregulation in T-cells in an inflammatory bowel disease model [212]. Ten years later, Grbic et al confirmed in cell culture and in mice with inflammatory bowel disease, that intestinal inflammation leads to an increase in $P2Y_6$-receptor expression [73]. Also in human patients with Crohn's disease and ulcerative colitis, the $P2Y_6$-receptor mRNA expression was shown to be 3 to 4-fold elevated [73].

In a rat model of brain injury through kainic acid Koizumi et al. could additionally show, that also in induced brain injury an upregulation of $P2Y_6$-receptor mRNA occurs [72].

1.7.2. Extracellular release and metabolism of UDP in inflammation

Inflammatory conditions evoke complex responses which are tightly regulated in matters of time and place [38]. The interplay of activation and deactivation of mechanisms and enzymes forms the individual responses [38]. During the last years of research it has become clear, that extracellular nucleotide signalling is changed under inflammatory conditions and it was assumed, that alterations of extracellular nucleotide levels might influence responses to inflammatory stimuli [213].

Measurement results provided the data for increased nucleotide release under inflammatory conditions (see above) [72-73,214]. Moreover it was observed, that nucleotide metabolization is impaired by inactivation of metabolizing enzymes [196] during the initial phase of an inflammation. Both circumstances lead to elevated nucleotide levels in inflammation.

In addition, it has been recognized, that extracellular nucleotides can initiate cells to produce and release proinflammatory cytokines and in this way, trigger inflammatory responses. Therefore, nucleotide release seems to be an endogenous mechanism of cells to amplify the signal of inflammatory mediators like LPS or TNF-α and might be a first attempt to counteract stressful events [103].

Because of the difficulties in measuring uridine nucleotide concentrations, data about uridine diphosphate release in inflammation are rarely available. In 2008, Grbic et al. could confirm an increase of extracellular UDP after an addition of 10ng/ml TNF-α and 10ng/ml IFN-γ for 6h to human intestinal Caco-2 cells with HPLC measurement of UDP in the supernatant [73]. In the supernatant of cells which were unstimulated, the UDP concentration was below the detection capacity of 10nM, but after stimulation with TNF-α and IFN-γ, the concentration of UDP was in the range of 110-125nM. The concerns expressed in the study, that these levels might not be sufficient to stimulate the $P2Y_6$-receptor which

was shown by Nicholas et al. to have an EC_{50} value of 190nM [47] might be negligible not only because of the mentioned underevaluation of nucleotide concentration in the bulk medium, but also in regard to the determined EC_{50} values at human $P2Y_6$-receptors, which are much lower at 13nM, 15nM, 47nM, and 86nM [110-113]. The measured values of UDP in the supernatant should therefore be sufficient, with or without underestimation of the pericellular UDP, to induce a response at the $P2Y_6$-receptor.
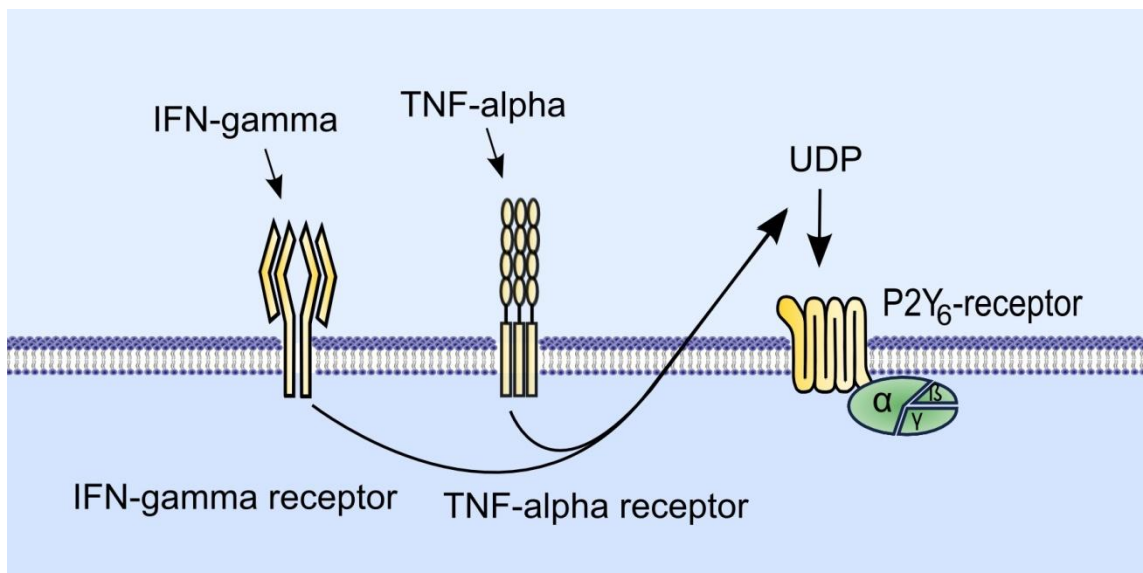

Illustration 25: _TNF-α and IFN-γ induce an increase of extracellular UDP_

Robson et al. impressively showed a rapid loss in activity of nucleotide metabolizing enzymes in endothelial cells from 1 to 8h after stimulation with human recombinant TNF-α through the production of oxygen radicals [196]. Burch et al. could confirm these results in primary bronchial epithelial cells, which were exposed to LPS. They showed a profound decrease of NTPDase 1 and 3 activity after 1h, and still a small decrease after 8h of exposure [215]. In this early phase of inflammation, therefore, an accumulation of extracellular nucleotides occurs.

In later stages of an inflammatory reaction however, the nucleotide metabolizing activity of extracellular enzymes increases.

After 18h Robson et al. could show, that the nucleotide metabolizing activity of extracellular enzymes on endothelial cells was completely restored [196]. Burch et al. even could demonstrate that the activity of NTPDase 1 and 3 was significantly elevated after 24h of exposure to LPS as well as their mRNA expression levels [215]. Besides the E-NTPDases, also the ecto-5'-nucleotidase transcript is elevated in inflammation [216].

1.7.3. LPS and TNF-α potentiate their effect through autocrine stimulation at the P2Y$_6$-receptor

In current studies it is increasingly expressed, that extracellular inflammatory stimuli like LPS or TNF-α might use nucleotide release to the extracellular space to subsequently increase the release of chemokines via autocrine stimulation of P2-receptors [134,138,208], especially in the early phase of the inflammatory reaction.

For nucleotides like ATP experimental data are available for the multiple genes, like IL-1α [209] and IL-1ß [208]. However, knowledge about LPS and TNF-α mediated actions through UDP and the P2Y$_6$-receptor is limited to only a few genes, like IL-8 [71,134].

In 2001 Warny et al. found, that LPS induced IL-8 release in monocytes is affected by extracellular nucleotides [71]. LPS-stimulation in the presence of apyrase, which quickly metabolizes nucleotide tri- and diphosphates into nucleotide monophosphates, inhibited LPS induced IL-8 release [71,134], furthermore P2Y$_6$-receptor antisense experiments showed a sustained inhibition of IL-8 release and P2Y$_6$-receptor overexpression led to increased IL-8 reporter activity [71]. Kukulski et al. could demonstrate, that the specific P2Y$_6$-receptor

antagonist MRS2578 is also able to reduce LPS-induced IL-8 release [134]. They concluded therefore, that LPS-induced release of IL-8 might be mediated by autocrine signalling through extracellular nucleotides which act on $P2Y_6$-receptors [71,134].
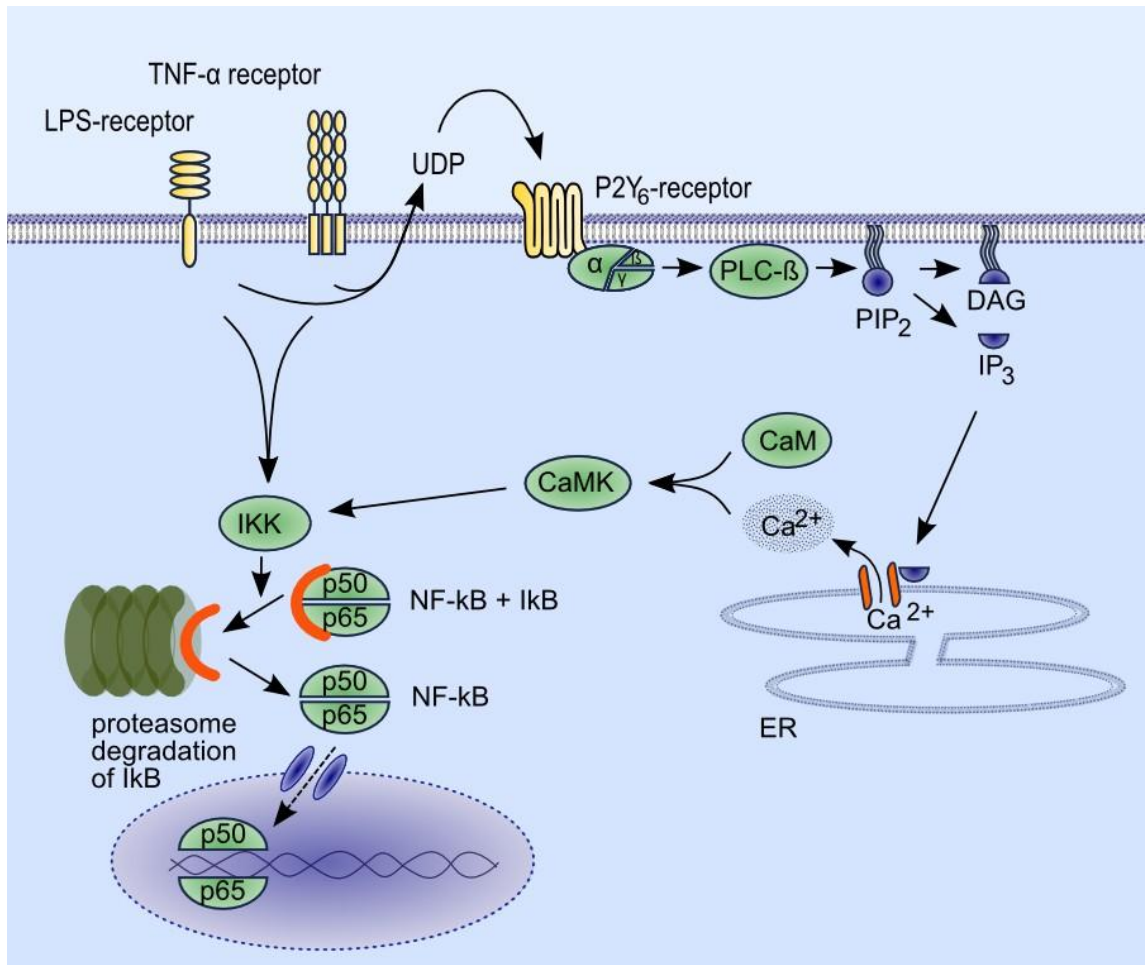

Illustration 26: *Supposed LPS and TNF-alpha influence on nucleotide mediated induction of proinflammatory genes.*
LPS and TNF-α stimulation lead to extracellular accumulation of UDP which acts on $P2Y_6$-receptors. $P2Y_6$-receptor stimulation leads to a rise an intracellular calcium

IL-8 is the most powerful chemoattractant to neutrophils [62] and LPS and TNF-α as well as UDP were shownto be able to induce a secretion of IL-8 in monocytes and intestinal epithelial cells [71,73,90,134,217]. Own studies with a NF-κB reporter assay supplement these findings, showing that stimulation of endothelial cells with TNF-α induces a robust induction of NF-κB reporter activity which can be abolished by $P2Y_6$-receptor antagonist MRS2578 in the first hours after onset of TNF-α stimulation.

These findings lead to the assumption, that LPS and TNF-α induce a release of nucleotides, especially UDP, which stimulates the $P2Y_6$-receptor and by this way, induces ERK phosphorylation and NF-κB activation and subsequently IL-8 induction.

1.8.   Clinical relevance

Regarding the proinflammatory role of $P2Y_6$-receptor signalling under inflammatory conditions, a supportive effect of $P2Y_6$-receptor antagonists seems to be likely in conditions of excessive inflammation.

As TNF-α has been implicated to be an important and possibly pathogenetic factor in Kawasaki disease [218], $P2Y_6$-receptor antagonists migt be helpful in attenuating the inflammatory response. In this case, it might be advantageous, that the $P2Y_6$-receptor could be targeted with a chemical compound, which might avoid allergic reactions induced by standard IVIG treatment of Kawasaki disease.

So far clinical trials with immunomodulating antiinflammatory substances failed to reduce mortality in sepsis [219] however it will be of interest, how $P2Y_6$-receptor signalling influences the development and progression of sepsis.

Moreover, the understanding of the mechanism of $P2Y_6$-receptor signalling in inflammation also seems to be important in cystic fibrosis, which is associated with bacterial infections of the lung and an increase of IL-8 in bronchoalveolar lavage fluid [220-222].

Since also in inflammatory bowel disease a markedly upregulation of $P2Y_6$-receptor and IL-8 was shown [73], evaluation of $P2Y_6$-receptor antagonists in attenuating chronic inflammatory bowel diseases also might be of interest.

1.9.   Rationale of the thesis

In inflammation extracellular nucleotides are released and act as autocrine and paracrine mediators on diverse cell types. UDP acting on $P2Y_6$-receptors is thereby assumed to be responsible for signal amplification of inflammatory mediators like TNF-α and LPS in immune and epithelial cells.

Moreover, it was reported that $P2Y_6$-receptor activation plays a role in regulating the activity of NF-κB in osteoclasts. However up to now no involvement of endothelial $P2Y_6$-receptors in inflammation was confirmed.

Since the endothelium is an important partner for immune cells in intravascular inflammatory responses, we wanted to study the role of $P2Y_6$-receptor signalling on endothelium in inflammation.

The reported signal amplification of inflammatory mediators through $P2Y_6$-receptor signalling and the reported NF-κB activation caused us to investigate, if NF-κB activation by TNF-α might be at least partially mediated through $P2Y_6$-receptor signalling.

Furthermore we wanted to examine, if P2Y$_6$-receptor upregulation occurs not only in vitro, but also in vivo in a mouse model of systemic intravascular inflammation.

2. Materials and Methods

2.1. Cell culture

Used materials:	Product No.; Company; Registered office
HMEC-1	Gift from F. Candal; Centers for Disease Control, Atlanta, GA, USA
HCAEC	301-75a; Cell Applications, Inc.; San Diego, CA, USA
Endothelial Cell Growth Medium MV	C-22020; Promocell; Heidelberg, Germany
Supplement Mix / Endothelial Cell Growth Medium MV	C-39225; Promocell; Heidelberg, Germany
L-Glutamine 200mM 100x	25030-024; Gibco BRL; Gaitherburg, MA, USA
EGF	354052; BD; 354052
Hydrocortisone	H-0888; Sigma; Taufkirchen, Germany
Accutase	L11007; PAA; Pasching, Austria
MCDB 131	10372-019; Gibco; Gaitherburg, MA, USA
G418-Bc Sulfate Powder Subst.	A291-25; Biochrom; Berlin, Germany
Dulbecco's PBS 1x without Ca^{2+} and Mg^{2+}	H15-002; PAA; Pasching, Austria
DPBS 1x with Ca^{2+} and Mg^{2+}	14040; Gibco; Gaitherburg, MA, USA
FCS Gold	A15-151; PAA; Pasching, Austria
Antibiotic-Antimycotic Solution	A5955; Sigma; Taufkirchen, Germany
Tissue culture Flasks 70ml	Becton Dickinson, Le Pont De Claix, France
Tubes 50ml/15ml	Becton Dickinson, Le Pont De Claix, France

Human microvascular endothelial cells (HMEC-1) were a kind gift from F. Candal (Centers for Disease Control, Atlanta, GA, USA). Cells were grown in MCDB-131 medium containing 10% FCS, 0,01µg/ml EGF, 1µg/ml hydrocortisone, 10mM L-glutamine, 1%antibiotic / antimycotic solution.

Human coronary artery endothelial cells (HCAEC) were purchased from Cell Applications, Inc. (San Diego, CA, USA). Endothelial cell growth medium was complemented with the supplement mix and contained 5% FCS, 0.4% ECGS/H, 10ng/ml EGF and 1µg/ml hydrocortisone.

Cells were incubated at 37°C with 5% CO_2. Confluent cells were harvested with Accutase and passaged three times a week.

2.2. Inflammatory and P2Y$_6$-receptor antagonist stimulation of cell culture cells

Used materials:	Product No.; Company; Registered office
rHu TNF-α	C-63720; Promokine; Heidelberg, Germany
Albumin from bovine serum	A7906-50G; Sigma; Taufkirchen, Germany
LPS from E.coli O26:B6	L2654; Sigma; Taufkirchen, Germany
CD14	ALX 201-133; Alexis Biochemicals; San Diego, CA, USA
IL-1α	C-61112; Promokine; Heidelberg, Germany
DPBS 1x with Ca^{2+} and Mg^{2+}	14040; Gibco; Gaitherburg, MA, USA
MRS2578	2146, Tocris, Bristol, UK
DMSO	Sigma, Taufkirchen, Germany

Preparation of stock solutions

Lyophilized human recombinant TNF-α was reconstituted in sterile, ultra-pure water to a concentration of 10µg/ml. As a carrier protein, bovine serum albumine was added at a concentration of 0,1%. Aliquots were stored at -20°C. Lyophilized lipopolysaccharide from E.coli O26:B6 was reconstituted with 1ml phosphate buffered saline to a concentration of 1mg/ml. Aliquots were stored at -20°C. Lyophilized human recombinant CD14 was reconstituted with 20µl sterile, ultra pure water to a concentration of 500µg/ml. Aliquots were stored at -80°C.

Lyophilized human recombinant IL-1α was reconstituted with 100µl sterile, ultra pure water to a concentration of 100µg/ml. This solution was further diluted with 900µl of PBS to a concentration of 10µg/ml. Aliquots were stored at -20°C.

10mg of lyophilized P2Y$_6$-receptor antagonist MRS2578 (MW: 472.66) was reconstituted with 212µl DMSO to a concentration of 100mM. Aliquots were stored at -80°C.

Inflammatory stimulation:

The inflammatory stimulation of cells was performed in normal serum containing cell culture medium for 2, 6, 12, or 24h with concentrations between 1 and 10ng/ml TNF-α.

Stimulation with the P2Y$_6$-receptor antagonist MRS2578:

Stimulation with the P2Y$_6$-receptor antagonist MRS2578 was performed at a concentration of 1 to 10µM. Stimulation of controls with the same amount of solvent was always performed simultaneously. Inflammatory stimulation with TNF-α was performed after a preincubation period of 30min with the P2Y$_6$-receptor antagonist MRS2578.

2.3.   RNA-Isolation, transcription, PCR

Used materials:	Product No.; Company; Registered office
Ultra PURE Distilled Water, DNAse, RNAse Free	10977; Gibco; Gaitherburg, MA, USA
Ethanol absolut	A3678.1000; AppliChem; Darmstadt, Germany
iScript cDNA Synthesis Kit	170-8891; Bio-Rad Laboratories, Inc.; Munich, Germany
iQ^{TM} SYBR$^{®}$ Green Supermix	170-8884; Bio-Rad Laboratories, Inc.; Munich, Germany
NucleoSpin RNA II	740955.250; Macherey & Nagel; Düren, Germany
FlashGel^{TM} DNA Marker 100bp-4kb	50473; Lonza; Basel, Switzerland
FlashGel^{TM} Loading Dye	50462; Lonza; Basel, Switzerland
FlashGel^{TM} DNA Cassette, 1,2% agarose, 12+1 Single	57023; Lonza; Basel, Switzerland
FlashGel^{TM} DNA Cassette, 1,2% agarose, 16+1 Double	57029; Lonza; Basel, Switzerland
iCycler	Bio-Rad Laboratories, Inc.; Munich, Germany
FlashGel^{TM} Dock	57025; Lonza; Basel, Switzerland
Photometer Ultrospec 3000	Biochrom; Berlin, Germany
Thermocycler	Bio-Rad Laboratories, Inc.; Munich, Germany

Total RNA was extracted from cell culture and murine tissue with the use of the total RNA isolation kit NucleoSpin RNA II. Concentration and purity of isolated RNA were determined through photometric measurement. 1µg of RNA was reverse transcribed with iScript$^{™}$cDNA Synthesis Kit, that consists of a blend of

oligo(dT) and random hexamer primers. The reaction protocol was as follows: a 5 min step at 25°C, a 30 min step at 42°C and a 5 min step at 85°C.

A total amount of 25ng transcribed mRNA was finally used for real-time quantitative PCR. Real-time RT-PCR was performed with the use of iQ$^{™}$Sybr$^{®}$ Green Supermix on iCycler. The PCR reaction contained 10 pM of each sense and antisense primer (see tables below).

The primer set was amplified using a protocol optimized by Bio-Rad technologists as followed:

Step 1:		3:00min	95°C	Denaturation
		0:15min	95°C	Denaturation
Step 2: (40 cycles)		0:30min	60°C	Annealing temperature: different between the primers, see table
		0:10min	72°C	Elongation
Step 3:		1:00min	72°C	Final elongation
Step 4:		0:10min	51°C-102°C	Melting curve analysis

Annealing temperature was varied between different genes according the results of their gradient PCRs (see table below).
Quality of PCR data was ensured through standard curve analysis, melting curve analysis and blank control samples. Relative quantification was performed with the housekeeping gene ß-actin.

2.4. Primer

Primers were designed with the use of Primer3 (http://frodo.wi.mit.edu), Netprimer and Primer Designer software. Wherever possible, primers were designed to anneal to exons on both sides of an intron or within the exon/exon boundary of the gene, to ensure that only cDNA and no genomic DNA was amplified through the PCR reaction. To optimize the efficiency of real-time RT-PCR, lengths of amplificats were chosen between 80 and 250bp. BLAST search was performed to ensure that the primer recognizes only the target gene.

The primer for murine ß-actin was taken from literature [223-225].

Most primers were analyzed with gradient PCRs for ideal annealing temperature. Analysis of amplificats was performed through visualization on fast gels with most primers. All primers were analyzed through melting curve analysis.

Primer of human ß-actin

Length: 190bp

annealing temperature: 58-66°C

Sense: GGAGAAAATCTGGCACCACA

Peak (melting curve): 90,5°C

Antisense: AGAGGCGTACAGGGATAGCA

Exon spanning: Yes

Melting curve:

Gel:
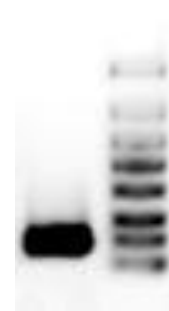
- 4000bp
- 2000bp
- 1250bp
- 800bp
- 500bp
- 300bp
- 200bp
- 100bp

Primer of human P2Y$_1$-receptor

Length: 117bp

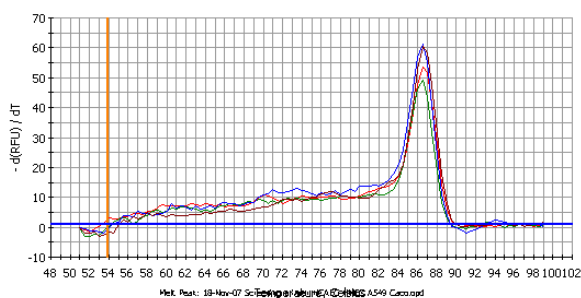annealing temperature: 62°C

Sense: ACCTCTATGGCAGCATCTTG

Peak (melting curve): 87°C

Antisense: CGCTGATACAGATCGCATTC

Exon spanning: No, P2Y$_1$

Expressed in / Positive control: HMEC-1

consists of only one exon.

Melting curve:

Gel:

- 4000bp
- 2000bp
- 1250bp
- 800bp
- 500bp
- 300bp
- 200bp
- 100bp

Primer of human P2Y₂-receptor

Length: 100bp

Sense: CCGTGGCGCTCTACATCTTC
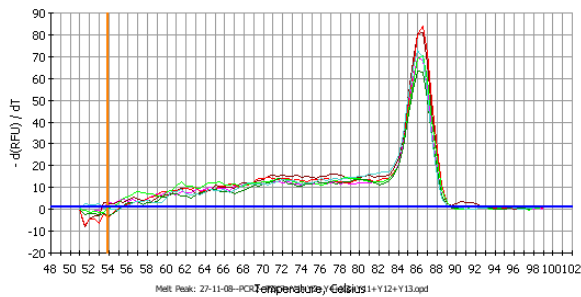
Antisense: GAGGCCGCATACAGTGCATC

Expressed in / Positive control: HMEC-1

Annealing temperature: 60°C

Peak (melting curve): 86,5°C

Intron spanning: No, lies in
    coding exon.

Melting curve:

Primer of human P2Y₄-receptor

Length: 136bp

Sense: ATCCTGCTGCCTGTGAGCTA
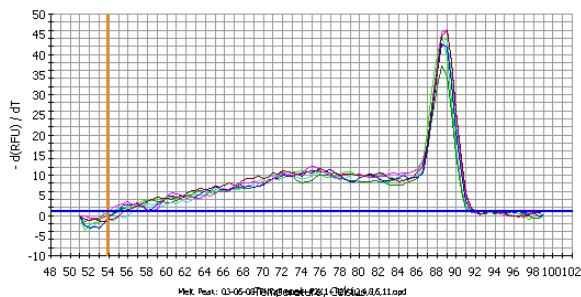
Antisense: CTGACAATGCCAGGTGGAAC

Expressed in / Positive control: HMEC-1

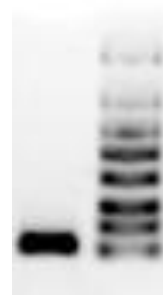annealing temperature: 61-
62°C

Peak (melting curve): 88,5°C

Intron spanning: No, lies in
    coding exon.

Melting curve:

Gel:

- 4000bp
- 2000bp
- 1250bp
- 800bp
- 500bp
- 300bp
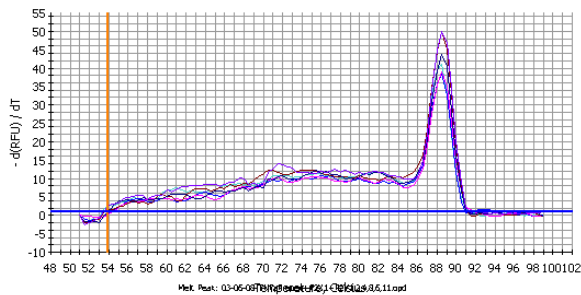- 200bp
- 100bp

Primer of human P2Y$_6$-receptor

Length: 110bp

annealing temperature: 63°C

Sense: CCACAGGCATCCAGCGTAAC

Peak (melting curve): 88,5°C

Antisense: CAGCAGGAAGCCGATGACAG

Intron spanning: No, lies in

Expressed in / Positive control: HMEC-1

coding exon.

Melting curve:

Gel:

- 4000bp
- 2000bp
- 1250bp
- 800bp
- 500bp
- 300bp
- 200bp
- 100bp

Primer of human P2Y$_{11}$-receptor

Length: 133bp

Annealing temperature: 60°C

Sense: GAGCATGGCAGCCAACGTCT

Peak (melting curve): 90°C

Antisense: CACGGCCACCAGGAACTCAA
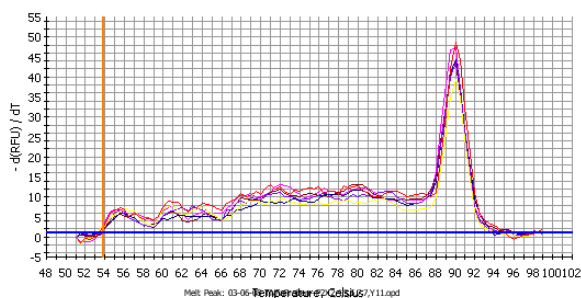
Intron spanning: Yes

Expressed in / Positive control: Caco-2

Melting curve:

Primer of human P2Y$_{12}$-receptor

Length: 146bp   Annealing temperature: 61°C

Sense: CAGATGCCACTCTGCAGGTT   Peak (melting curve): °C

Antisense: CTGGTGCACAGACTGGTGTT   Intron spanning: Yes

Primer of human P2Y$_{13}$-receptor

Length: 106bp   Annealing temperature: 60°C

Sense: CCAGCTCCTCCACCTTCATC   Peak (melting curve): 85°C

Antisense: GGTGCCAGGTGTGAGTCAGA   Intron spanning: No, in coding

                                                                          exon.

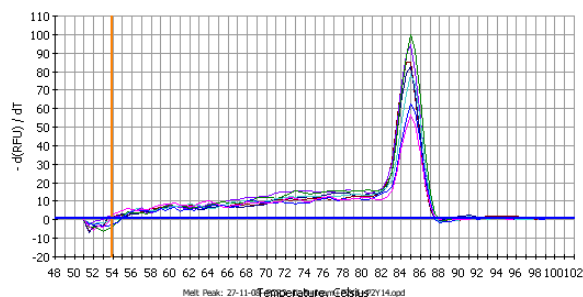## Primer of human P2Y$_{14}$-receptor

Length: 156bp   Annealing temperature: 60°C

Sense: GGCCTCTGCCTTCAGAAGTT   Peak (melting curve): 85°C

Antisense: CCTGACACTCCATTGAGTAG   Intron spanning: Yes (1bp)

Expressed in / Positive control: A549

Melting curve:

Primer of human P2X$_1$-receptor

Length: 102bp

Annealing temperature: 61°C

Sense: GGCTACGTGGTGCAAGAGTC

Peak (melting curve): 88°C

Antisense: GTGCCAGTCCAGGTCACAGT

Intron spanning: Yes

Expressed in / Positive control: Caco-2

Melting curve:

Gel:
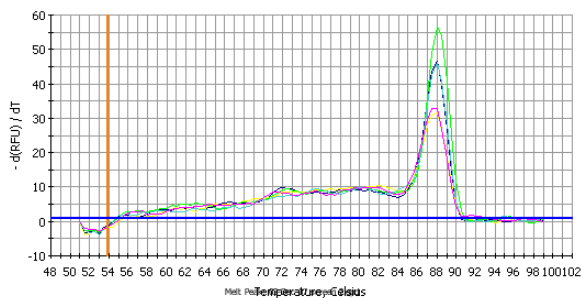
- 4000bp
- 2000bp
- 1250bp
- 800bp
- 500bp
- 300bp
- 200bp
- 100bp
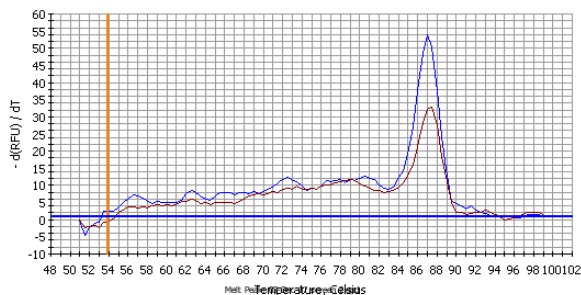
Primer of human P2X$_2$-receptor

Length: 116bp

Annealing temperature: 61°C

Sense: GCTTCACAGAGCTCGCACAC

Peak (melting curve): 87°C

Antisense: GTCAAGCCTCCGGAAGGAGT

Intron spanning: Yes

Melting curve:

Primer of human P2X$_3$-receptor

Length: 140bp

Sense: CCAAGTCGGTGGTTGTGAAG

Antisense: CGAGGACTCAATGGCTGTGT   Intron spanning: Yes

Primer of human P2X$_4$-receptor

Length: 120bp   Annealing temperature: 60°C

Sense: GGATGTGGCGGATTATGTGA   Peak (melting curve): 86,5°C

Antisense: GTGGTCGCATCTGGAATCTC   Intron spanning: Yes

Expressed in / Positive control: HMEC-1

Melting curve: Gel:

Primer of human P2X$_5$-receptor

Length: 120bp

Sense: TGGCGTACCTGGTCGTATGG

Antisense: GATCCGAGGTGTTGGTGAAG
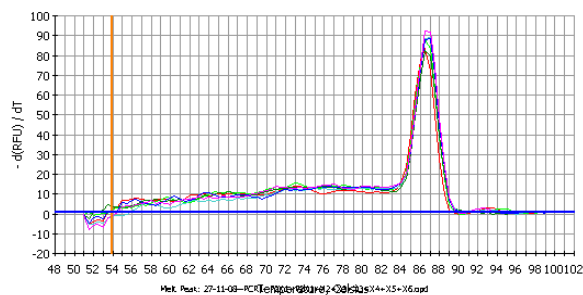
Expressed in / Positive control: HMEC-1
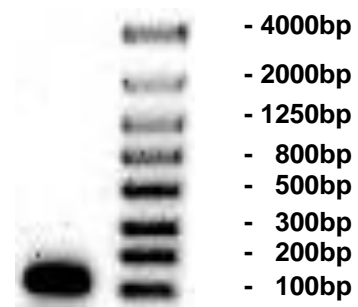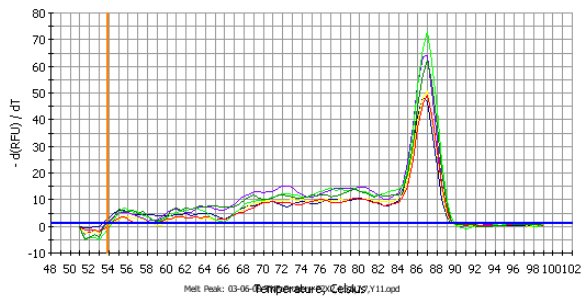
Annealing temperature: 64°C

Peak (melting curve): 87°C

Intron spanning: yes

Melting curve:

Gel:

- 4000bp
- 2000bp
- 1250bp
- 800bp
- 500bp
- 300bp
- 200bp
- 100bp

Primer of human P2X$_6$-receptor

Length: 209bp

Sense: TGACCTGCTACTGCTGTATG

Antisense: ATCCTGGTGTCTGTGTCTGA

Expressed in / Positive control: A549, Caco?
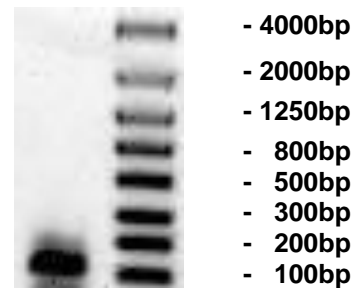
Annealing temperature: 63°C

Peak (melting curve): 92°C

Intron spanning: Yes
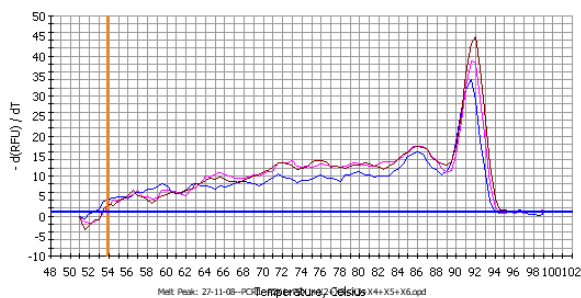
Melting curve:

Primer of human P2X$_7$-receptor

Length: 166bp

Sense: CAAGAGCAGCGGTTGTGTCC

Antisense: GGCACCAGGCAGAGACTTCA

Expressed in / Positive control: HMEC-1

Annealing temperature: 60°C

Peak (melting curve): 87,5°C

Intron spanning: No, in coding
  exon

Melting curve:

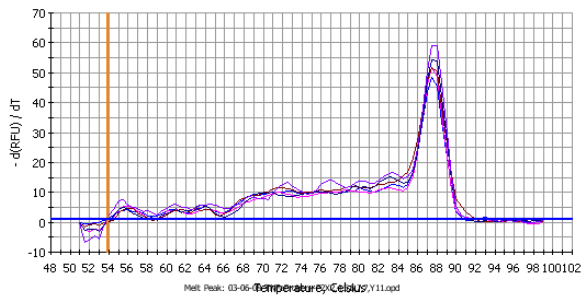## Primer of human VCAM

Length: 229bp

Sense: GACCACATCTACGCTGACAA

Antisense: GTCTATCTCCAGCCTGTCAA

Annealing temperature: 60°C

Peak (melting curve): 85,5°C

Intron spanning: Yes
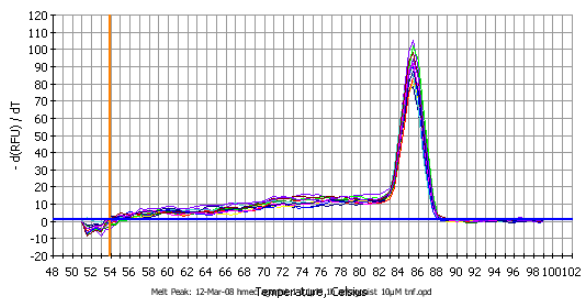
Melting curve:

Primer of human ICAM

Length: 117 bp

Sense: AACCAGAGCCAGGAGACACT

Antisense: GGCCTCACACTTCACTGTCA

annealing temperature: 60°C

Peak (melting curve): 87,5°C

Intron spanning: Yes

Melting curve:

Gel:
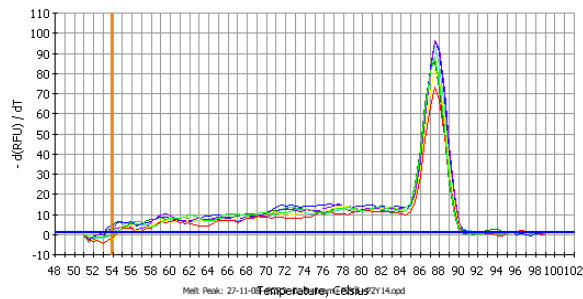
- 4000bp

- 2000bp
- 1250bp
- 800bp
- 500bp
- 300bp
- 200bp
- 100bp

Primer of human IL-8

Length: 171 bp

Sense: CTCTTGGCAGCCTTCCTGAT

Antisense: TTCTGTGTTGGCGCAGTGTG

annealing temperature: 60°C

Peak (melting curve): 85°C

Intron spanning: Yes
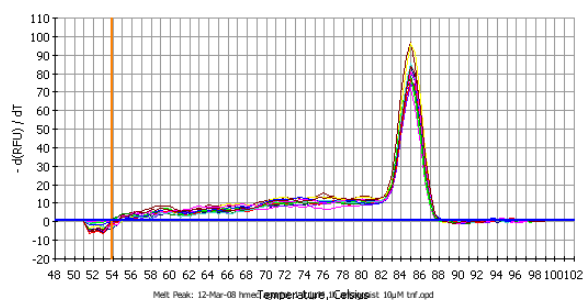
Melting curve:

Gel:

- 4000bp

- 2000bp
- 1250bp
- 800bp
- 500bp
- 300bp
- 200bp
- 100bp

Primer of murine ß-actin

Length: 107 bp

annealing temperature: 58-68°C

Sense: GGCTCCTAGCACCATGAAGA

Peak (melting curve): 86,5°C

Antisense: ACATCTGCTGGAAGGTGGAC
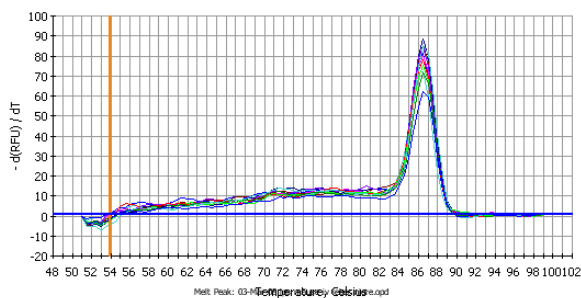
Intron spanning: Yes

Melting curve:

Gel:

- 4000bp
- 2000bp
- 1250bp
- 800bp
- 500bp
- 300bp
- 200bp
- 100bp
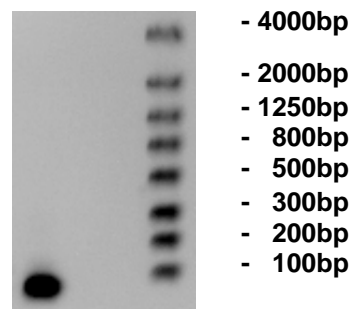
Primer of murine P2Y$_6$-receptor

Length: 265bp

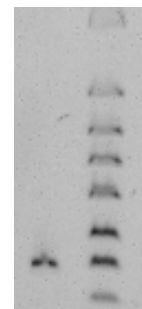annealing temperature: 58-66°C

Sense: AGGCGGTTGCTGTGTCAGAG

Peak (melting curve): 88°C

Antisense: CCTATGCTCGGAGAGTCTGT
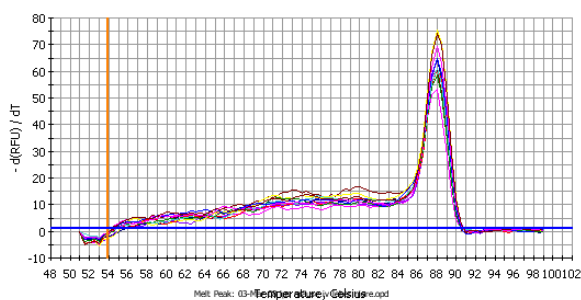
Intron spanning: Yes

Melting curve:

2.5. Microarray

Used materials:	Product No.; Company; Registered office
rHu TNF-α	C-63720; Promokine; Heidelberg, Germany
8th passage HMEC-1 cells	Gift from Francisco Candal
NucleoSpin RNA II	740955.250; Macherey & Nagel; Düren, Germany
Petri dishes	Cellstar, Frickenhausen, Germany
Agilent RNA 6000 Nano Kit	Agilent Technologies; Santa Clara; CA; USA
Agilent 2100 Bioanalyzer platform	Agilent Technologies; Santa Clara; CA; USA
Agilent 2100 Bioanalyzer expert software	Agilent Technologies; Santa Clara; CA; USA
Agilent Low RNA Input Linear Amp Kit	Agilent Technologies; Santa Clara; CA; USA
Agilent Gene Expression Hybridization Kit	Agilent Technologies; Santa Clara; CA; USA
Agilent DNA microarray scanner	Agilent Technologies; Santa Clara; CA; USA
ND-1000 Spectrophotometer	NanoDrop products; Wilmington; DE; USA
Agilent Whole Human Genome Oligo Microarrays 4x44K	Agilent Technologies; Santa Clara; CA; USA
Agilent's hybridization chamber	Agilent Technologies; Santa Clara; CA; USA
Agilent's hybridization oven	Agilent Technologies; Santa Clara; CA; USA
Agilent Gene Expression Wash Buffer 1	Agilent Technologies; Santa Clara; CA; USA
Agilent Gene Expression Wash Buffer 2	Agilent Technologies; Santa Clara; CA; USA
Microarray Scanner System G2505B	Agilent Technologies; Santa Clara; CA; USA
Scan Control Software version A.8.1.3	Agilent Technologies; Santa Clara; CA; USA

| Agilent Feature Extraction Software (FES) version 10.2.1.3 | Agilent Technologies; Santa Clara; CA; USA |
| Rosetta Resolver® gene expression data analysis system | Rosetta Inpharmatics LLC.; Seattle; WA; USA |

Early passage HMEC-1 cells (passage 8) were seeded and grown to confluence. Then cells were exposed to 100ng/ml TNF-α for 4 and 24h. Three separate Petri dishes were pooled and total RNA was isolated as described in chapter 2.1.3 "RNA isolation". Probes were shipped on dry ice to Miltenyi Biotec, where the further parts of the experiments were performed.

Via the Agilent 2100 Bioanalyzer platform, RNA samples were quality-controlled on the one hand by visualization in a gel image and electropherogramm and on the other hand by the generation of an RNA Integrity Number (RIN), which provides information about integrity and overall quality of the RNA samples. According to the manufacturer, RNA with a RIN >6 is considered sufficient quality for microarray experiments. In our experiments, all RNA samples revealed a RIN between 9,9 and 10.

As starting material, 1µg of total RNA per sample was utilized. Control samples were labeled with Cy3, experimental samples were labeled with Cy5 using the Agilent Low RNA Input Linear Amp Kit according to the manufacturer's protocol. Dye incorporation rate and cRNA output were quantified with the ND-1000 Spectrophotometer. Hybridization was conducted with the use of the Agilent Gene Expression Hybridization Kit according to the Agilent 60-mer oligo microarray processing protocol. 825ng of cRNA of each sample were combined and hybridized for 17 hours at 65°C to Agilent Whole Human Genome Oligo Microarrays 4x44K with the use of Agilent's recommended oven and hybridization chamber.

At the end, the microarrays were washed several times. The first time they were washed with 6x SSPE buffer containing 0,005% N-lauroylsarcosine for 1 minute at room temperature, the second time with pre-heated 0,06x SSPE buffer at 37°C containing 0,005% N-lauroylsarcosine for another minute. Finally the microarrays were washed with acetonitrile for 30 seconds.

Signal detection was performed with Agilent's DNA microarray scanner system G2505B with the Scan Control Software version A.8.1.3. The 2 scanned monochrome images for the dyes Cy3 and Cy5 of each microarray are combined to create the "false color" image of the microarray shown in Illustration 27. In this context "false color" means, that the measured intensities for the dyes Cy3 and Cy5 are translated into an image with green, yellow and red spots for the analyst to read.
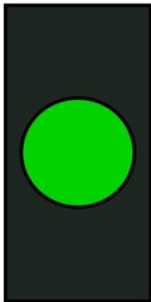
Green spot:

Polynucleotides expressed in the control sample, labeled with the green Cy3, hybridized abundantly to this spot on the microarray. Whereas in the experimental sample, labeled with the red Cy5, the polynucleotide isn't expressed.
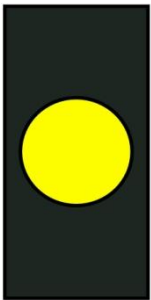
Yellow spot:

Polynucleotides, which bind to this spot, are expressed in both, the green Cy3 labeled control sample and the red Cy5 labeled experimental sample.
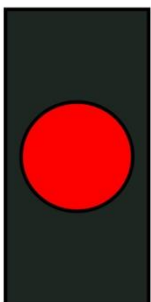
Red spot:

Polynucleotides expressed in the experimental sample, labeled with the red Cy5, hybridized abundantly to this spot on the microarray. Whereas in the control sample, labeled with the green Cy3, the polynucleotide isn't expressed.

Black spot:

Neither polynucleotides expressed in the control sample nor in the experimental sample hybridized abundantly to this spot on the microarray.
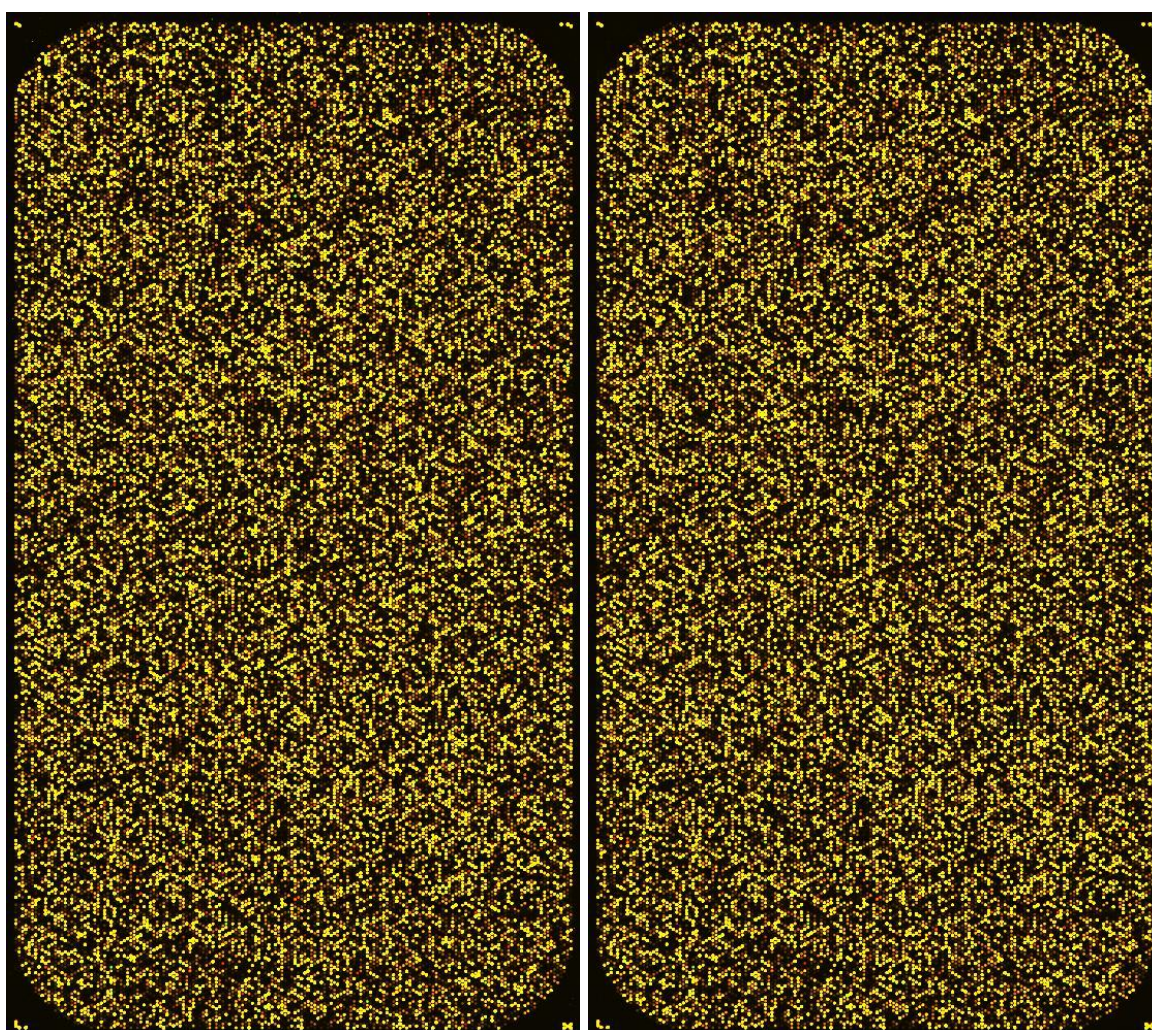
HMEC-1 Control vs. TNF-α 4h   HMEC-1 Control vs. TNF-α 24h

Illustration 27: *"False color" image of microarrays.*

Images were then read out and quantified using Agilent Feature Extraction Software version 10.2.1.3. Feature Extraction was performed according to the Agilent Feature Extraction Software Reference Guide v9.5. Linear and Lowess data normalization was performed.

Received data were arranged and published on the GEO platform GPL 4133 of NCBI over GEO archive. Therefore, the raw data file for 4h TNF-α stimulated cells (US22502695_251485035141_S01_GE2-v5_95_Feb07_1_1.txt) and the raw data files for 24h TNF-α stimulated (US22502695_251485035141_ S01_GE2-v5_95_Feb07_1_3.txt) were submitted to NCBI GEO. Selected data were provided in a Matrix table:

- ID_REF: Agilent Feature Extraction Feature Number
- VALUE :   Linear-lowess normalized log 10 ratio =
  Cy5 [stimulated cells] /Cy3 [control]
- LogRatioError: Log ratio error of linear-lowess normalized
  log10 ratio
- PValueLogRatio: Significance level of the Log Ratio computed for a
  Feature.
- gProcessedSignal: The Cy3 (green) signal left after all the feature
  extraction processing steps have been completed.
- rProcessedSignal: The Cy5 (red) signal left after all the feature
  extraction processing steps have been completed
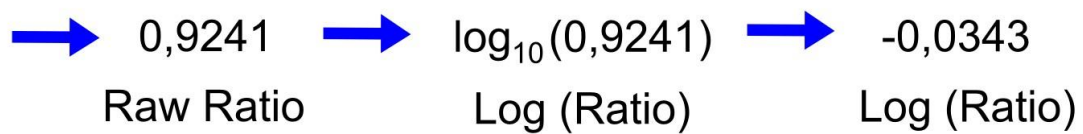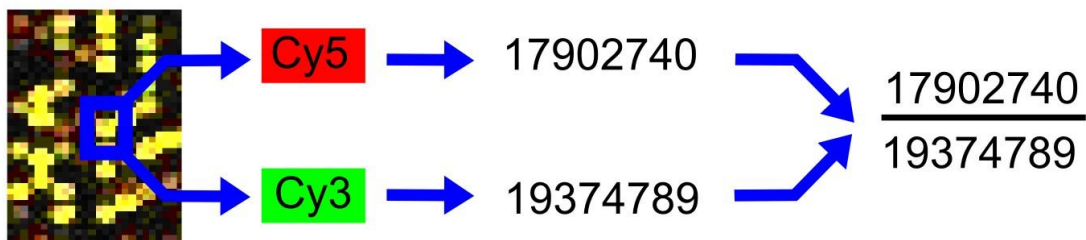
Illustration 28: *Calculation of Log (Ratios)*

Background signals (bright and dark corners) were labeled as "null" through Excel programming according to the requirements of NCBI GEOI. Microsoft Visual Basic was opened through the key combination "Alt" + "F11". Measured values in rows of "dark corners" were substituted through following text per each desired column.

```
Sub labelnullDC()
    For i = 1 To 46000
        If Tabelle1.Cells(i, 2) = "DarkCorner" Then
            Tabelle1.Rows(i, 3) = "null"
        End If
    Next i
End Sub
```

Measured values in rows of "bright corners" were substituted through following text, per each desired column.

```
Sub labelnullBC()
   For i = 1 To 46000
      If Tabelle1.Cells(i, 2) = "BrightCorner" Then
         Tabelle1.Cells(i, 3) = "null"
      End If
   Next i
End Sub
```

2.6.   Western Blot

Used materials:	Product No.; Company; Registered office
Skim milk	A0830.0500; AppliChem; Darmstadt, Germany
Magic Mark XP Western Standard	LC5602; Invitrogen; Carlsbad, CA, USA
SeeBlue Plus 2, Prestained Standard	LC5925; Invitrogen; Carlsbad, CA, USA
Immun-Blot-PVDF-Membrane	162-0177; Bio-Rad Laboratories, Inc.; Munich, Germany
Petri dishes	Cellstar, Frickenhausen, Germany
Restore Western Blot Stripping Buffer	21059; Pierce; Bonn, Germany
Tris Glycine Buffer 10x	161-0771; Bio-Rad Laboratories, Inc.; Munich, Germany
Tris Glycine SDS Buffer 10x	161-0772; Bio-Rad Laboratories, Inc.; Munich,

	Germany
Triton$^{®}$ X-100	Sigma; Taufkirchen, Germany
Cell Scraper	Costar, NY, USA
Eppendorf Cups	Eppendorf AG, Hamburg, Germany
Gel-Cassettes	Invitrogen, Karlsruhe, Germany
BCA Protein Assay Kit	23225; Pierce; Bonn, Germany
Complete	11836145001; Roche; Basel; Switzerland
Tween$^{®}$	AppliChem; Darmstadt; Germany
Microtiter Plate 96 well	nunc$_{TM}$, Brand Products, Denmark
Methanol	1.06009.2511; Merck; Darmstadt; Germany
EDTA	Sigma; Taufkirchen, Germany
NaCl	Merck; Darmstadt; Germany
Tris-base (pH7.4)	AppliChem; Darmstadt; Germany
H_2O_2	1.08597; Merck; Darmstadt; Germany
KCl	4936; Merck; Darmstadt; Germany
TrisHCl	A3452; AppliChem; Darmstadt; Germany
p-Coumeric-acid	C9008; Sigma; Taufkirchen, Germany
Luminol	O9253; Sigma; Taufkirchen, Germany
$NaHPO_4$	AppliChem; Darmstadt; Germany
Ammoniumpersulfate	A9164; Sigma; Taufkirchen; Germany
Sodium dodecyl sulphate, 99%	L4390; Sigma: Taufkirchen; Germany
Rotiphorese® Gel = 30% Acrylamide	3029.1; Roth; Karlsruhe; Germany
Igepal	I-7771; Sigma; Taufkirchen, Germany
Bromphenol blue	A33640; Applichem; Darmstadt; Germany
Temed	T9281;Sigma; Taufkirchen, Germany
Beta-Actin (13E5) Rabbit mAb	4970; Cell Signaling; Danvers, MA, USA
Anti human P2Y$_6$ receptor antibody (H-70) rabbit polyclonal IgG	Sc-20127; Santa Cruz Biotechnologies; Santa Cruz, CA, USA
Goat anti-rabbit IgG-HRP	Sc-2004; Santa Cruz Biotechnologies; Santa Cruz, CA, USA

Novex Mini-Cell	Invitrogen; Carlsbad, CA, USA
Mini Trans-Blot Electrophoretic Transfer Cell	170-3930; Bio-Rad Laboratories, Inc.; Munich, Germany
GENios microplate reader	Tecan Group Ltd.; Männedorf; Germany
Magellan software	B01760001; Tecan Group Ltd.; Männedorf; Germany
Restore Western Blot Stripping Buffer	Pierce, Bonn, Germany
Diana luminescence imaging system	Raytest; Straubenhardt, Germany

HMEC-1 were grown to confluence and were exposed for indicated times to 10ng/ml of TNF-α. Thereafter, Petri dishes were placed on ice, supernatant was removed quickly and pre-cooled RIPA buffer with 25x complete was added. Cells were immediately scraped and transferred to a sterile tube. Subsequently, cells were lysed for 45min at 4°C on a tube rotator in a cold room. After spinning for 15min at 12,000g to remove cell debris, the supernatant was transferred to a fresh tube. Protein concentrations were determined using Pierce BCA Protein Assay kit and Magellan Reader. Concentrations were adjusted with RIPA buffer and non-reducing 4 fold sample buffer was added. Samples were heated to 70°C for 10 min and either frozen at -20°C or directly used on a 12% polyacrylamide gel for SDS-gelelectrophoresis. Electrophoresis was performed with 110V for 90min using Novex Mini-cell chambers and Running buffer. Gels were quarried out of the gel cassettes and were laid down on PVDF membranes, which were preincubated for 1 min in acetone and 10min in running buffer. On both sides, two layers of filter paper were added. The transfer of the proteins was performed in a Mini Trans-Blot Electrophoretic Transfer Cell with 110V for 60min. The membranes were blocked for 1 h at room temperature in blocking buffer and incubated with polyclonal rabbit anti-human P2Y$_6$ receptor antibody over night at 4°C. The P2Y$_6$-receptor antibody was diluted 1:200 in blocking buffer. After washing the PVDF membrane in 0.1% TBST 3 times for 5 minutes, membranes were incubated with the HRP-

conjugated goat anti-rabbit IgG secondary antibody (SantaCruz, CA, USA) for 1h at room temperature. After incubation, the membranes were washed again 3 times for 5 minutes. The wash was repeated and proteins were detected by enhanced chemiluminescence. Therefore the ECL-detection buffer was added on the membranes for 1,5min.

As a control for equal protein loading, membranes were stripped and reprobed for ß-actin using rabbit anti-human ß-actin monoclonal antibody and the above mentioned secondary antibody.

Preparation of buffers and reagents for Western Blotting:

RIPA-buffer:

Tris-Base (MW: 121,14g/mol)	3,95g
NaCl	4,5g
1M HCl	For adjustment of pH to 7,4
10% Igepal	50ml
500mM EDTA	1ml
Aqua dest.	500ml

We added 3,95g TRIS-base and 4,5g NaCl to 375ml of Aqua dest.. The solution was stirred until all solids were dissolved. Thereafter the pH was adjusted to 7,4 with 1M HCl. 50ml of 10% Igepal was added, as well as 1ml of 500mM EDTA. The volume was adjusted to 500ml and the RIPA buffer was either stored at +4°C for up to 2 weeks or was frozen at -20°C for long term storage.

Lower-buffer for SDS Page, pH 8,8:

1,5M Tris-Base (MW: 121,14g/mol)	90,85g
0,4% SDS	2,0g
500ml Aqua dest.	

90,85g of 1,5M TRIS-Base and 2,0g of 0,4% SDS were added to 500ml Aqua dest. Adjustment of pH was performed with HCl and NaOH to pH 8.8.

Upper-buffer for SDS Page, pH 6,8:

0,5M Tris-Base (MW: 121,14g/mol)   30,3g

0,4% SDS   2,0g

500ml Aqua dest.

30,3g of 0,5M TRIS-Base and 2,0g of 0,4% SDS were added to 500ml Aqua dest.. Adjustment of pH was performed with HCl and NaOH to pH 8.8.

Preparation of gels:

Separating gel (12% acrylamide):

30% Acrylamide   10ml ($\triangleq$ 12%)

Lower buffer   10,8ml

Aqua dest.   13,2ml

10% APS   200µl

TEMED   80µl

A 12% acrylamide gel was used to ensure optimal protein separation for proteins between 20 and 50 kDa (MW of $P2Y_6$-receptor = 36kDa). Firstly, we prepared cassettes and ridges for 1,5mm gels. After that, we added the acrylamide and lower buffer to the Aqua dest. Subsequently we added 200µl of APS and 80µl of TEMED. Because of fast polymerization, the solution was poured into the gel cassettes with the use of 10ml stripettes. Immediately a layer of aqua dest. was added on top of the separating gel and the gel was allowed to polymerize for 90min at room temperature.

Stacking gel (5% acrylamide):

30% Acrylamide	2,5ml ($\triangleq$ 5%)
Upper buffer	3,8ml
Aqua dest.	8,7ml
10% APS	60µl
TEMED	20µl

After polymerization of the stacking gel, the stacking gel was prepared. Acrylamide and lower buffer were added to the Aqua dest., as well as 60µl of APS and 20µl of TEMED. Aqua dest. layer on the top of the separating gel was removed and the gel cassette was filled up with stacking gel. The ridge was inserted and the gel was allowed to polymerize another 60min at room temperature. If not immediately used, the gel was stored at +4°C up to one week. For this purpose, the gel was airtight welded in foil with approximately 10ml of running buffer.

Loading-buffer (4x) (non-reducing):

1M Tris HCl pH 6,8	2,5ml
Glycerol	4ml
SDS	0,8g
Bromphenolblue	0,01g
Aqua dest.	1ml

For the preparation of the loading-buffer a mask was used because of the weighing of the SDS. Substances were added together and stored in aliquots at 4°C.

<u>Running buffer:</u>

200ml 10x Tris/Glycin/SDS buffer and 1800ml Aqua dest. were merged.

<u>Transfer buffer:</u>

200ml 10x Tris/Glycin buffer, 400ml Methanol and 1400ml Aqua dest. were merged.

<u>TBS 10x, pH 7,6:</u>

24,2g Tris-Base and 80g NaCl were dissolved in 1l aqua dest..

<u>0,5% TBST:</u>

100ml of TBS 10x were diluted with 900ml aqua dest. 500µl of Tween were added and solution was stirred.

<u>Blocking-buffer:</u>

1,5g of skim milk were added to 50ml of 0.1% TBST resulting in a 3% skim milk containing blocking buffer.

<u>ECL detection system:</u>

250mM Luminol	0,44g in 10ml DMSO
90mM p-Coumeric acid	0,15g in 10ml DMSO

Luminol and p-Coumeric acid were dissolved in DMSO and aliquotted in 50µl aliquots (Luminol) and 22µl aliquots (p-Coumeric acid). Aliquots were stored at -20°C.

Solution A:

1M Tris-HCl pH 8,5	500µl
Aqua dest.	4,4ml
250mM Luminol	50µl
90mM p-Coumeric acid	22µl

Solution B:

1M Tris-HCl pH 8,5	500µl
Aqua dest.	4,5ml
H_2O_2	3µl

Solution A and B had to be freshly prepared before application and had to be stored in the dark after preparation. For detection of a western blot membrane we mixed 2ml of each solution and incubated the membranes for 1,5min. After the incubation period, the membranes were immediately scanned at the luminometer (Diana).

2.7. Transfection and Reporter Assay

Used materials:	Product No.; Company; Registered office
Gene Juice Transfection Reagent	70967; Novagen / Merck; Darmstadt, Germany
Luciferase Assay System	E1501; Promega; Madison, WI, USA
Passive Lysis Buffer 5x	E1941; Promega; Madison, WI, USA
Disposable Cuvettes	E2371; Promega; Madison, WI, USA
pNF-κB-Luc	631904; Clontech; Mountain View, CA, USA

Luciferase Assay Substrate	E151A; Promega; Madison; WI; USA
Luciferase Assay Buffer	A152A; Promega; Madison; WI; USA
TD-20/20 Luminometer	Turner BioSystems, Inc.; Sunnyvale, CA, USA
Spreadsheet interface software	Turner BioSystems, Inc.; Sunnyvale, CA, USA
MRS2578	2146, Tocris, Bristol, UK
DMSO	Sigma, Taufkirchen, Germany
rHu TNF-α	C-63720; Promokine; Heidelberg, Germany
Cellstar 24 well plates	Greiner bio-one; Frickenhausen, Germany

Transfection was performed in 24 well plates with GeneJuice transfection reagent. Per well 0,75µl transfection reagent were added to 20µl serum free medium and were mixed thoroughly by vortexing (for 4x 24well plates: 2ml SFM and 75µl transfection reagent). The mixture was incubated for 5 min at room temperature. For each well, 0,25µg plasmid DNA (NF-κB reporter plasmid, positive or negative control) were added and mixed by gentle pipetting. GeneJuice/DNA mixture were incubated for 10min at room temperature and filled up to 500µl serum free medium per well. Medium on 24well plates was removed and substituted by 500µl of GeneJuice/DNA mixture.

4h after transfection, reagents were removed. Normal cell culture medium or medium with 10µM P2Y$_6$-receptor antagonist MRS-2578 or medium with the equal amount of the solvent DMSO were added on the cell layer. After 30min of preincubation, cells were either stimulated with TNF-α or the equal amount of solvent (PBS with 0,1%BSA) was added to the medium. After the indicated time points, cells were washed twice and 100µl of 1x passive lysis buffer were added per well. Additional mechanical lysis was performed. Plates were done on a plate shaker for at least 15min at room temperature. Cells were mixed well by pipetting and 20µl cell suspension were added in 100µl of LAR II. Again, mixture was performed by pipetting up and down. Luciferase activity was measured by a luminometer and subsequently total protein concentrations were determined using Pierce BCA Protein Assay kit and Magellan Reader.

The NF-κB plasmid used in the transfections contained the NFκB response element NF-κB 1 = GGGAATTTCC, which is inserted four fold one after another.

2.8.   Immunofluorescence

Used materials:	Product No.; Company; Registered office
Skim milk	A0830.0500; AppliChem; Darmstadt, Germany
Glass Slides	NalgeNuc International, Naperville, IL, USA
Methanol	1.060018; Merck; Darmstadt; Germany
Anti human P2Y$_6$ receptor antibody (H-70) rabbit polyclonal IgG	Sc-20127; Santa Cruz Biotechnologies; Santa Cruz, CA, USA
Alexa Fluor 488 goat anti-rabbit IgG antibody	Invitrogen; Carlsbad, CA, USA
Prolong® Gold Antifade Reagent	P36930; Invitrogen; Carlsbad, CA, USA
Confocal microscope	Zeiss; Jena, Germany
Zen software	Zeiss; Jena, Germany
DPBS 1x with Ca^{2+} and Mg^{2+}	14040; Gibco; Gaitherburg, MA, USA

HMEC-1 were seeded on glass coverslips and stimulated with 10ng/ml TNF-α. After 24h of incubation, cells were fixed with cooled methanol (-20°C) for 10min. Cells were washed with PBS and blocked with 0.1% TBST and 3% skim milk (see "2.6 Western Blot") for 1h at room temperature. 5µg/ml anti-human P2Y$_6$-receptor antibody diluted in 0.1% TBST and 1,5% skim milk, 5µg/ml negative control rabbit immunoglobulin fraction or 1,5% skim milk without antibodies was administered for 2h. After the incubation period, cells were washed three times

and incubated with Alexa Fluor 488 goat anti-rabbit IgG antibody for 45min in the dark to prevent fluorochrome fading. Again, cells were washed three times with PBS and glass coverslips were transferred onto microscope slides. Subsequently 20µl Prolong® Gold Antifade Reagent was added on each glass coverslips and probes were sealed under further glass coverslips. Probes were kept in the dark and on ice until they were measured by confocal laser scanning.

2.9.   Intravenous LPS injection

Used materials:	Product No.; Company; Registered office
C57Bl/6 mice	Own breeding
LPS from E.coli O26:B6	L-8274; Sigma; Taufkirchen
Ketamin ratiopharm® 50mg/ml	Ratiopharm; Ulm; Germany
Atropinsulfat 0,5mg/ml	566/0403;B.Braun; Melsungen, Germany
Rompun® 2% = Xylazin	Bayer Vital GmBH; Leverkusen; Germany
1ml syringes, BD Plastipak^{TM}	300013; Becton Dickinson; Franklin Lakes; New Jersey; USA
10ml syringes	B.Braun; Melsungen, Germany
25 G needles, BD Microlance 3	300400; Becton Dickinson; Franklin Lakes; New Jersey; USA
27 G needles; BD Microlance 3	302200; Becton Dickinson; Franklin Lakes; New Jersey; USA
0,9% NaCl	B.Braun; Melsungen, Germany
Coagulator	Erbe Elektromedizin, Tübingen, Germany
Bipolar forceps	Erbe Elektromedizin, Tübingen, Germany
5-0 Vicryl filaments	B.Braun; Melsungen, Germany

All animal protocols were in accordance with the German guidelines for Use of

Living Animals and were approved by the Institutional Animal Care and Use Committee of the Universtity Hospital and the Regierungspräsidium Tübingen. All mice were matched by sex, age and weight. C57BL/6 mice weighing 20-25g were used.

Prearrangement:

Preparation of anaesthetic:

200µl of Ketamine, 40µl of Atropine and 50µl of Rompun were drawn up in a 1ml syringe. 0,9% NaCl was added until a total volume of 1ml was achieved.

Preparation of LPS stock solution:

A LPS stock solution of 2,5mg/ml was prepared. 100mg of LPS from E.coli O26:B6 were solubilized in sterile 0,9% NaCl. Stock solution was either prepared immediately before the experiment and then stored in the refrigerator at +4°C between the injections or was frozen at -20°C for subsequent experiments.

Intravenous LPS Injection in C57Bl/6 mice:

Animals received 200µl of anaesthetic solution intraperitoneally. After their loss of consciousness, the left side of the neck was shaved and disinfected. An incision of the neck from left-lateral-apical to right-medial-dorsal was performed and fatty tissue and small fasciae were removed to have a good view on the left jugular vein.

Illustration 29: *Preparation of murine jugular vein.*

The area of preparation was set up under the microscope and 200µl of NaCl 0,9% or LPS (300µg/200µl) were injected. Complete injection volume was monitored under the microscope. Immediately after removing the syringe, the jugular vein was coagulated with a bipolar forceps.
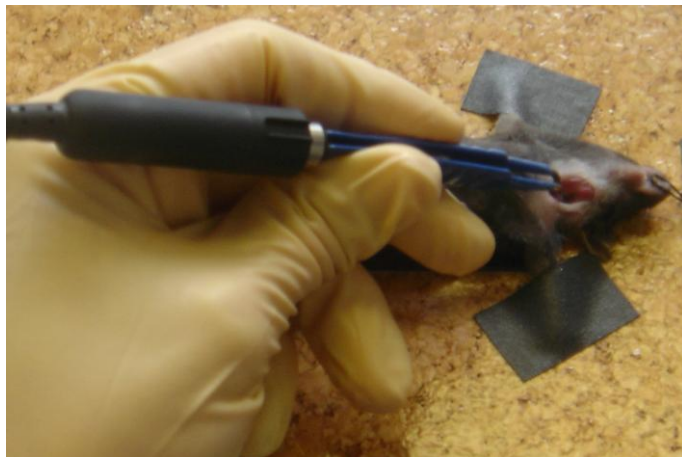

Illustration 30: *Coagulation of murine jugular vein after intravenous injection.*

The tissue was closed with 5-0 Vicryl filament with continuous stitches.

24h after administration of LPS mice were killed with an overdose of anesthetic and were exsanguinated with 10ml of 0.9%NaCl. Organs were immediately frozen in liquid nitrogen. RNA extraction, transcription and PCR were performed as described in "2.3 RNA-Isolation, transcription, PCR". For PCR murine ß-actin and murine $P2Y_6$-receptor primer were used. They are listed in the chapter "2.4 Primer".

2.10. Statistical Analysis, programming, illustrations and references

Used materials:	Product No.; Company; Registered office
Prism 4 for Windows Version 4.03	Graphpad Software Inc.; San Diego, CA, USA
Microsoft Office Professional Edition 2003	Microsoft Corporation; Redmond, WA, USA
AIDA software	Raytest; Straubenhardt, Germany
Gene expression macro	Bio-Rad Laboratories, Inc.; Munich, Germany
Inkscape	Open-source vector graphic editor
EndNote8	Thomson Reuters

First, PCR results were calculated in the gene expression macro from Bio-Rad Laboratories. Following statistical analysis of PCR results was then performed with the use of Microsoft Excel and Graphpad Prism. Standard deviation was calculated in Excel, p-values as well as upper and lower 95% confidence limit were calculated by Graphpad Prism by an unpaired t-test.

The western blot was evaluated with AIDA software. Illustrations in this document were drawn with the open-source vector graphic editor inkscape. As a reference manager, EndNote8 was used.

3. Results

3.1. The endothelial P2Y$_6$-receptor is selectively induced after TNF-α exposure

Since P2 receptors are known to have multiple functions on immune cells as well as vascular cells under inflammatory conditions, and are therefore suspected to be important molecules in regulating inflammatory responses, we pursued transcriptional responses of previously described nucleotide receptors in vascular endothelia. We gained first insight from a microarray study performed by Dr. Rosenberger. HMEC-1 endothelia cells were exposed to 100 ng/mL TNF-α for 24h. For the purpose of studying extracellular nucleotide signaling, we compiled all data for known nucleotide P2Y and P2X receptors (Table 11; http://www.ncbi.nlm.nih.gov/geo/ query/acc.cgi?acc = GSE18102). Surprisingly, these studies showed a selective induction of the P2Y$_6$ receptor transcript after TNF-α stimulation. As such, this screening experiment pointed us toward a potential role of P2Y$_6$ as a candidate receptor for nucleotideelicited alteration of vascular inflammation.

Table 11: *P2Y$_6$-receptor is upregulated in microarray results.*
Human microvascular endotelial cells (HMEC-1) were exposed to 100ng/ml
TNF-α for 4 and 24h. For each Microarray, 3 different samples were pooled.
RNA was isolated and an Agilent Whole Human Genome Microarray was
performed by MACS molecular.

Gene	Fold Induction (4h)	Fold Induction (24h)	Agilent Array Designation
P2RY$_1$	0,76	0,97	A_23_P382835
P2RY$_2$	1,22	1,24	A_23_P24903
P2RY$_4$	1,24	1,08	A_23_P114307
P2RY$_6$	1,50	2,06 *	A_23_P64611
P2RY$_{11}$	0,83	0,90	A_23_P4696
P2RY$_{12}$	1,07	0,66	A_23_P143902
P2RY$_{13}$	0,76	0,95	A_23_P211948
P2RY$_{14}$	0,80	0,95	A_24_P165864
P2RX$_1$	0,91	1,04	A_23_P372848
P2RX$_2$	0,96	1,14	A_23_P124003
P2RX$_3$	1,01	1,08	A_23_P127721
P2RX$_4$	0,82	1,17	A_23_P53623
P2RX$_5$	0,81	0,84	A_23_P413760
P2RX$_6$	0,94	0,98	A_23_P355980
P2RX$_7$	0,72	0,92	A_24_P319113

Intrigued by this observation, we used RT-PCR to confirm our initial microarray findings. For this purpose, we stimulated HMEC-1 cells with TNF-α over 24 hours and assessed transcript levels of all known P2 receptors (Figure 1). Consistent with our microarray studies, we observed a selective and robust (4.2 $\pm$ 2; $P < 0.05$) induction of P2Y$_6$ receptor transcript.
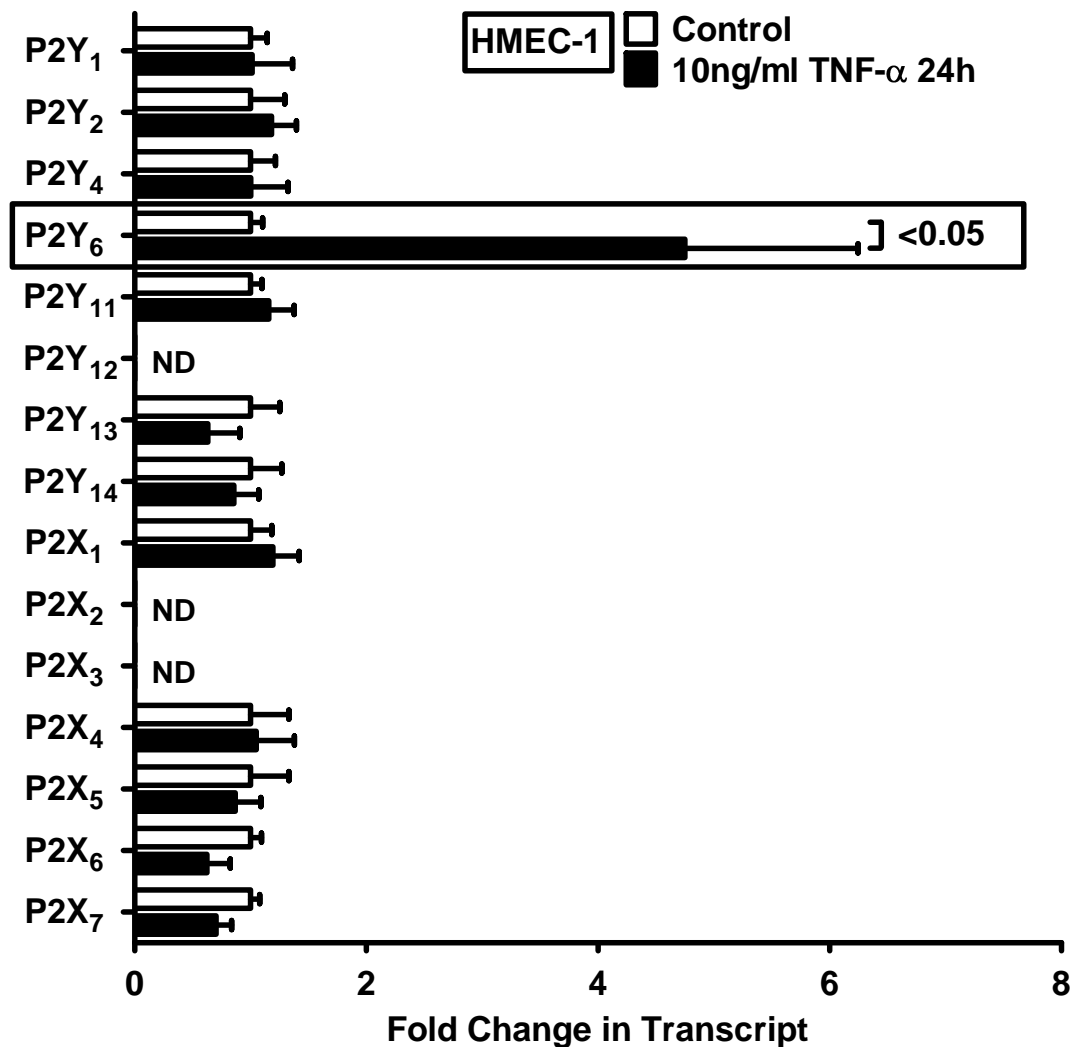
Figure 1: *Endothelial P2 receptor expression after inflammatory stimulation.*
Human microvascular endothelial cells (HMEC-1) were exposed to 10ng/ml TNF-α for 24h. Receptor expression levels of the 15 known P2 receptors were determined in controls and TNF-α stimulated cells by real-time RT-PCR (n= three to four separate experiments). Data were calculated relative to the internal housekeeping gene β-actin and are expressed as mean ± SD fold change compared to control.

To confirm these these initial screening experiments, we exposed cultured endothelial cell lines (HMEC-1 and HCAEC) to different doses and time periods of TNF-α stimulation and assessed $P2Y_6$ transcriptional responses.

Here, we found time- and dose-dependent increases of $P2Y_6$ transcript levels in HCAECs (Figure 2A-B) and in HMEC-1 cells (Figure 2C-D). Moreover, P2Y6 receptor induction was also observed on IL-1α or LPS exposure of HMEC-1 cells (Figure 2E).

In addition, $P2Y_6$ protein levels after inflammatory stimulation of vascular endothelia were elevated with the use of immunofluorescence (Figure 2F) or Western blotting (Figure 2G).

*__Figure 2:__ Endothelial $P2Y_6$ receptor upregulation after TNF-α stimulation.*
HCAECs (A-B) or HMEC-1 cells (C-D) were exposed to TNF-α for the indicated time periods or with indicated doses. $P2Y_6$ receptor transcript was determined by real-time RT-PCR. Data were calculated relative to the internal housekeeping gene (β-actin) and are expressed as mean $\pm$ SD fold change compared with controls (without TNF-α) (n= 3-4). (E) HMEC-1 cells were exposed to LPS, IL-1α, and TNF-α for 24 hours. (F) HCAECs were grown to confluence on cover glasses and exposed to 10 ng/mL TNF-α for 24 hours. Cell layers were stained with antibodies specific for human $P2Y_6$ receptor and Alexa Fluor 488-coupled secondary antibody (green) or isotype controls and Alexa Fluor 488-coupled secondary antibody or Alexa Fluor 488–coupled secondary antibody only. DAPI (4',6-diamidino-2-phenylindole) was used as nuclear counterstain (blue). Slides were kept on ice before image acquisition. Probes were analyzed by confocal microscopy with the use of Zeiss Laser Scanning Microscope LSM 510 and the Plan-Apochromat 63x/1.40 Oil Dic M27 objective lens with oil immersion. Zen software was used for acquisition and image processing (γ adjustment) applied equally to all images. One representative image of 3 is displayed. (G) HMEC-1 cells were exposed to TNF-α for the indicated time periods, and $P2Y_6$ receptor protein was determined by Western blotting. The same blot was stripped and reprobed for human β-actin as a control for protein loading. One of 3 representativeWestern blots is displayed.
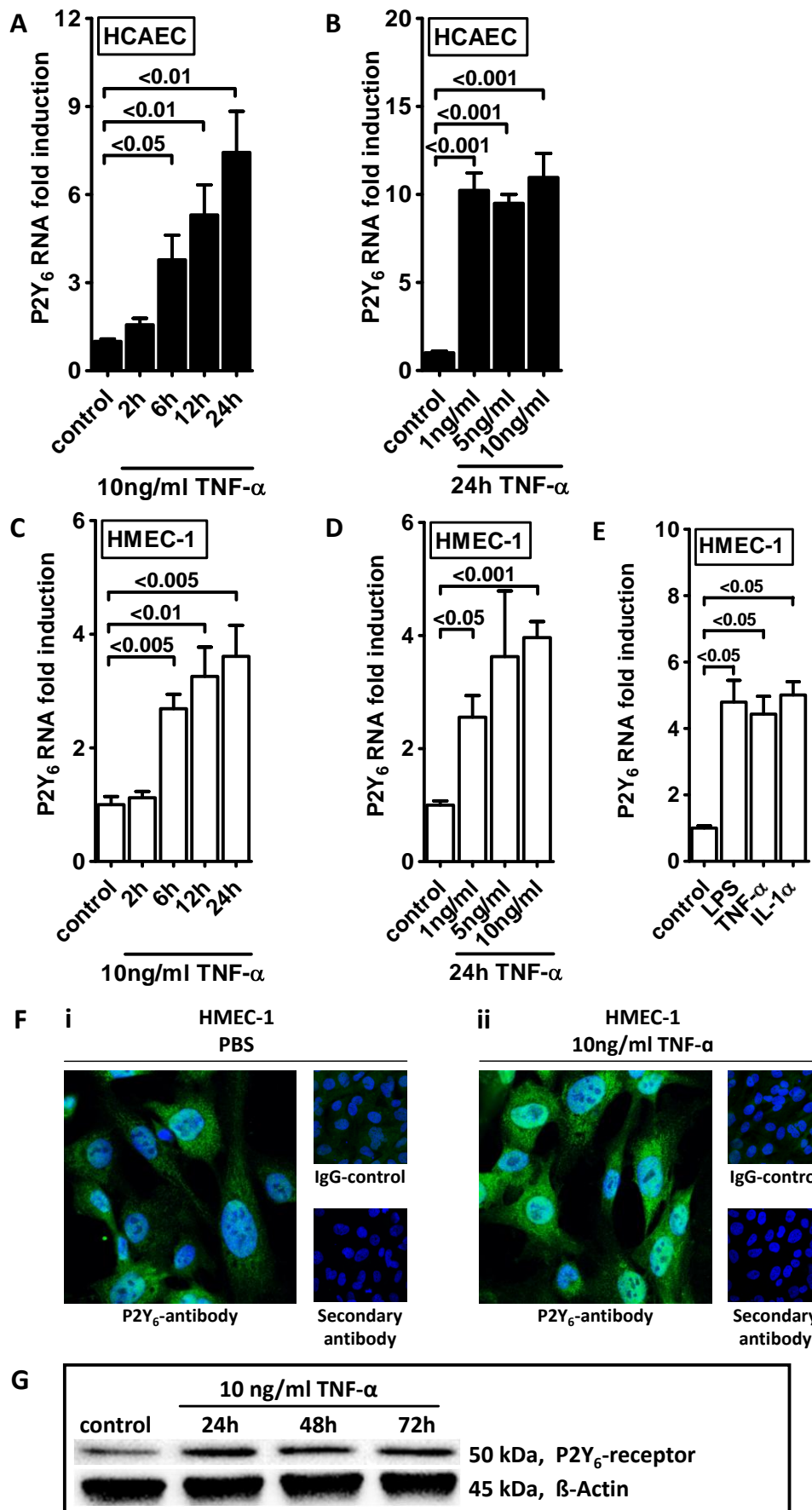
3.2. P2Y$_6$-receptor antagonist MRS2578 dampens mediator-induced inflammation of vascular inflammation of vascular endothelia in vitro

After having shown that P2Y$_6$ receptor transcript and protein expressions are selectively increased on inflammatory stimulation, we next studied functional consequences of endothelial P2Y$_6$ signaling in vitro. As in the in vitro model, we transfected HMEC-1 cells with a NF-κB reporter plasmid containing the binding sites for p50/p65. Treatment of HMEC-1 cells with the P2Y$_6$ agonist uridine diphosphate only resulted in a modest increase in NF-κB activity (1.64-fold $\pm$ 0.45; P <0.05; data not shown). However, and consistent with previous studies implicating P2Y$_6$ signaling in NF-κB activity [103,163] we observed a profound inhibition of basal NF-κB activity in the presence of the P2Y$_6$ antagonist MRS 2578. This reduction of NF-κB activity was time (Figure 3A) and dose (Figure 3B) dependent. As such, these studies indicate that downstream targets of P2Y$_6$ activation are necessary but not sufficient for NF-κB activation and speak for an indirect effect in enhancing vascular inflammation. Next, we examined if NF-κB activation by proinflammatory mediators (eg, TNF-α) was attenuated after pharmacologic inhibition of the P2Y$_6$ receptor. For this purpose, we again used HMEC-1 endothelia that were transfected with a NF-κB reporter. Although treatment with 10 ng/mL TNF-α significantly increased NF-κB reporter activity in media alone or in vehicle-treated cells (DMSO), this response was completely abolished in HMEC-1 cells pretreated with the P2Y$_6$ receptor antagonist MRS 2578 (Figure 3C). Together, these studies show a profound inhibition of TNF-α -induced NF-κB activation after pharmacologic blockade of the P2Y$_6$ receptor.
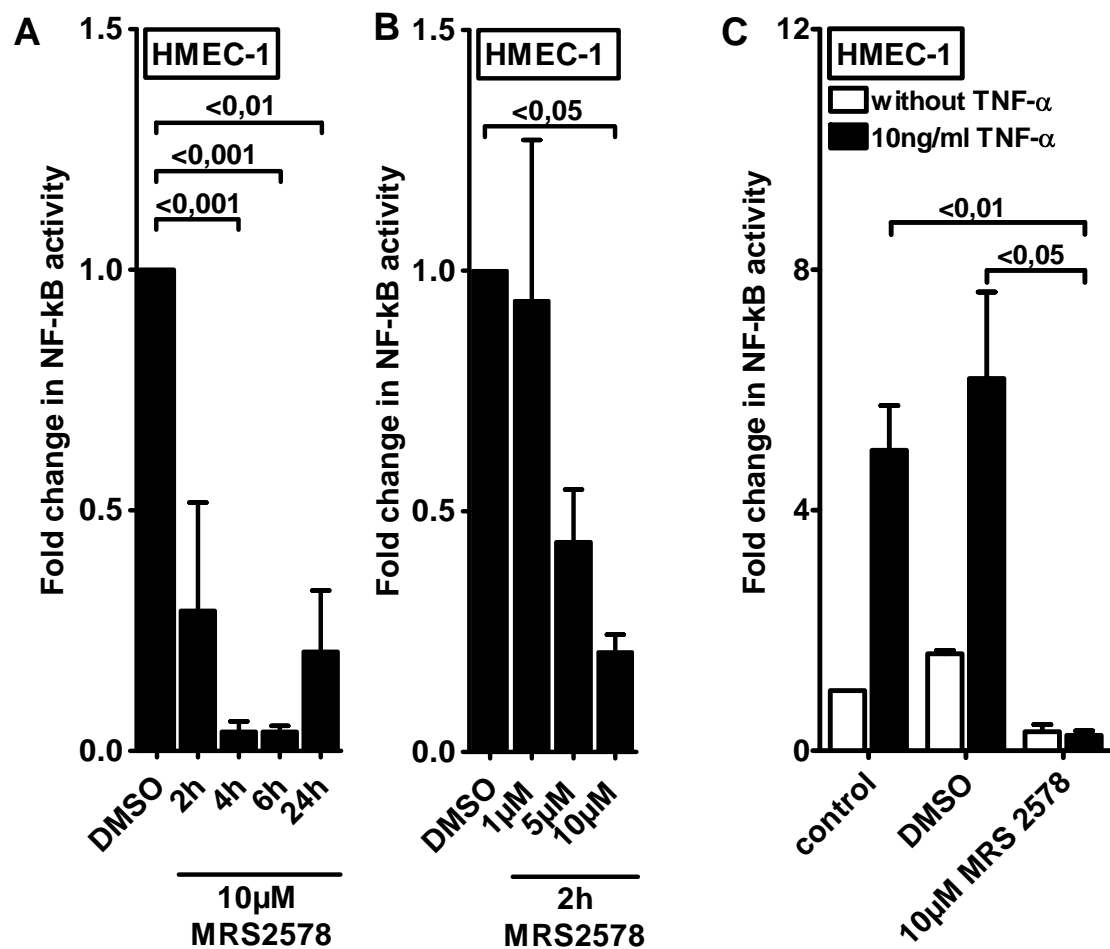
Figure 3: _Effect of P2Y6 receptor antagonist MRS 2578 on endothelial NF-κB activity._

To measure the NF-κB activity, vascular endothelia (HMEC-1) were transfected with 0.25 μg of either NF-κB promoter reporter (Clontech) or control pGL3 vector. Cells were exposed to the P2Y$_6$ receptor antagonist MRS 2578 either with or without TNF-α for the indicated time periods. As readout for NF-κB activity cells were lysed, and luciferase activity was determined relative to the total protein concentration. (A-B) Unstimulated NF-κB activity after exposure to indicated times or concentrations of MRS 2578. (C) NF-κB activity after stimulation with MRS 2578 (30 minutes) and after stimulation with TNF-α (10 ng/mL) for 2 hours. Results are displayed as mean ± SD (n = 3).

After these studies, we examined the effect of treatment with the P2Y$_6$ receptor antagonist on TNF-α - induced expression of proinflammatory cytokines and adhesion molecules. For this purpose we examined the transcript levels of IL-8 (Figure 4A), vascular cell adhesion molecule 1 (VCAM-1; Figure 4B), and intercellular adhesion molecule 1 (Figure 4C) after TNF-α exposure. In fact, these studies showed significant reduction of TNF-α - induced proinflammatory gene expression with pharmacologic inhibition of P2Y$_6$. Taken together, these studies show that inhibition of P2Y$_6$ dampens the inflammatory response of vascular endothelia in vitro.

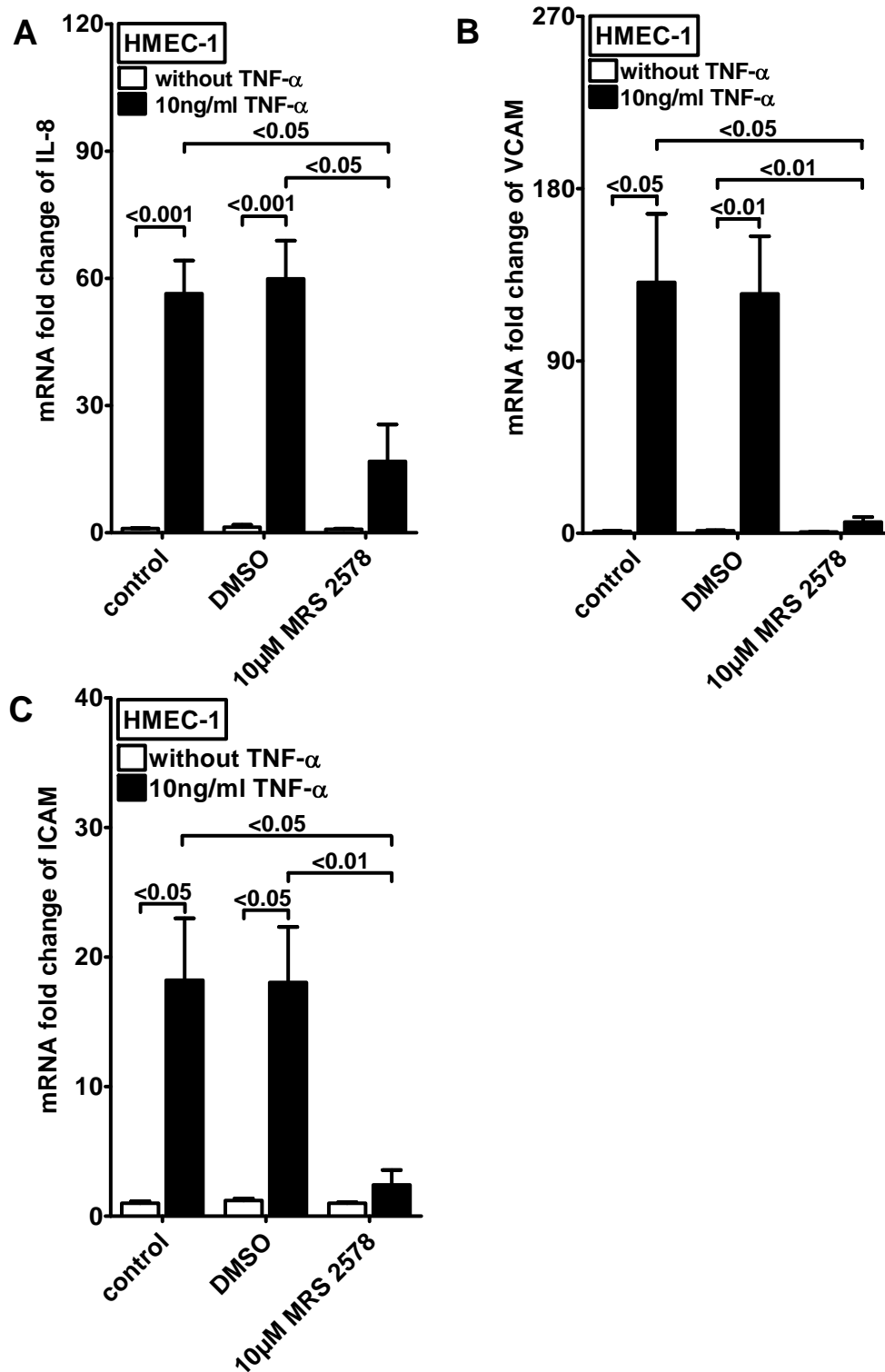**_Figure 4 A) – C):_** **_Reduction of NF-κB activity by P2Y_$_6$_-receptor antagonist MRS2578._**
Basal measurement of NF-κB activity of HMEC-1 cells after the addition of P2Y$_6$
antagonist. Reduction of NF-κB activity is time and dose dependent.

3.3.   P2Y$_6$ receptor is induced in vivo after intravenous LPS treatment

After having shown a selective induction of the P2Y$_6$ receptor in conjunction with a functional role in vascular inflammation in vitro, we next examined changes in P2Y$_6$ receptor expression levels under vascular inflammatory conditions in vivo. As a murine model of vascular inflammation, we treated mice with an intravenous injection of 300 µg of LPS or vehicle given into the jugular vein and examined P2Y$_6$ expression patterns at 24 hours after LPS treatment. Examination of P2Y$_6$ transcript levels showed a robust induction of P2Y$_6$ in vascular organs such as the kidney (Figure 5A) and the heart (Figure 5B). Moreover, immunofluorescence of the abdominal aorta (Figure 5C) showed vascular induction of the P2Y$_6$ receptor after intravenous LPS treatment. Although these studies showed induction of the P2Y$_6$ receptor in multiple cells (eg, vascular smooth muscle cells), this response was most prominent in the vascular endothelia.

3.4.   Pharmacologic inhibition or genetic deletion af the P2Y$_6$ receptor convey protection from LPS-induced inflammation

After having shown that murine P2Y$_6$ receptor is induced after systemic LPS treatment, we next pursued pharmacologic and genetic studies to address a functional in vivo role of P2Y$_6$ signaling. Dr. Idzko performed the experiments shown in figure 5D) to I) at the University of Freiburg, where P2Y$_6$ knockout mice were available. They exposed wild-type mice treated with the P2Y$_6$ receptor antagonist MRS 2578 or vehicle (DMSO) to intravenous LPS (300 µg) and compared corresponding serum keratinocyte derived chemokine levels by enzyme-linked immunoabsorbent assay. We observed that KC serum protein levels were dramatically increased in LPS-treated wild-type mice exposed to vehicle alone. In contrast, serum KC elevations were attenuated in mice treated with the P2Y$_6$ receptor antagonist (Figure 5D). Next, they examined previously described gene-targeted mice for P2Y$_6$ [140] or littermate control mice matched in weight, sex, and age. Two hours after administering 300 µg of intravenous LPS

serum KC levels examined by enzyme-linked immunoabsorbent assay were significantly lower in $P2Y_6^{-/-}$ mice than in the corresponding control mice (Figure 5E). Next, they examined transcript levels of VCAM-1 in vascular organs of LPS-treated $P2Y_6^{-/-}$ mice or littermate controls matched in age, sex, and weight. In fact, these studies showed that renal (Figure 5F) and cardiac (Figure 5G) VCAM-1 levels were induced in LPS-treated control animals 2 hours after exposure to LPS. Importantly, however, increased VCAM-1 expression was almost completely abolished in $P2Y_6^{-/-}$ mice. As additional readout for vascular inflammatory responses they used the albumin marker Evans blue. Here, we observed that LPS-induced increases in albumin leakage into vascular organs were significantly attenuated in $P2Y_6^{-/-}$ mice (kidney, Figure 5H; heart, Figure 5F). Taken together, these studies provide genetic and pharmacologic in vivo evidence for a role of $P2Y_6$ signaling in vascular inflammation after LPS treatment.

Figure 5: _P2Y$_6$-receptor expression after intravenous LPS treatment and attenuated inflammatory responses in P2Y$_6^{-/-}$ mice after intravenous LPS exposure._

(A-B) C57BL/6 mice received an intravenous injection of LPS (300 µg; E coli O26:B6) or vehicle control (PBS). Induction of P2Y$_6$ mRNA was seen in kidney (A) and heart (B). (C) C57BL/6 mice received an intravenous injection of LPS (300 µg; E coli O26:B6) or vehicle control (PBS). Abdominal aorta was stained with antibodies specific for murine P2Y$_6$ receptor and Alexa Fluor 488-coupled secondary antibody (green) or isotype controls and Alexa Fluor 488-coupled secondary antibody or Alexa Fluor 488-coupled secondary antibody only. DAPI (4',6-diamidino-2-phenylindole) was used as nuclear counterstain (blue). Slides were kept on ice before image acquisition. Probes were analyzed by confocal microscopy with the use of Zeiss Laser Scanning Microscope LSM 510 and the Plan-Apochromat 20x/0.8 M27 objective lens with oil immersion. Zen software was used for acquisition and image processing (γ adjustment) applied equally to all images. White arrows indicate luminal side. One representative image of 3 is displayed. (D) Determination of plasma KC levels by enzyme-linked immunoabsorbent assay (ELISA) in C57Bl/6 wild-type mice treated with MRS 2578 and DMSO (solvent of MRS 2578) 2 hours after intravenous injection of LPS (300 µg; E coli O26:B6) or vehicle control (PBS) (wild-type control, n = 8; wild-type LPS, n = 8; P2Y$_6^{-/-}$ control, n = 6; P2Y$_6^{-/-}$ LPS, n = 6). (E) Determination of plasma KC levels by ELISA in C57Bl/6 wild-type versus P2Y$_6^{-/-}$ mice 2 hours after intravenous injection of LPS (300 µg; E coli O26:B6) or vehicle control (PBS) (wild-type control, n = 10; wild-type LPS, n = 10; P2Y$_6^{-/-}$ control, n = 8; P2Y$_6^{-/-}$ LPS, n = 14). (F-G) Two hours after the LPS injection, mice were killed, and vascular organs (heart, kidney) were harvested. Transcript levels of VCAM were determined by real-time RT-PCR relative to the housekeeping gene -actin in vascular organs (F) kidney (control, n = 3; LPS, n = 6) or (G) heart (control, n = 4; LPS, n = 6). All results are displayed as mean ± SD. (H-I) Determination of Evans blue concentration in kidney and heart tissue of C57Bl/6 wild-type versus P2Y$_6^{-/-}$ mice 2 hours after intravenous challenge with 300 µg of LPS. (H) Kidney (wild-type control, n = 4; wild-type LPS, n = 10; P2Y$_6^{-/-}$ control, n = 6; P2Y$_6^{-/-}$ LPS, n = 12). (I) Heart (wild-type control, n = 4; wild-type LPS, n = 10; P2Y$_6^{-/-}$ control n = 6; P2Y$_6^{-/-}$ LPS n = 11).
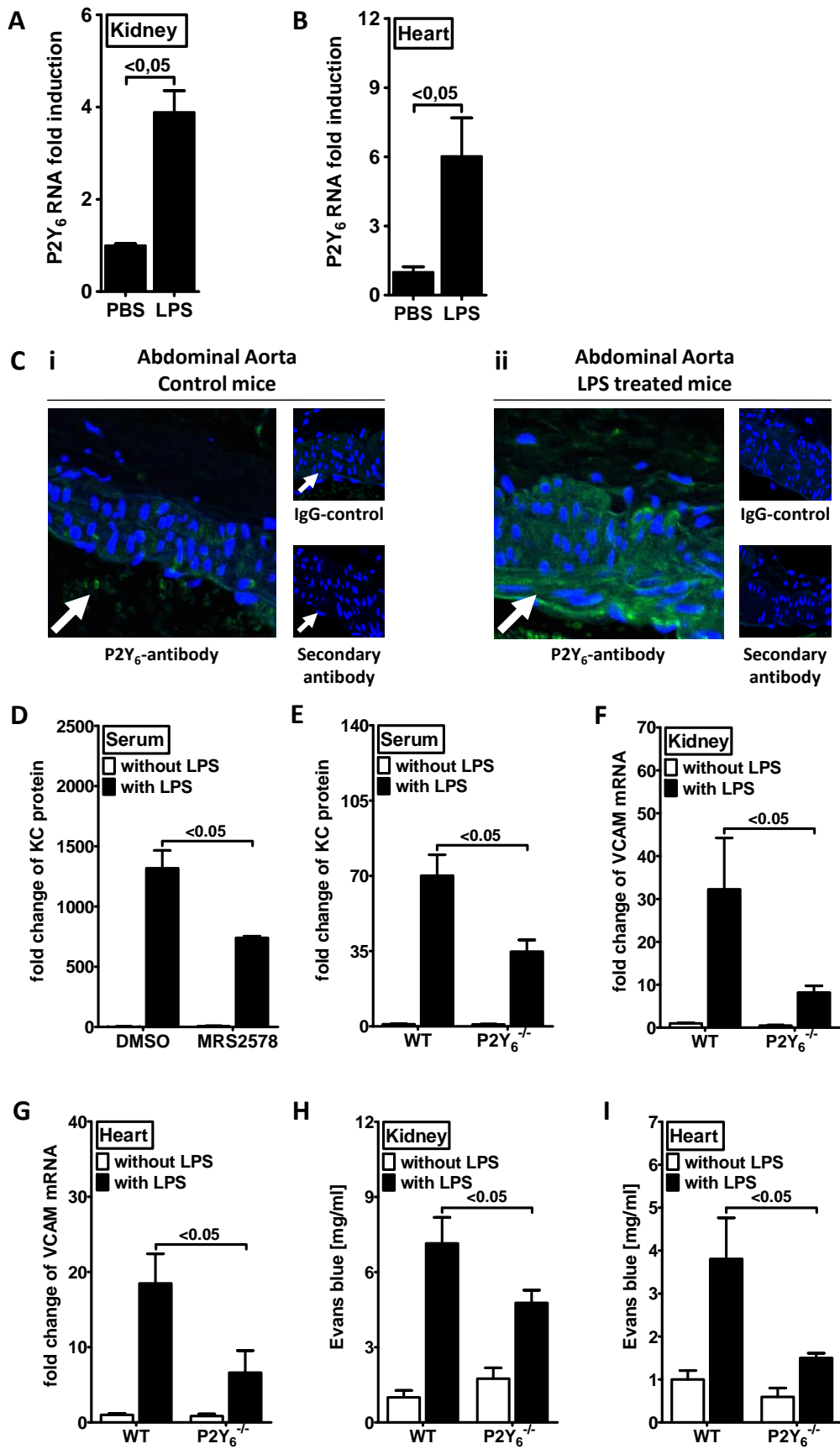
4. Discussion

Although research work over the past decades has clearly identified several groups of vascular inflammatory proteins that regulate inflammatory cell trafficking across the endothelial monolayer, biochemical processes that govern vascular inflammatory responses are less clearly defined. On the basis of the observation that extracellular nucleotide levels are dramatically increased after inflammatory stimulation, ischemia, or hypoxia, we pursued the hypothesis that extracellular nucleotide receptor signaling could play a role in vascular inflammation. Initial microarray studies pointed us toward a selective induction of the $P2Y_6$ receptor after inflammatory stimulation. With the use of real-time RT-PCR and Western blotting, we were able to confirm a robust induction of $P2Y_6$ transcript and protein levels on inflammatory stimulation of model endothelia. Moreover, functional in vitro and in vivo studies combining pharmacologic or genetic approaches have shown a role of $P2Y_6$ signaling in enhancing vascular inflammation. Taken together, these studies provide evidence for a selective role of the $P2Y_6$ receptor in enhancing vascular inflammatory responses and implicate the $P2Y_6$ antagonist as a treatment form for conditions characterized by excessive vascular inflammation. Although the present studies clearly indicate that endothelial $P2Y_6$ receptors are transcriptionally induced on inflammatory stimulation, the transcriptional mechanism remains unknown. This could at least in part be related to the diversity of $P2Y_6$ receptor variants. In fact, 4 variants of the $P2Y_6$ receptor have been identified [(1) NM_176797.1, (2) NM_176798.1, (3) NM_176796.1, (4) NM_004154.3; http://www.ncbi.nlm.nih.gov/gene/5031]. Studies that used different RT-PCR primers indicate the likelihood that all 4 variants are induced by inflammatory stimuli (Figure 6).
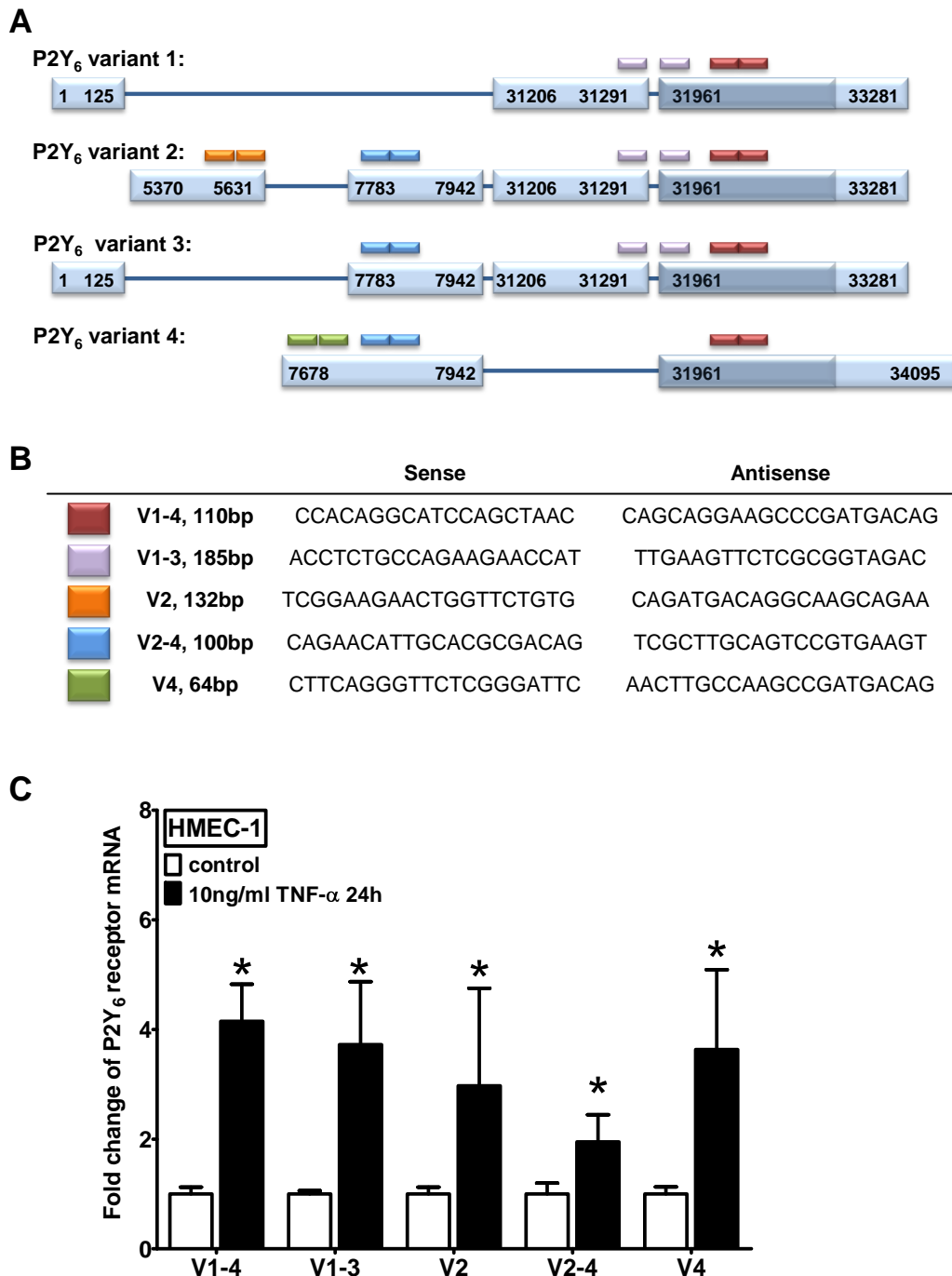
A

P2Y$_6$ variant 1:

P2Y$_6$ variant 2:

P2Y$_6$ variant 3:

P2Y$_6$ variant 4:

B

		Sense	Antisense
	V1-4, 110bp	CCACAGGCATCCAGCTAAC	CAGCAGGAAGCCCGATGACAG
	V1-3, 185bp	ACCTCTGCCAGAAGAACCAT	TTGAAGTTCTCGCGGTAGAC
	V2, 132bp	TCGGAAGAACTGGTTCTGTG	CAGATGACAGGCAAGCAGAA
	V2-4, 100bp	CAGAACATTGCACGCGACAG	TCGCTTGCAGTCCGTGAAGT
	V4, 64bp	CTTCAGGGTTCTCGGGATTC	AACTTGCCAAGCCGATGACAG

C

Figure 6 A) – C): P2Y$_6$ receptor transcript variants and expression.
In order to narrow down the promoter region of the P2Y$_6$ receptor which is relevant for the induction of P2Y$_6$ transcription after TNF-α stimulation, we designed primers for the four different variants of P2Y$_6$ receptor. However, we saw an upregulation of all variants as far as we could distinguish them with our approach. Data were calculated relative to the internal housekeeping gene beta-actin and are expressed as mean fold change compared to the control (without TNF-α) ± SD (n= 3-4).

However, only P2Y$_6$ receptor variant 1 and variant 3 share homology in their promoter region. In fact, transcription factor binding searches indicate binding sites for different transcription factors for the individual variants (eg, only the promoter of variant 2 contains a binding site for NF-κB), indicating that the transcriptional regulation of P2Y$_6$ during inflammatory conditions is complex and could involve different transcription factors (Figure 6). Consistent with the present results, a previous study addressed the role of nucleotide receptor signaling in epithelial inflammation.[73] That study determined the effect of intestinal inflammation on P2Y$_6$ receptor expression by PCR in the mouse, rat, and human. The investigators of that study found that epithelial inflammation induces epithelial P2Y$_2$ and P2Y$_6$ receptors in the mucosa of the colon of colitic mice. As such, that study was among the first to show a role of P2Y receptors in the inflammatory response of intestinal epithelia. Other studies have shown a role of P2Y$_6$ receptor activation in enhancing NF-κB activity in osteoclasts and an associated increase in their survival.[99] Further studies have shown that P2Y$_6$ receptor activation mediates the release of proinflammatory cytokines and chemokines in monocytic cells.[102,140] Taken together, these studies are consistent with the present in vitro and in vivo findings that implicate P2Y$_6$ signaling in vascular inflammation.

In fact, it is conceivable that enhanced inflammatory responses could also involve cross-talk pathways between epithelial cells and vascular endothelia.[226] For instance, nucleotide release has been previously implicated in the cross talk between different cell types.[227-228] Moreover, it is somewhat unclear whether the role of P2Y6 in inflammation and barrier function is direct or indirect. The fact that we observed only mild increases in inflammatory activation with P2Y$_6$ agonist treatment of cultured endothelia (data not shown) argues for an indirect effect. As such, P2Y$_6$ signaling could represent an additional mechanism for "finetuning" inflammatory pathways in the setting of a systemic stimulus (eg, LPS).

Although the present studies show that activation of the $P2Y_6$ receptor can enhance vascular inflammation, extracellular nucleotides also represent the metabolic substrate for extracellular generation of adenosine and uridine. In fact, extracellular adenosine is known to be an important anti-inflammatory signaling molecule. This paradigm goes back to studies showing an antiinflammatory effect of adenosine signaling on inflammatory cells.[229-230]

As such, adenosine triphosphate (ATP) or adenosine diphosphate (ADP) can be rapidly hydrolyzed to adenosine in a 2-step enzymatic process that involves the ectonucleoside triphosphate diphosphohydrolase 1 (CD39; conversion of ATP/ADP to adenosine monophosphate [AMP]) [228,231-234] and the ecto-5′-nucleotidase (CD73; conversion of AMP to adenosine). [234-238] Several studies have implicated extracellular adenosine signaling in attenuating acute inflammatory responses during conditions such as hypoxia-induced vascular leakage and pulmonary edema,[228,239-241] sepsis,[242-243] or acute lung injury.[216,243-245] As such, experimental approaches that will enhance extracellular nucleotide breakdown and their subsequent conversion to adenosine will simultaneously dampen activation of nucleotide receptors (such as the $P2Y_6$) and increase extracellular adenosine signaling. Consistent with this concept, previous studies have shown that treatment with apyrase (enhances ATP/ADP phosphohydrolysis) or nucleotidase (enhances AMP phosphohydrolysis) are effective in the treatment of acute inflammatory disease such as acute lung injury,[216,244,246-247] hypoxia-induced vascular permeability changes,[238] or organ ischemia.[232-233,236,238,248] Interestingly, also extracellular uridine (derived from the P2Y receptor agonist uridine triphosphate or uridine diphosphate) is implicated as an anti-inflammatory signaling molecule. [249]

Taken together, the present studies show selective induction of the vascular $P2Y_6$ receptor after inflammatory stimulation in conjunction with enhanced systemic inflammation. These findings implicate pharmacologic strategies that dampen $P2Y_6$ signaling (eg, apyrase treatment or selective $P2Y_6$ receptor blockade) in the treatment of inflammatory disorders that involve a vascular

phenotype, such as systemic inflammatory response syndrome, or sepsis. Future challenge will involve the definition of pharmacologic approaches, their translation from murine models toward the treatment of patients, and the identification of potentially unwanted side effects, such as alterations in coagulation or platelet function,[250-251] heart rate, and blood pressure,[252] or the consequences of chronic elevation of extracellular adenosine levels.[246,253]

5. Summary

Nucleotide signaling plays an important role during vascular inflammation. Here, we determined the $P2Y_6$-receptor expression changes under inflammatory conditions. We found, that different inflammatory stimuli like TNF-α and LPS lead to a notable increase in $P2Y_6$-receptor mRNA in vitro and in vivo. Moreover, $P2Y_6$-receptor antagonist studies revealed, that signaling through $P2Y_6$-receptors is important for amplification of TNF-α and LPS signals.

It is known, that inflammatory stimuli lead to an increased accumulation of extracellular UDP which act on $P2Y_6$-receptors. Pretreatment with the $P2Y_6$-receptor antagonist MRS2578 however reduced TNF-α induced NF-κB activation and TNF-α and LPS induced transcription of IL-8, VCAM and ICAM. Taken together, these studies implicate an important proinflammatory role of $P2Y_6$-receptor signaling in signal amplification in inflammation.

6. Summary in German

Nukleotid Signaluebertragung spielt eine wichtige Rolle waehrend vaskulaeren Entzuendungsreaktionen. In dieser Arbeit haben wir Veraenderungen im Expressionsmuster endothelialer Nukleotid-Rezeptoren (P2-Rezeptoren) untersucht und konnten eine selektive Hochregulation des $P2Y_6$-Rezeptors feststellen. Wir konnten zeigen, dass verschiedene infammatorische Stimuli wie TNF-α und LPS zu einer signifikanten Hochregulation der $P2Y_6$-Rezeptor mRNA in vitro und in vivo fuehrt.Darueber hinaus konnten wir durch Antagonisten-Studien zeigen, dass die Signaluebertragung durch den $P2Y_6$-Rezeptor wichtig fuer die Amplifikation der durch TNF-α induzierten NF-κB Aktivierung ist. Zusammengefasst deuten unsere Daten darauf hin, dass der $P2Y_6$-Rezeptor eine wichtige Rolle bei der Signalamplifikation in Entzuendungsreaktionen spielt.

7. Index of Illustrations, Figures and Tables

8. Glossary

Apyrase	Enzyme that catalyzes the hydrolysis of nucleoside triphosphates and diphosphates to monophosphates: $NTP \rightarrow NDP + P_i \rightarrow NMP + 2\ P_i$
Desensitization	A decrease in response to persistent or intermittent application of an agonist that is a consequence of alterations at the level of the receptor.
Excitable cells	Cells, which exhibit all-or-none action potentials in response to depolarization. Excitable cells are neurons and muscle cells.
RGD sequence	An amino acid sequence containing the 3 amino acids: arginine – glycine – asparagine (Arg-Gly-Asp). In the one-letter amino acid code: R – G – D. Integrins are able to bind on this sequence, mostly found in the extracellular matrix and mediate cell adhesion.
Non-excitable cells	Cells, which cannot generate all-or-none action potentials in response to depolarization, because of their lack of voltage-gated Na^+ or Ca^{2+} channels. In non-excitatory cells action is mediated by an intracellular change of Ca^{2+} concentration. Ca^{2+}-permeable channels in non-excitable cells are not gated by depolarization.
SN50	Cell-permeable inhibitor of NF-κB, composed of a nuclear localization sequence (NLS) for NFkB p50 linked to a cell-permeable carrier. SN50 inhibits NFkB by interfering with its translocation through the nuclear pore.

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10. Publication / Poster

Parts of this work have already been published in:

Ann K. Riegel, Marion Faigle, Stephanie Zug, Peter Rosenberger, Bernard Robaye, Jean-Marie Boeynaems, Marco Idzko, and Holger K. Eltzschig. Selective induction of endothelial $P2Y_6$ nucleotide receptor promotes vascular inflammation. Blood. 2011

Poster presentation:

A.-K. Stenz, P. Rosenberger, M. Faigle, S. Zug, and H.K. Eltzschig. Pro-inflammatory role of $P2Y_6$-receptor signalling during vascular inflammation. Interdisciniplinary Center for Clinically Research (IZKF) yearly meeting, Tuebingen, Germany 2009

Prizes:

Interdisciniplinary Center for Clinically Research (IZKF) poster prize 2009

Commented by:

Michael R. Blackburn. $P2Y_6$ and vascular inflammation. Blood 2011

"Talent wins games,
but teamwork and intelligence
win championships."
Michael Jordan

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12. Curriculum Vitae

Education

USMLE 1	*07/24/2011*
Final State Examination in Medicine, University of Tübingen	*05/18/2010*
Studies of medicine, University of Tübingen	*10/12/2004 – 05/18/2010*
Studies of medicine, University of Regensburg preliminary medical examination	*10/10/2002-10/11/2004*
High school diploma, Königin Katharina Stift, Stuttgart, Germany	*06/21/2002*

Clinical rotations, practical training

Anesthesiology department, University of Tuebingen	*December 2009*
Ear, Nose and Throat department, University of Tuebingen	*04/06/2009 - 07/12/2009*
Children surgery department, University of Tuebingen	*02/07/2009 - 04/03/2009*
Surgical intensive care unit, University of Tuebingen	*12/15/2008 - 02/06/2009*
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Cardiology department, University of Tuebingen	*08/25/2008 - 10/19/2008*
Anesthesiology department, University of Colorado Health Science Center	*07/03/2008 - 07/31/2008*

Research fellowship

Scholarship of the Interdiscipliary Centre of Clinical Research (IZKF; project number 1681-0-0)	*01/01/2007 - 12/31/2007*

Awards

Award for the presentation of the poster: "Proinflammatory role of $P2Y_6$-receptor signaling during vascular inflammation"	*10/13/2009*
Scheffelpreis	*06/21/2002*