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Investigation of the role of the P2X7 receptor in inflammatory bowel disease

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Summary

Inflammatory bowel diseases (IBD) are fairly common, have a high economic importance and are associated with strong physiological and psychological strains for patients. Currently there are no specific treatments available despite the relevance of the disease. The P2X7 receptor could be a promising pharmaceutical target in order to establish a new strategy for anti-inflammatory treatment. Therefore we aimed to illuminate the role of the receptor in inflammatory processes of the bowel. In our study we focused on macrophages, since these cells play an important role in maintaining gut homeostasis and strongly express the P2X7 receptor. In order to study the properties of the receptor we compared a P2X7 knock-out (KO) and a BAC transgenic mouse model to wildtype mice. We analysed the role of the receptor on three different levels: on the cellular level, concerning cell count and inflammatory polarisation of cells, on the humoral level, concerning circulating cytokines, and on the functional level. In contrast to the vast amount of evidence concerning the role of the P2X7 receptor in IBD, we could not detect any relevant differences in immunity between our genotypes. It is up to discussion if this is due to an insufficient experimental design or due to a minor role of the P2X7 receptor in IBD. For future experiments we suggest to repeat our immunological assays on single cells and in a colitis model that provides an inflammatory state and may unmask the properties of the P2X7 receptor.

Zusammenfassung

Chronisch entzündliche Darmerkrankungen (CED) treten häufig auf. Sie sind von großer ökonomischer Bedeutung und mit hohen physischen und psychischen Belastungen für die Patienten verbunden. Trotz der Relevanz der Erkrankung sind aktuell keine spezifischen Therapien verfügbar. Der P2X7 Rezeptor könnte ein vielversprechender pharmakologischer Angriffspunkt im Rahmen einer anti-inflammatorischen Therapie sein. Daher war es unser Ziel seine Rolle in entzündlichen Erkrankungen zu beleuchten. Unsere Forschungsarbeiten konzentrierten sich auf Makrophagen, da diesen Zellen ein hoher Stellenwert in der Erhaltung der Homöostase zukommt und sie eine hohe Dichte an P2X7 Rezeptoren aufweisen. Zur Untersuchung des Rezeptors verglichen wir ein P2X7 knock-out (KO) und ein BAC transgenes Mausmodell mit Wildtyp Mäusen. Wir untersuchten hierzu drei verschiedene Ebenen: die zelluläre, d.h. die Zellzahl und die entzündliche Polarisation der Zellen, die humorale, welche sich auf zirkulierende Zytokine konzentriert, sowie eine funktionelle Ebene. Entgegen der zahlreichen wissenschaftlichen Belege für eine tragende Rolle des P2X7 Rezeptors bei CED konnten wir keine relevanten immunologischen Unterschiede zwischen unseren verschiedenen Genotypen erkennen. Es stellt sich die Frage, ob dies an einer unzureichenden Experimentplanung liegt oder ob der P2X7 Rezeptor bei CED nur eine geringe Rolle spielt. In zukünftigen Experimenten sollten immunologische Tests an einzelnen Zellen und in einem Colitis-Modell, welches ja einen entzündlichen Zustand bietet, durchgeführt werden. So könnten etwaige Effekte des P2X7 Rezeptors demaskiert werden.

Chapter 1

Introduction

1.1 Inflammatory bowel diseases

1.1.1 Motivation

Inflammatory bowel diseases (IBD) consist of the two major clinical subtypes "Crohn's disease" (CD) and "Ulcerative colitis" (UC) plus some minor clinical subtypes. This group of diseases is fairly common with an incidence of 6.6 cases per 100000 inhabitants per year for CD and 3.9/100000 for UC [33]. The highest incidence appears in the third decade of life. Therefore the disease starts for most patients with IBD during their education and continues during their working life. This causes high direct and indirect costs generated for instance by sick days thus making CD and UC economically important diseases [45]. In contrast to their importance their etiopathogenesis is still not completely understood. Therefore there are no options for a specific treatment of IBD.

1.1.2 The role of macrophages

Recent evidence indicates a complex interplay of a deregulated immune response against microbial antigens in the lumen of the intestine. Environmental triggers and a genetic predisposition are also seen to be responsible for the genesis of IBD [1]. Furthermore, a deregulated intestinal barrier seems to be an important factor that enables inflammation in response to commensal bacteria. The intestinal barrier is partly maintained by a large group of immunological cells: intestinal macrophages. Since the intestine is the largest immune compartment of the human body, [28] it also contains the largest pool of macrophages [31]. Macrophages are part of the innate immune system and eliminate

pathogens as a first line defence by phagocytosis and induce inflammation by the production of cytokines. In the intestine, however, invading pathogens are being eliminated without initiating inflammatory response. In the face of the microbiota and the constant exposure to food antigens, this process is essential for maintaining gut homeostasis [31]. Intestinal macrophages control this homeostasis by the production of a variety of cytokines such as PGE2 that contributes to the integrity of the epithelial barrier by stimulating proliferation of epithelial progenitor cells. Moreover, they produce IL-10, an anti-inflammatory cytokine, that among other factors contributes to the generation of homeostatic regulatory T-cells (T_{reg}) thus creating an anti-inflammatory environment [31]. The significance of IL-10 becomes obvious in IL-10 receptor deficiency models that are associated with an early onset and a severe form of IBD. Another cytokine that may be involved in this mechanism is TGF-β that suppresses inflammatory response and is abundant in the intestinal wall. In contrast to that, an accumulation of pro-inflammatory macrophages can be observed in IBD producing pro-inflammatory cytokines such as IL-1, IL-6 or TNF-α [31]. This evidence led to the interest in targeting the macrophage lineage for therapeutic purposes.

1.1.3 The role of purinergic signalling

Recent evidence indicates a connection between purinergic signalling and IBD. Molecules such as ATP are considered to be a damage associated molecular pattern (DAMP) that can regulate immune responses and inflammation [9]. In experimental colitis elevated levels of extracellular ATP can be measured compared to non-inflamed tissue preparing the basis for purinergic signalling [3]. Especially the P2X7 receptor seems to play an important role in detecting those danger signals and is directly involved in the regulation of pro-inflammatory cytokines and apoptosis [23]. Furthermore, this receptor seems to be involved in the pathophysiology of IBD. For instance, elevated levels of P2X7 were detected in tissue samples of IBD patients. Moreover, P2X7 KO mice did not develop colitis in IBD models and the treatment with the P2X7 antagonist OxATP ameliorated inflammation in experimental colitis [1]. But purinergic signalling is also connected to physiological roles in the gastrointestinal tract, for instance with motility. Adenosine seems to take an important part in the fine-tuning of enteric neuromuscular functions [49]. It is therefore not surprising that a link between purinergic signalling and chronic gut dysfunction in IBD has been found in recent studies [17]. According to them the P2X7 receptor is involved in neuronal death in the myenteric plexus. The specific agonist BzATP led to decreased neuronal packing density, whereas antagonising the P2X7 receptor had a neuroprotective effect in

experimental colitis. This pathway was closely connected to pannexin-1 dependent ATP release and to caspase-1 associated cell death mediated by the inflammasome. However, in this study the protective effect did not concern macroscopic damage scores indicating a specific role in neuronal damage [17]. Apart from neuronal death, in inflammation the P2X7 receptor seems to be involved in the inhibitory control of colonic motility mediated by the inhibition of cholinergic pathways. Remarkably, it does not affect motility under physiological conditions emphasising its role in inflammation [3]. In summary, the properties of the P2X7 receptor in intestinal inflammation make it an interesting target for the treatment of IBD.

1.1.4 The role of the enteric nervous system

Besides, the enteric nervous system (ENS) itself plays a role in the pathogenesis of IBD and is therefore connected to neuronal loss in the myenteric plexus [5]. Abnormalities of the ENS have been reported in inflamed as well as in noninflamed tissue of CD patients as well as in animal models. Furthermore, transmitter colocalization patterns seem to be altered in non-inflamed tissue of IBD patients which supports the occurence of adaptive alterations in the ENS. The mechanisms for these abnormalities of the ENS are up to discussion. On the one hand, there is evidence for an interaction with immune cells. The fundamental role of macrophages and T cells has already been described. Neutrophils, while rare in the MP in physiological conditions, seem to accumulate in enteric ganglia early in inflammation and are associated with neuronal damage [40]. Moreover, treatment with anti-neutrophil antibodies reduced the loss of enteric neurons in experimental colitis [6]. On the other hand, a pathway apart from immune cells, working directly by means of purinergic signalling as described above, has been stated [17]. Interestingly, the ENS can also affect immune reactions reciprocally. An anti-inflammatory role has been described for the vagus nerve: When stimulated, it activates sympathetic neurons in mesenteric ganglia that release noradrenalin, activating T cells. These T cells in turn release acetylcholine that inhibits the release of pro-inflammatory cytokines from macrophages expressing acetylcholine receptors. Other studies claimed that this vagal effect on macrophages was rather mediated by cholinergic neurons of the MP [5].

Whereas the exact mechanisms of interaction between the ENS and IBD remain unclear, they could be an important theurapeutical target in order to reduce IBD associated dysmotility and to ameliorate inflammation.

1.2 Purinergic signalling

In multicellular organisms there is a need for a communication system among cells. This is realised by certain molecules that migrate through the intercellular space and bind to specific receptors in order to induce a signal. Purine nucleotides represent a primordial system of cell communication and are integrated in many different signalling pathways. ATP is the universal energy source of cells and therefore participates in a large number of biochemical reactions within the cells. However, in sites of inflammation and tissue damage large amounts of ATP pass into the extracellular space where it acts as a signalling molecule. It is considered to be a damage associated molecular pattern (DAMP) that mediates inflammation. Almost all cells of the innate and adaptive immune system express purinergic receptors and are therefore able to react accordingly. The receptor mostly involved in inflammation and immunity is the P2X7 receptor [13].

The purinergic receptors are divided into two families. The P1 receptors are gated by the breakdown product adenosine and the P2 receptors are nucleotide sensitive. The P2 receptors can be divided again into two subgroups: the metabotropic P2Y receptors and the ionotropic P2X receptors. The P2Y subgroup consists of eight different receptors that have a rather mixed ligand selectivity from ADP and UDP to ATP and UTP. In contrast, P2X receptors have seven different family members and are specific to ATP [22].

1.2.1 The P2X7 receptor

Structure and functional features

P2X receptors are trimeric ligand-gated, non-selective ion channels with a high permeability for Ca^{2+}. They are present in virtually all mammalian tissues and mediate a large variety of responses. The P2X7 is the largest subtype of this group, consisting of 595 amino acids (aa). The monomer has a short intracellular N-terminal domain (26 aa), a bulky extracellular domain (282 aa), two transmembrane helices (about 24 aa each), and a long cytoplasmic carboxy-terminal tail (239 aa) that is unique to this receptor subtype. Multiple binding motifs for lipids and proteins have been identified in this region. For instance, they show homology with tumour necrosis factor receptor 1 or the LPS-binding region of LPS-binding protein. However it is not clear if these homologies are physiologically relevant [13]. In the past the formation of a large pore with high conductance and the ability to increase the pore size during prolonged exposure to the agonist has been considered

typical for the P2X7 receptor. This macropore was supposed to allow a rapid inward flux of Na^+ and Ca^{2+}, as well as an outward flux of K^+. However there is an ongoing debate about the mechanism or even the existence of such a macropore. Possibly these ion fluxes occur through the P2X7 receptor itself [14]. The ion fluxes are crucial for P2X7 mediated responses. Subsequent desensitisation occurs rather slowly, possibly mediated by effects on the intracellular domain [30], and is followed by recovery [23, 13].

ATP is the physiological agonist of the P2X7 receptor. This receptor is only activated by high, millimolar concentrations of ATP occuring, for instance, during pathological conditions like cell death. However, many synthetic agonists and antagonists for purinergic receptors have been produced. Benzoyl-ATP (BzATP) is a very potent agonist and relatively specific in activating the P2X7 receptor, although it also stimulates P2X1 and P2X3 [22]. Due to the rising evidence for the involvement of the P2X7 receptor in pain and inflammation processes, many P2X7 selective antagonists have been discovered in the past years [23].

A splice variant of the P2X7 receptor, named P2X7k, can be found on T-cells in mice that can be activated by an ATP-independent pathway. An ADP-ribosylation of the P2X7 can be performed in the presence of nicotinamide adenine dinucleotide (NAD^+). This leads to a long-lasting activation signal that may alter the T cell phenotype and even induce apoptosis. In fact the P2X7 receptor negatively regulates peripheral T_{reg} cells, whereas P2X7 deficient mice exhibit higher numbers of T_{reg} cells. Therefore the P2X7 receptor is thought to play a role in T cell homeostasis. Since NAD^+ is released in sites of inflammation, but also during cell preparation, this effect may have an unintended impact on immunological assays. The classical P2X7 variant found on macrophages, named P2X7a, was found to be eight to ten times less sensitive to agonists than the P2X7k on T-cells and can not be activated by NAD^+. The question if the P2X7 receptor also plays a role in macrophage homeostasis can currently not be answered [37].

Downstream signalling

The pore formation of the P2X7 receptor enables rapid fluxes of different cations. This process mediates different downstream reactions. The most important process for its role in inflammation and immunity may be its association with the NLRP3 inflammosome. The NLRP3 inflammasome is a multiprotein complex appearing mostly in cells of the innate immune system. Its activation occurs upon stimulation of pattern recognition receptors (PRR) that can detect pathogen associated molecular patterns (PAMPs) as well

as DAMPs, which are signs of infection or cell damage. One of those DAMPs is ATP that stimulates the P2X7 receptor [12]. This activation leads to the assembly of NLRP3, the adaptor molecule apoptosis-associated speck-like protein (ASC) and procaspase-1. The inflammasome reacts by autocleavage of procaspase-1 and thus processes progenitors of pro-inflammatory cytokines such as IL-1β and IL-18 [4]. Therefore, the inflammasome plays a crucial role in inflammation.

1.2.2 Functional properties

The P2X7 receptor is well known for its association with immunity and inflammation. This was not only shown by its role in releasing pro-inflammatory cytokines, but also in a more complex context, such as inflammatory diseases. Genetic ablation could ameliorate symptoms in several disease models [13]. But on the other hand the receptor plays an important role in restraining the replication of certain viruses and the immune response against bacterial and parasitic infections [41]. The properties of the receptor, however, go way beyond inflammation. It seems to play a complex role in the host response against tumors [13]. In recent studies the depletion or the inhibition of the P2X7 receptor led to increased tumor development which was associated with alteration of tumor infiltrating immune cells [37]. Another important capacity of this receptor is the induction of cell death. Recent evidence suggests that the receptor is able to influence immune cell distribution by this mechanism, especially in T cells, and therefore plays a crucial role in immune tolerance [37]. For instance, it was shown that the depletion of P2X7 receptor led to increased numbers of T follicular helper cells in Peyer's patches causing increased IgA secretion with a high affinity to commensal bacteria attenuating the intestinal microbiota [35]. We know that the P2X7 receptor plays a crucial role in all kinds of signalling pathways and diseases, but many other questions remain unanswered.

1.3 Aim of the study

In summary the vast properties of purinergic signalling in therapy stand in contrast to our current knowledge about its mechanisms. Vieira et al. have already stated:

> "Although purinergic signaling modifications underlying inflammatory responses of the GI tract are not fully understood, the extreme plasticity of the purinergic system and its pathophysiological impact on immune reactions, enteric neuronal

networking and cellular communication make drugs targeting the purinergic cascade ideal candidates for treating inflammatory GI diseases" [49].

Therefore we aim to illuminate at least a part of purinergic signalling in the GI tract by studying the role of the P2X7 receptor in intestinal inflammation and focus especially on macrophages as one of the main actors in IBD.

Chapter 2

Material and Methods

2.1 Materials

2.1.1 Chemicals and materials

The following table lists all the used chemicals and materials (2.1). All chemicals were purchased from Carl Roth GmbH and Co. KG or Sigma-Aldrich if not stated otherwise.

2.1.2 Commercial kits and solutions

The following table lists the commercial kits and solutions used in this study (2.2).

2.1.3 Buffers and media

The following table lists the buffers and medias used in this study (2.3).

2.1.4 Antibodies

The following tables list the primary and secondary antibodies for immunohistochemistry (IHC) (2.5, 2.6) as well as for FACS (2.4).

2.1.5 Oligonucleotides

The following table lists all the primers used for qPCR in this study (2.7).

Name	Supplier
Dulbecco'sModifiedEagle'sMedium(DMEM)	Sigma-Aldrich
FcR blocking reagent	Miltenyi Biotec
Fetal bovine serum	Thermo Fisher
Gel Loading Dye, Purple (6X)	New England Biolabs
Gentamicine	Carl Roth
Goat serum	Thermo Fisher
HBSS	Thermo Fisher
HEPES	Sigma-Aldrich
Isoflurane (IsoFlo®)	Abbot
LightCycler 480 multiwell plate 96	Roche
NGS	Thermo Fisher
Ni-NTA Agarose	Qiagen
Odyssey® blocking buffer (TBS)	LI-COR
Penicillin-Streptomycin (100x)	Sigma-Aldrich
PermaFluor mounting medium	Thermo Fisher
Poly-D-Lysin	Sigma-Aldrich
Quick-Load® Purple 2-Log DNA Ladder	New England Biolabs
Rat serum	Thermo Fisher
RC-26 imaging chamber	Warner Instruments
Roti®-GelStain	Carl Roth
SHD-26KIT slice anchor	Warner Instruments

Table 2.1: List of used materials

Name	Supplier
APC Annexin V	BioLegend
Cellular ROS Assay Kit (Red)	Abcam
DyLight 594 labeled Lycopersicon Esculentum (Tomato) Lectin	Vector Laboratories
ELISA MAX™ Deluxe Set Mouse TNF-α	BioLegend
ELISA MAX™ Deluxe Set Mouse IL-1β	BioLegend
Fura-2 acetoxymethyl ester	Sigma-Aldrich
LightCycler 480 SYBR green I master	Roche
QuantiNova reverse transcription kit	Qiagen
RNeasy plus mini kit	Qiagen

Table 2.2: List of commercial kits and solutions

Name	Composition
Cell culture medium	DMEM supplemented with 10% (v/v) (heat-inactivated) fetal bovine serum and Penicillin-Streptomycin
External solution	Na-Ringer (pH 7.20, 285 mOsm/kg) 0.2% (w/v) Glucose 2 mM $CaCl_2$ 1 mM $MgCl_2$
FACS buffer	PBS supplemented with 0.5% BSA and 2 mM EDTA
IHC blocking solution	PBS 0.5% Triton X-100 0.1% NaN_3 4% NGS
Krebs buffer	1.2 mM $MgCl_2$ 2.5 mM $CaCl_2$ 1.2 mM NaH_2PO_4 117 mM NaCl 25 mM $NaHCO_3$ 11 mM Glucose 4.7 mM KCl
PBS	137 mM NaCL 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4
PFA	Phosphate buffer 4% Paraformaldehyd 1.2% picric acid
Phosphate buffer 0,1M	0.075 M $Na_2PO_47H_2O$ 0.025 M $NaH_2PO_4H_2O$
SDS sample buffer (5x)	0.3 M Tris/HCL; pH 6.8 5% (w/v) SDS 50% (v/v) glycerol 0.1% bromphenol blue
SDS-PAGE running buffer	25 mM Tris; 192 mM glycine 0.1% SDS
Staining solution for calcium imaging	External solution 0,1% BSA 5µM Fura-2
Western blot transfer buffer (10x)	250 mM Tris; 1.92 M Glycin 7 mM SDS
Western blot blocking buffer	50% (v/v) Odyssey® blocking buffer (TBS)

Table 2.3: List of buffers and media

Name	Supplier	Identifier	Dilution
CD 11b APC rat	BioLegend	Cat# 101212 RRID: AB_312795	1:100
F4/80 PE rat	BioLegend	Cat# 123110 RRID: AB_893486	1:100
K-Isotype control APC rat IgG2b	BioLegend	Cat# 400611	1:100
K-Isotype control PE rat IgG2a	BioLegend	Cat# 400507	1:100

Table 2.4: Antibodies for FACS

Name	Supplier	Identifier	Dilution
CD 3 rat	Abcam	Cat # ab11089 RRID: AB_369097	1:250
CD 206 goat	R&D Systems	Cat # MAB2535 RRID: AB_2063008	1:100
iba 1 rabbit	WAKO	Cat # 019-19741 RRID: AB_839504	1:500
iNOS mouse	R&D Systems	Cat # MAB9502 RRID: AB_2152874	1:100
P2X7 rabbit	Synpaptic Systems	Cat # 177003 RRID: AB_887755	1:200

Table 2.5: Primary antibodies for IHC

Name	Supplier	Identifier	Dilution
AF 488 goat anti-mouse	Thermo Fisher	Cat # A-11001 RRID: AB_2534069	1:400
AF 488 goat anti-rabbit	Thermo Fisher	Cat # A-11008 RRID: AB_143165	1:400
AF 594 goat anti-rabbit	Thermo Fisher	Cat # A-11037 RRID: AB_2534095	1:400
AF 594 goat anti-rat	Thermo Fisher	Cat # A-11007 RRID: AB_10561522	1:400
AF 633 donkey anti-goat	Thermo Fisher	Cat # A-21082 RRID: AB_141493	1:400

Table 2.6: Secondary antibodies for IHC

Gene	Sequence
RPLP0-fwd	GGACCGCCTGGTTCTCCTAT
RPLP0-rev	ACGATGTCACTCCAACGAGG
PPIA-fwd	AGGGTGGTGACTTTACACGC
PPIA-rev	CTTGCCATCCAGCCATTCAG
P2X7-fwd	CTGGTTTTCGGCACTGGA
P2X7-rev	CCAAAGTAGGACAGGGTGGA
P2X4-fwd	CCAACACTTCTCAGCTTGGAT
P2X4-rev	TGGTCATGATGAAGAGGGAGT
IL-1β-fwd	TGCCACCTTTTGACAGTGATG
IL-1β-rev	TGATGTGCTGCTGCGAGATT
TNF-α-fwd	CTGTAGCCCACGTCGTAGC
TNF-α-rev	TTGAGATCCATGCCGTT
IFN-γ-fwd	CAAGTTTGAGGTCAACAACCCA
IFN-γ-rev	CGAATCAGCAGCGACTCCTT
IL-6-fwd	CTCATTCTGCTCTGGAGCCC
IL-6-rev	CAACTGGATGGAAGTCTCTTGC
IL-10-fwd	GCAGGACTTTAAGGGTTACTTGG
IL-10-rev	GCTCCACTGCCTTGCTCTTA
TGF-β-fwd	ACCGCAACAACGCCATCTAT
TGF-β-rev	TGCTTCCCGAATGTCTGACG

Table 2.7: List of primers

Designation	Reference	Information
P2X7-EGFP(FVB/N-Tg(RP24-114E20P2X7-StrepHis-EGFP)Ani)	[24]	Lines: 46, 59 (also in BL/6N), 61
P2X7451P-EGFP(FVB/N-Tg(RP24-114E20P2X7451P-StrepHis-EGFP)Ani)	[24]	Lines: 15, 17 (also in BL/6N)
B6-P2rx7$^{tm1a(EUCOMM)Wtsi}$	European Mutant Mouse Archive	
P2rx4$^{-/-}$	[43]	Gift from Prof. Dr. Mederos y Schnitzler

Table 2.8: Mouse lines

Device	Manufacturer
BD LSRFortessa™	BD Biosciences
Guava® easyCyte 5	Merckmillipore
LightCycler® 480 system	Roche
LSM 880 with Airyscan	Zeiss
NanoDrop™2000c	Thermo Fisher Scientific
Odyssey® FC imaging system	LI-COR
Precellys® 24 homogenizer	Bertin Instruments
Typhoon™Trio	GE Healthcare
Trans-Blot® SD Semi-Dry Transfer Cell	Bio-Rad

Table 2.9: List of devices

2.1.6 Mouse lines

The following table lists the mouse lines used in this study (2.8).

2.1.7 Special equipment

The following table lists the devices used in this study (2.9).

2.2 Methods

2.2.1 Confocal Microscopy

Confocal microscopy allows high resolution even of subcellular structures with a minimum of unspecific signalling. It is usually combined with fluorescent microscopy. Compared to fluorescent microscopes unspecific signalling is reduced. We used a Zeiss LSM 880 Airyscan confocal microscope to perform immunohistochemistry and live cell imaging.

2.2.2 Immunohistochemistry

Immunohistochemistry is a method to stain tissues and cells by means of antibodies. We used this method to characterize cells and localize certain proteins such as the P2X7 receptor in the murine myenteric plexus. For this purpose, we removed the upper layers of the tissue to expose the myenteric plexus. To keep the tissue alive, we used Krebs buffer, which contains glucose to support the tissue, electrolytes to sustain osmolarity and bicarbonate to buffer acidic metabolites of the tissue. The buffer needs to be bubbled for 15 minutes and the pH value must be adjusted to 7,44. To maintain its function, the buffer must be changed every 10 minutes. In order to obtain colonic tissue, mice at the age of eight to ten weeks were sacrificed. We opened the abdomen and removed the second centimeter of the distal colon which can be distinguished by its morphology. The tissue was immediately transferred to a sylgard coated petri-dish which was filled with Krebs buffer. The colon was opened longitudinally along the mesenterium and pinned flat on the dish. After the removal of the mucosa and submucosa we fixed the tissue by incubation with 4% PFA containing 1,2% picric acid for four hours at 4°C following three washes for ten minutes with phosphate buffer. For these washing steps the dishes were put on an orbital shaker and shaken with 200rpm. We stored the tissue in PBS and removed the circular muscule layer. For staining, the resulting longitudinal muscle myenteric plexus (LMMP) was blocked with a blocking solution containing PBS 0,5% Triton X-100, 0,1% NaN3 and 4% NGS for one hour at room temperature. This step is necessary to reduce background staining because certain sites of the tissue can bind unspecifically to proteins. After that the tissue was incubated with the primary antibody which was diluted in blocking solution at room temperature over night followed by three washes for 10 minutes with PBS. All incubation steps were performed in an Eppendorf tube on a shaker with 30rpm. The washing steps were performed in a 24-well plate and shaken at 30rpm. The incubation

with the secondary antibody took two hours in the dark at room temperature. Since antibodies have different affinities to their binding site, an optimal dilution ratio needs to be investigated for each single one. The dilutions for primary and secondary antibodies are described in 2.5 and 2.6. We washed the stained tissue three times for ten minutes with PBS and embedded it on a microscope slide with permafluor mounting medium (ThermoFisher). All images were acquired with a Zeiss LSM 880 Airyscan confocal microscope.

2.2.3 Peritoneal Lavage

In order to obtain peritoneal macrophages, we performed a peritoneal lavage on sacrificed mice. We uncovered the peritoneum and injected 5ml of HBSS with a 21G needle. In order to increase the amount of dissolved cells, we massaged their peritoneum for one minute. Afterwards we gained the fluid by means of a syringe. This procedure was repeated twice. The dissolved cells were centrifuged at 1000rpm at a temperature of 4°C for ten minutes and resuspended in 5ml DMEM with 10% FBS and 1% Penicillin/Streptomycin. This procedure usually resulted in 10^6 cells/ml. For Fluorescence-activated cell sorting (FACS) analysis we centrifuged the cells at 1500rpm, 4°C for five minutes. We resuspended the pellet in 90µl MACS buffer and incubated it with 10µl FcR Blocking reagent for ten minutes at 4°C in order to reduce unspecific binding to antibodies. Afterwards we centrifuged the cells at 1500rpm, 4°C for five minutes and resuspended the pellet in 100µl MACS buffer per staining condition. For staining we incubated 100µl of the dissolved cells with 1µl of FACS-antibodies at 4°C in the dark for twenty minutes. We washed the cells three times and resuspended them in 200µl MACS buffer. For Calcium-Imaging and ROS-Assay we transferred 100µl of dissolved cells onto Poly-L-Lysin coated cover slips in 35mm petri dishes. This resembled about 10^5 cells. We incubated the cells for fifteen minutes so that they could adhere to the cover slips and filled the petri dish with 190ml DMEM. The cells were incubated overnight at 37°C, 95% O2, 5% CO2 before use.

2.2.4 Apoptosis Assay

As the stimulation of the P2X7 receptor induces apoptosis, we aimed to determine the effect of P2X7 receptor by stimulating KO, WT and TG mice with ATP. For this purpose we used Annexin V. This protein detects phosphatidylserine which is a marker of apoptosis since it is expressed on the outer leaflet of the plasma membrane upon apoptosis. We gained cells by peritoneal lavage as described above and incubated them with 100µM, 300µM

or 1mM ATP for fifteen minutes at room temperature to induce apoptosis. Afterwards we centrifuged the cells at 1500rpm, 4°C for five minutes and resuspended them in 95µl Annexin V buffer provided by the manufacturer. For staining we added 5µl of Annexin V conjugated to APC and 1µl FcR-blocker and incubated the cells for fifteen minutes at 4°C in the dark. We washed the cells (1500rpm, 4°C, 5min), resuspended them in 200µl Annexin V buffer and measured apoptosis by means of FACS. Signifance between the groups was analysed by using an ANOVA.

2.2.5 Live Cell Imaging

Live cell imaging is a commonly used method to analyse processes in living cells and tissue. It enables researchers to study biochemical processes such as Calcium influx in cell culture or cell movement and invasion in tissue. We aimed to investigate macrophages and especially their movement in the myenteric plexus. For this purpose a setting for live cell imaging needed to be constructed. To keep the tissue alive, it needs be perfused with fresh buffer to support it with nutrients and to relieve it from metabolites. Therefore, we put the longitudinal muscle myenteric plexus (LMMP) into an open bath imaging chamber (Warner Instruments) that we attached to tubes. These tubes are connected to two different bottles and a waste bottle respectively. One bottle contains Krebs buffer and the other one Krebs buffer with 3mM ATP. A heating plate kept the buffer at 37°C. The incubator of the microscope additionally kept the tissue at 37°C. We controlled the influx into the chamber with an infusion system (one drop per 2 seconds) and the efflux with a peristaltic pump (Gilson Minipuls 2). During the experiment we switched the perfusion from Krebs buffer to Krebs buffer containing 3mM ATP using a three-way stopcock. EGFP fluorescence was visualized using a 20 times objective and a 488nm laser of a Zeiss LSM 880 Airyscan confocal microscope. Images were acquired every two minutes.

2.2.6 Calcium Imaging

Since the P2X7 receptor provokes calcium influx upon stimulation, we used calcium imaging to examine the functionality of the P2X7R-EGFP. In order to measure the calcium influx, we used Fura-2, a fluorescent dye which binds to free intracellular calcium. Upon binding calcium, it changes its fluorescent characteristics. It is excited at 340nm and 380nm and the ratio of the emissions at those wavelengths correlates with the amount of the intracellular calcium. For calcium imaging with a fluorescent microscope we gained peritoneal

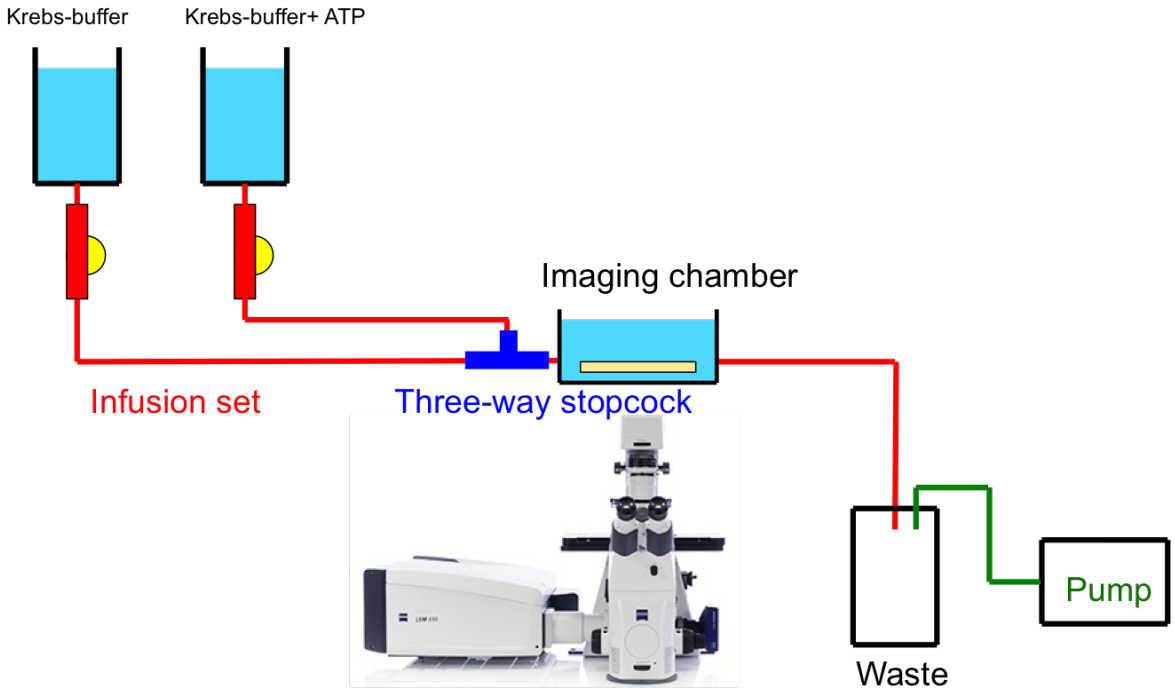


Figure 2.1: **Setup for live cell imaging.** Tissue in the imaging chamber was continiously perfused by Krebs buffer in order to keep it alive. Stimulation was performed by perfusion with an ATP containing Krebs buffer solution.

macrophages as described above. The cover slips were incubated in a staining solution for forty minutes at room temperature in the dark. Afterwards the cover slips were washed twice with the external solution and transferred into the measuring chamber of the microscope in 360µl external solution. We determined the macrophages by their morphology in light microscopy and marked them as regions of interest. After starting the measurement at 340nm and 380nm, we added 40µl of 3mM BzATP at frame 100 (corresponding to 50 seconds) in order to stimulate the cells with 300µM BzATP. In order to normalise our data and to exclude falsification by different basal activity of different genotypes, we calculated the ratio of the data and the average basal activity for each cell. Significance compared to our wildtype mice was analysed by using an unpaired Student's t-test. We analysed 108 to 121 cells in 3 different animals per group.

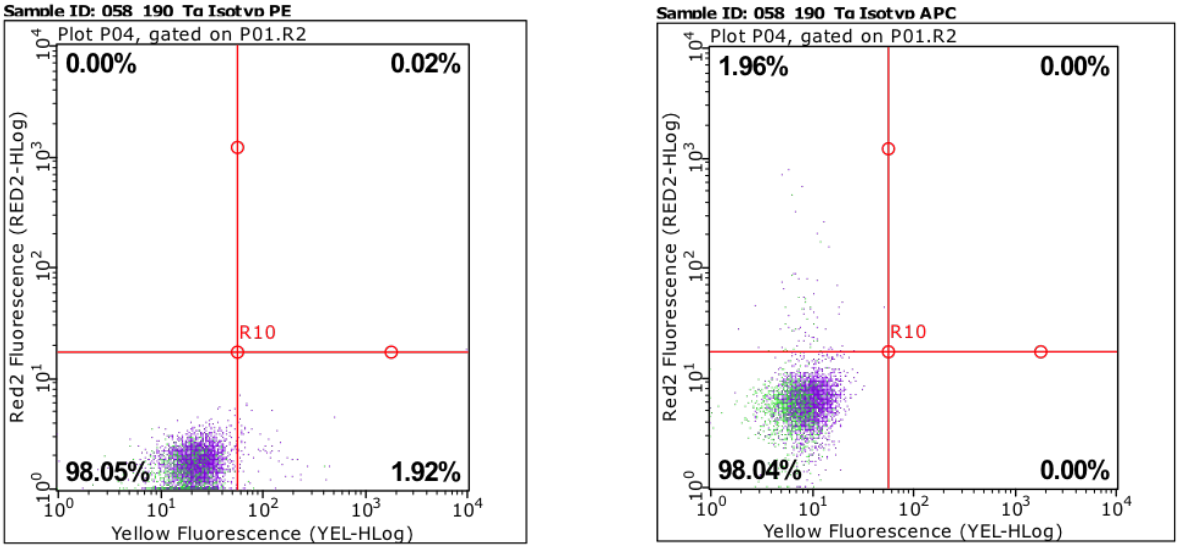
2.2.7 ROS assay

For our ROS (reactive oxygen species) assay we gained macrophage cultures using peritoneal lavage as described above. For macrophage staining we washed the cells once with

the imaging solution plus 0,1% BSA and incubated them in a 2% Tomato Lectin DyLight 594 solution for 30 minutes at 37°C, 95% O_2, 5% CO_2. Afterwards we washed the cells twice in the imaging solution. For ROS staining we incubated the cells in a 0,2% working solution (consisting of ROS stock solution and assay buffer) for 60 minutes at 37°C, 95% O_2, 5% CO_2. It was important to reduce commotion on the cell cultures in order to prevent unspecific ROS production. The first experiments on plate readers provided very volatile results. Therefore we used a confocal microscope in order to measure the ROS production of single macrophages. The Tomato Lectin DyLight 594 signal was measured in a DY-590 channel, the ROS-signal was measured in a DY-650 channel. We identified macrophages by the tomato lectin staining and drew a region of interest around those cells. Since commotion can provoke unspecific ROS production, we excluded all cells that provided a ROS signal before stimulation. Furthermore, we performed an injection control with the imaging solution right before stimulation in order to exclude unspecific stimulation. We stimulated the cells with 3mM ATP. For analysis we substracted background noise from our ROS-signal.

2.2.8 FACS

Fluorescence-activated cell sorting (FACS) is a method to perform measurements on cells and particles in a liquid suspension. It relies on the scattering of light that is caused by cells passing through a laser beam. This enables the analysis of multiple characteristics of single cells by multicolour labeling. We aimed to analyse changes in the amount of immune cells that are caused by the overexpression and knock-out of the P2X7-receptor in the colon and in peritoneal lavage. We performed Fluorescence activated cell sorting (FACS) in order to analyse changes in the numbers of immune cells that are caused by overexpression and knock-out of the P2X7-receptor. For the analysis of the colon we dissociated the cells of the murine colon by enzymatic digestion using the Miltenyi lamina propria dissociation kit. Furthermore the tissue was shred by a Gentle MACS Dissociator. 100µl of the resulting cell pellet were stained at 4°C in the dark for 20 minutes and fixed with 4% paraformaldehyde for 20 minutes. FACS analysis was performed within a few days with a BD LSR Fortessa and FlowJo software (Tree Star, Ashland, OR). For the analysis of the peritoneal lavage we isolated the cells as described above. After we had stained 10^5 cells at 4°C in the dark for 20 minutes with the corresponding antibodies, we analysed them with a Guava easyCite Flow Cytometer. Mononuclear phagocytes were characterized by their surface antigens CD11b and F4/80. As fluochromes we used phycoerythrin (PE) that has a primary absorbance



(a) Isotype control for PE.

(b) Isotype control for APC.

Figure 2.2: **Isotype control for FACS analysis.** Cells were obtained by peritoneal lavage and stained by an isotype control. (a) Isotype control for PE (yellow). (b) Isotype control for APC (red).

maxima at 565 nm and a single emission maxima at 574 nm, and allophycocyanin (APC) that has aprimary absorbance maxima at 651 nm and a single emission maxima at 660 nm. The gating was performed by means of an isotype control for each single genotype in order to exclude signals from unspecific antibody binding. Less than 2% were supposed to be positive in the isotype control (2.2a, 2.2b). Significance between the groups of three animals each was analysed by using an ANOVA.

2.2.9 RNA isolation

In order to obtain mRNA for our qPCR analysis, we removed the colon of the sacrificed mice and flushed it with PBS. Afterwards the tissue was frozen in liquid nitrogen and stored at -80°C. For the RNA isolation we used the RNeasy® Mini Kit following the manufacturer's instructions. Briefly, we dissolved 30mg of the colonic tissue by using a Precellys® 24 homogenizer and added the solution to a spin column. In a last step we washed the column with 50µl water in order to obtain the mRNA. We assessed the concentration and the purity of the product by means of the optical density at 260/280nm using a NanoDrop™2000c UV-Vis spectrophotometer by loading 1,5µl of the sample. The concentration was calculated in comparison to a blank solution (milli-Q® H_2O).

2.2.10 Reverse transcription

The extracted mRNA was converted into cDNA using the QuantiNova reverse transcription kit following the manufacturer's instructions. Briefly, 2,21µg RNA was used for each sample in order to enable comparability in qPCR. The amount of produced cDNA was measured in order to guarantee comparability using a NanoDrop^{TM}2000c UV-Vis spectrophotometer as described above.

2.2.11 qPCR

Quantitative Real-Time Polymerase Chain Reaction (qPCR) is a commonly used technique to determine gene expression. We used a SYBR Green-Assay for our experiments and studied gene expressions in intestine, brain, and lung. In the intestine we investigated the genes of P2X7, P2X4, IL-10, IL-1β, TNF-α, IFN-γ, TGF-β and IL-6; in the brain and in the lung only P2X7, P2X4, IL-1β and TNF-α. RPLP0 and PPIA were used as housekeeping genes as proposed by Krzystek-Korpacka et al. [27]. The quantitative polymerase chain reaction was performed using LightCycler 480 SYBR Green I Master (Roche) and LightCycler 480 Multiwell Plate 96 (Roche) following the manufacturer's instructions. The fluorescence was analysed by a LightCycler480 System. The reaction was performed in 20µl. Primers at a final concentration of 1µM and cDNA which was diluted ten times were used. 2µl of a ten times primer stock were laid into the wells before 18µl of Master Mix were added. The master mix for one reaction consisted of 3µl water, 10µl SYBR Master Mix and 5µl template. Afterwards the multiwell plate was centrifuged at 3000rpm for two minutes and loaded into the LightCycler. Data were analysed using the ΔΔCt-method. Significance of the ΔCt-values of TG and KO mice compared to the ΔCt-values of WT mice was analysed by using a two sided Mann Whitney U Test. We used two to three animals per group.

Primers for all genes were designed by using the NCBI Primer-Blast. These primers were tested by means of a negative control as non-template controls using agarose gel electrophoresis . A 1,5% gel was produced by boiling agarose in TAE buffer, supplementing it with Roti®-GelStain (8µl/100ml Agarose) and transferring it into a casting chamber. The DNA samples were supplemented with loading dye (2µl sample, 2µl loading dye, 8µl water) and transferred into the chamber. A molecular weight marker was additionally loaded for size comparison. A voltage of 90V was applied for thirty to sixty minutes. In order to analyse the products, fragments were visualized in the 600nm channel of an Odyssey® FC imaging system.

2.2.12 ELISA

The enzyme linked immunosorbent assay (ELISA) is a reliable method to specifically detect certain proteins. We used ELISA in order to measure the amount of the pro-inflammatory cytokines IL-1β and TNF-α in the intestinal tissue. In order to obtain the colonic tissue, we sacrificed mice and extracted 1cm of their distal colon. For stimulation we incubated the tissue in Krebs buffer for four hours with $1\frac{\mu g}{ml}$ LPS and one hour with 3mM ATP or with 3mM ATP alone for one hour at 37°C, 95% O2, 5% CO2. Unstimulated samples were incubated for the same time under the same conditions in Krebs buffer. After the incubation we dissociated the tissue using the Biorad Bioplex cell lysis kit according to the manufacturer's instructions and grinded it using a Precellys® 24 homogenizer at 4000rpm for 15s four times. The tissue was centrifuged at maximum speed for fifteen minutes and the supernatant was immediately frozen in liquid nitrogen. In order to use the same amount of protein for each sample, we measured the protein concentration and adjusted the samples. We performed the ELISA of IL-1β and TNF-α using the Biolegend ELISA kit according to the manufacturer's instructions. Significance compared to our wildtype mice or unstimulated groups was analysed by using a two sided Mann Whitney U Test. We analysed six to eight animals per group.

2.2.13 Western Blot

The Western Blot is an analytic technique to detect proteins depending on their molecular weight. The proteins undergo denaturation and gel electrophoresis before they are blotted onto a membrane and stained with an antibody. We used this method in order to distinguish between IL-1β and its precursor pro-IL-1β as this is not possible by means of ELISA. We obtained the tissue the same way as we did for ELISA. The stimulation was performed with 3mM ATP for one hour or additionally with $1\frac{\mu g}{ml}$ LPS for four hours. For the gel electrophoresis we fabricated a 15% acrylamid gel and denaturated the proteins at 40°C. The separation took place in an SDS-PAGE running buffer at 100 V for the stacking gel and 130 V for the separation gel. We performed the blotting at 100V, 250mA for one hour, we blocked the membrane in Odyssey Blocking buffer TBS for one hour and we incubated it with Anti-IL-1β (1:1000) at 4°C overnight. After staining the membrane with the secondary antibody (Anti-mouse 680 RD 1:15000) it was analysed using a Typhoon™Trio.

Chapter 3

Results

3.1 Cellular analysis

3.1.1 P2X7 positive cells in the colon

The distribution of the P2X7 receptor is not completely understood and is still the topic of research. While in the past our working group extensively studied the localisation of the P2X7 receptor on neurons [24], we now examined its localisation on immune cells in the myenteric plexus by immunohistochemistry.

T cells play an important role in the pathophysiology of IBD. In physiological conditions they are abundant in the lamina propria, but rarely seen in proximity to the ENS [5]. Since confocal microscopy revealed an elevated number of P2X7 positive macrophages in the MP of TG mice in the earlier studies of our working group, we analysed if there might be an altered interaction between immune cells and the ENS in our mouse model. However, corresponding to earlier studies T cells were very rare in myenteric plexus preparations and only sometimes merged with the P2X7 staining (3.1a), although T cells in general are described to be P2X7 positive [37]. The same applied to mast cells; we could not find any mast cells within our MP preparations (3.1c).

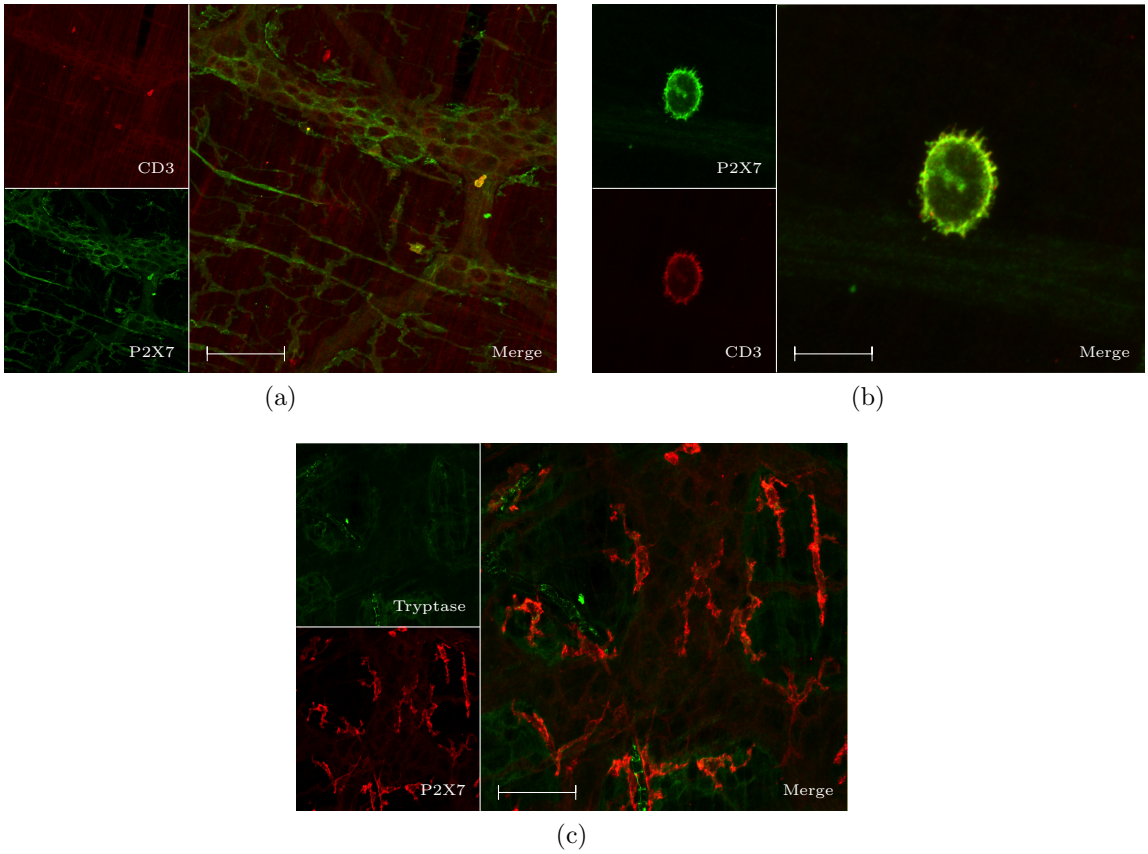


Figure 3.1: **Identification of P2X7 positive immune cells in the myenteric plexus.** MP preparations from TG mice were stained with antibodies against T cell marker CD3 or mast cell marker tryptase; (a) x40 magnification. Scale bar: 25µm. (b) 189x magnification. Scale bar: 5µm. Both marker proteins are shown in red. Anti-P2X7 antibodies are shown in green. (c) x40 magnification. Scale bar: 25µm. Marker protein is shown in green. Anti-P2X7 antibodies are shown in red.

3.1.2 Cellcount of macrophages

Macrophages are part of the innate immune system and vital in regulating the intestinal barrier function. Since studies show that a dysregulated barrier function is an important factor of IBD, macrophages could play a main part in this process. Furthermore, it was shown that IBD causes changes in the composition of immune cells and in the distribution as well as in the phenotypes of macrophages in the intestinal wall [28]. Therefore and because previous findings of our working group suggested an elevated number of macrophages in the myenteric plexus of P2X7 receptor overexpressing mice, we addressed the question

if the number of macrophages could be altered in our mouse model. In order to confirm those findings for the myenteric plexus determined by IHC and to examine if the macrophage numbers of the whole intestinal wall were elevated, we used FACS to count cells on a large scale. At first we tried to dissociate intestinal tissue using the Miltenyi lamina propria dissociation kit. However, this led to a high percentage of apoptotic cells. This could impair the results. The P2X7 expressing immune cells may be more likely to undergo apoptosis compared to the P2X7 negative cells since the P2X7 receptor can be activated during the tissue preparation and could induce apoptosis [37]. Therefore we used peritoneal lavage in order to obtain cells. As this method had a low number of apoptotic cells, it was suited for the analysis of macrophage numbers in different genotypes. However there is an ongoing controversial debate concerning the staining of macrophages and their discrimination from dendritic cells. Some studies suggest complex antibody panels [16] but there are papers which question the distinction of dendritic cells from macrophages [19] since there are no markers or functions in antigen presentation that distinguish them from macrophages. Thus we decided to consider them both as parts of the mononuclear phagocyte system along a continuum of activities and used a simpler antibody-panel. Dead cells were distinguished from living cells using the FSC-SSC-Scatter (3.2).

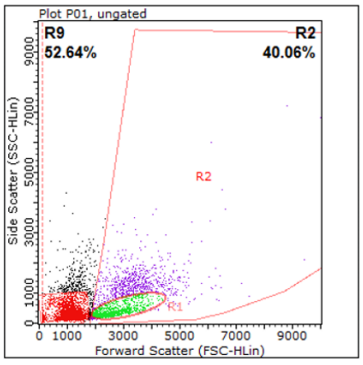
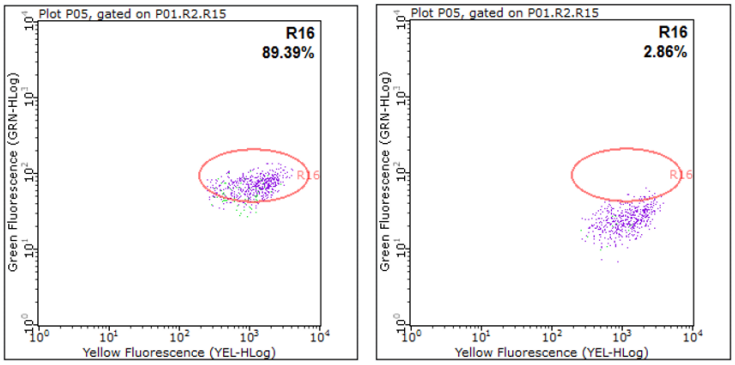


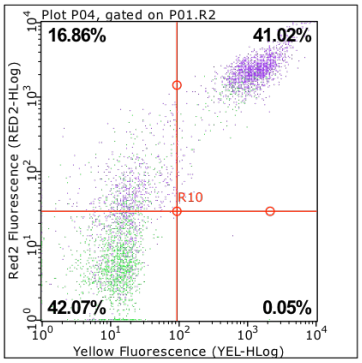
Figure 3.2: **Representative image of FACS analysis by FSC-SSC-Scatter.** Peritoneal cells from a TG mouse. R2 represents living cells, R9 represents dead cells.

First we reviewed the GFP-signal of our P2X7-EGFP and its location on mononuclear phagocytes (3.3a, 3.3b). We verified that peritoneal macrophages are P2X7 positive. Mononuclear phagocytes were characterized by their surface antigens CD11b (red) and F4/80 (yellow) (3.3c, 3.3d). Despite our previous findings in the MP, we found no significant differences in the number of macrophages in the different genotypes (3.3e). In each experiment the transgenic type was once again validated by controlling GFP positive cells.

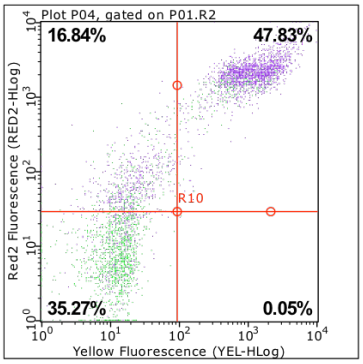


(a) TG mouse

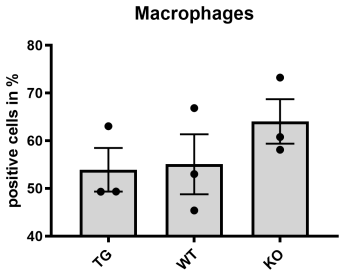
(b) WT mouse



(c) WT mice



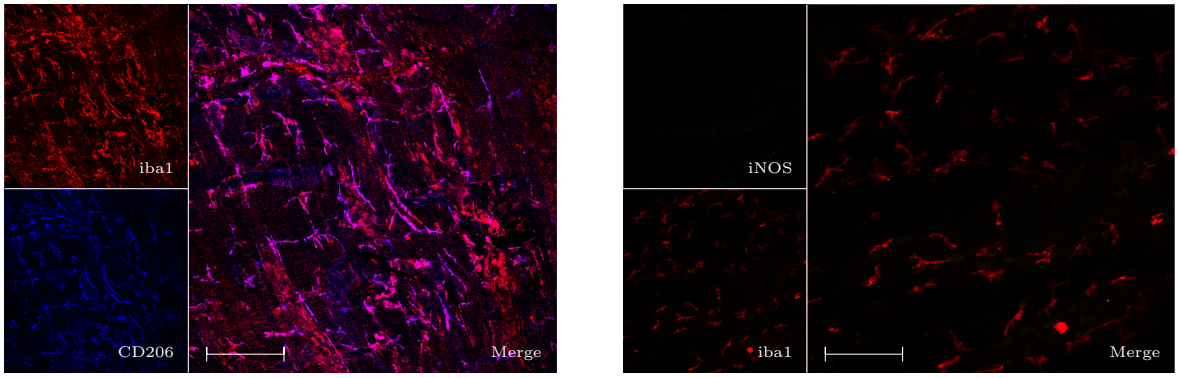
(d) TG mice



(e) Statistics

Figure 3.3: **Comparison of phagocytic cell numbers in KO, WT and TG mice by FACS analysis** Peritoneal mononuclear phagocytes of mice were obtained by peritoneal lavage and stained with F4/80 (yellow) and CD11b (red) antibodies. (a, b) Representative image of the signal of the P2X7-EGFP on mononuclear phagocytes gated on F4/80 (yellow). The EGFP signal of TG mice could be analysed in the green channel. (c, d) Representative image of F4/80 and CD11b positive mononuclear phagocytes (upper right corner). (e) Summary of results from three independent experiments as shown in (c) and (d). Significance between the groups of three animals each was analysed by using an ANOVA. Data are presented as mean ±SEM.

3.1.3 Macrophage polarisation



(a) (b)

Figure 3.4: **Polarization of macrophages in the myenteric plexus in steady state**. Tissue of TG mice stained with antibodies against iba1 as a marker for mononuclear phagocytes and markers for polarization. Representative images of three experiments are shown. Pictures were obtained by confocal microscopy (x20 magnification). Scale bars: 50µm. (a) Staining with CD206, a marker for the anti-inflammatory phenotype. (b) Staining with iNOS, a marker for the pro-inflammatory phenotype.

Intestinal macrophages (i.e. mononuclear phagocytes) are constantly exposed to commensal bacteria and food antigens. It is very important to prevent induction of inflammation in order to maintain homeostasis in the intestinal wall. At the same time those cells must clear bacteria from the tissue. Therefore macrophages establish an anti-inflammatory phenotype and contribute to a non-inflammatory steady state by the secretion of cytokines [16].

Previous findings of our working group showed that ATP stimulation of colonic tissue leads to a loss of neurons in the myenteric plexus. This effect was significantly stronger in P2X7 receptor overexpressing mice that also showed significantly more macrophages in the myenteric plexus compared to WT mice, thus suggesting an inflammatory cause. Therefore, we hypothesised that these macrophages tend to have a pro-inflammatory phenotype in TG mice in contrast to anti-inflammatory cells in WT and KO mice.

To examine this question, we performed IHC of myenteric plexus preparations. Macrophages were characterized by iba1 staining. We used anti-CD206 antibodies as a marker for an anti-inflammatory polarisation and anti-iNOS antibodies as a marker for pro-inflammatory polarisation as suggested for the intestinal wall by previous studies [21]. Furthermore, iNOS seems to play an important role in the neuronal loss during early inflammation [48]. In order to reduce the high background of the iNOS staining, we incubated the myenteric

plexus preparation in PBS with 0,1M glycin at room temperature for thirty minutes as an additional step before staining. All iba1-positive macrophages were CD206 positive thus indicating an anti-inflammatory polarisation of intestinal macrophages in all genotypes corresponding to previous studies (3.4a) [28]. However, there were no iNOS positive macrophages in any genotype neither in ATP-stimulated (not shown) nor in unstimulated tissue (3.4b). Thus we could not induce a change to pro-inflammatory phenotypes by ATP stimulation.

3.2 Humoral analysis

3.2.1 Gene expression of cytokines

In order to validate our TG mouse model, we assessed gene expression levels of the P2X7 receptor in colonic tissue and brain.

The P2X7 gene expression level in the brain of TG mice was in comparison to WT mice elevated by a factor of about 16 (3.5c); however this effect was not statistically significant. The P2X7 gene expression in the colon was also not significantly elevated (3.5a). Although we expected highly increased levels of P2X7, only a slight trend of about 1.6 fold changes was visible. Since only a small number of mice was used for this experiment, these results should be verified in further investigations. We can see a product in P2X7 KO mice since the deleted sequence in P2X7 KO mice was not within the amplicon of the primers (knock-out in exon 2, amplicon in exon 9) (3.5c). Its level was not significantly elevated compared to WT mice.

In previous studies a mutual interaction of P2X7 and P2X4 was described on mRNA and protein expression levels. In the kidneys of P2X7 KO mice a mutual decrease of expression was shown [10]. Therefore, we hypothesised that an elevated expression of P2X7 may result in increased levels of P2X4. Our data show that P2X7 overexpression in brain (3.5d) and colon (3.5b) did not result in an increased expression of P2X4, equivalent to our findings in lung tissue (data not shown).

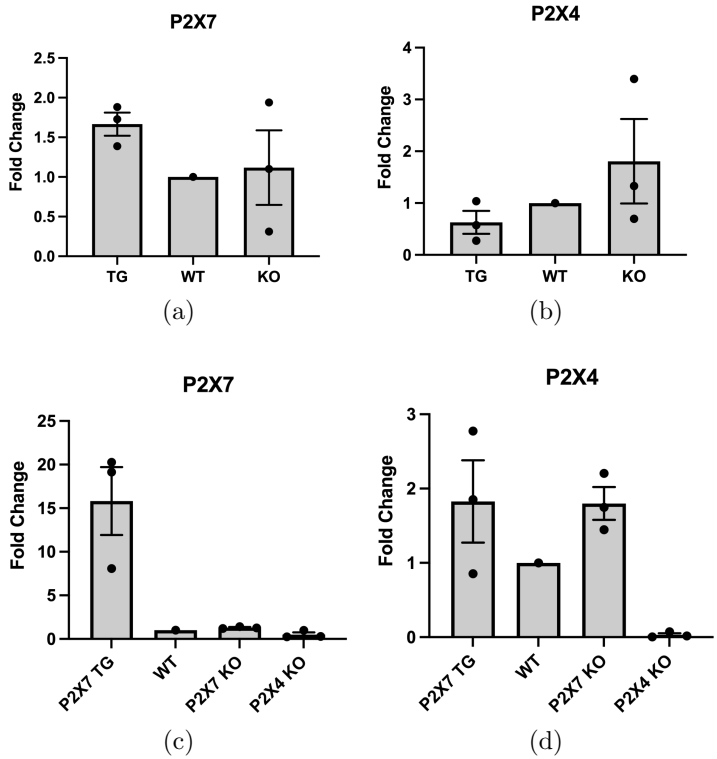
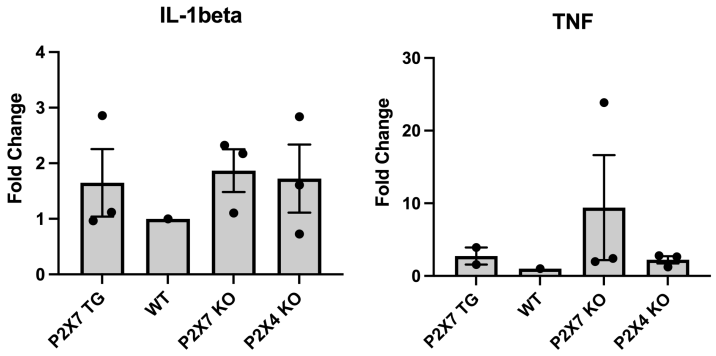


Figure 3.5: **Gene expression of P2X7 and P2X4 in colon and brain.** RNA was obtained from mechanically dissociated murine colon (a, b) and brain (c, d) and analysed by qPCR. The fold change compared to WT mice is shown. Significance of the ΔCt-values of TG and KO mice compared to the ΔCt-values of WT mice was analysed by using a two sided Mann Whitney U Test. Data are presented as mean ±SEM from three animals per group.

Based on our observation of higher numbers of macrophages and higher loss of neurons upon ATP stimulation in the MP of P2X7 receptor overexpressing mice, we hypothesised that the P2X7 receptor has an influence on immune homeostasis and inflammatory response. Since cytokines are the main mediators of this process, we aimed to measure their gene expression levels. The P2X7 receptor is known for its role in inflammasome activation and therefore its effect on IL-1β release in macrophages [11]. Previous studies showed an elevated basal level of IL-1β and TNF-α in colonoscopy samples of patients with CD. However, the inhibition of the P2X7 receptor led to a significant decrease of their levels [1]. This indicates a role of the P2X7 receptor in IL-1β and TNF-α production. Therefore, we expected elevated basal levels of those cytokines in TG mice and reduced levels in KO mice compared to WT mice. This is why we measured the expression of those cytokines in

brain and colon (3.6a, 3.6b, 3.7a, 3.7b). Furthermore, we quantified the gene expression of IFN-γ (3.7c). This is the signature cytokine of Th1-type immune response which has been shown to provoke upregulation of the P2X7 expression in human intestinal tissue [50]. IL-6 was used as an another example of a pro-inflammatory cytokine that is being upregulated in CD [8] (3.7d). Since IL-10 plays a crucial part in gut homeostasis and its basal levels are described to be reduced in patients with CD, [1] we investigated the effect of the P2X7 expression on the basal levels of IL-10 (3.7e). TGF-β is an important regulator of T_{reg}cells and reduces the inflammatory response of intestinal macrophages [28]. Thus it is an important factor for the homeostasis of the immune system. Previous studies show that the P2X7 receptor expression reduces the release of TGF-β [15]. Therefore, we investigated its levels in our mouse model (3.7f). In spite of our expectations the different genotypes showed none of the expected differences in gene expression.



(a) (b)

Figure 3.6: **Gene expression of IL-1β and TNF-α in brain.** RNA was obtained from mechanically dissociated murine brain and analysed by qPCR. The fold change compared to WT mice is shown. Significance of the ΔCt-values of TG and KO mice compared to the ΔCt-values of WT mice was analysed by using a two sided Mann Whitney U Test. Data are presented as mean ±SEM from three animals per group.

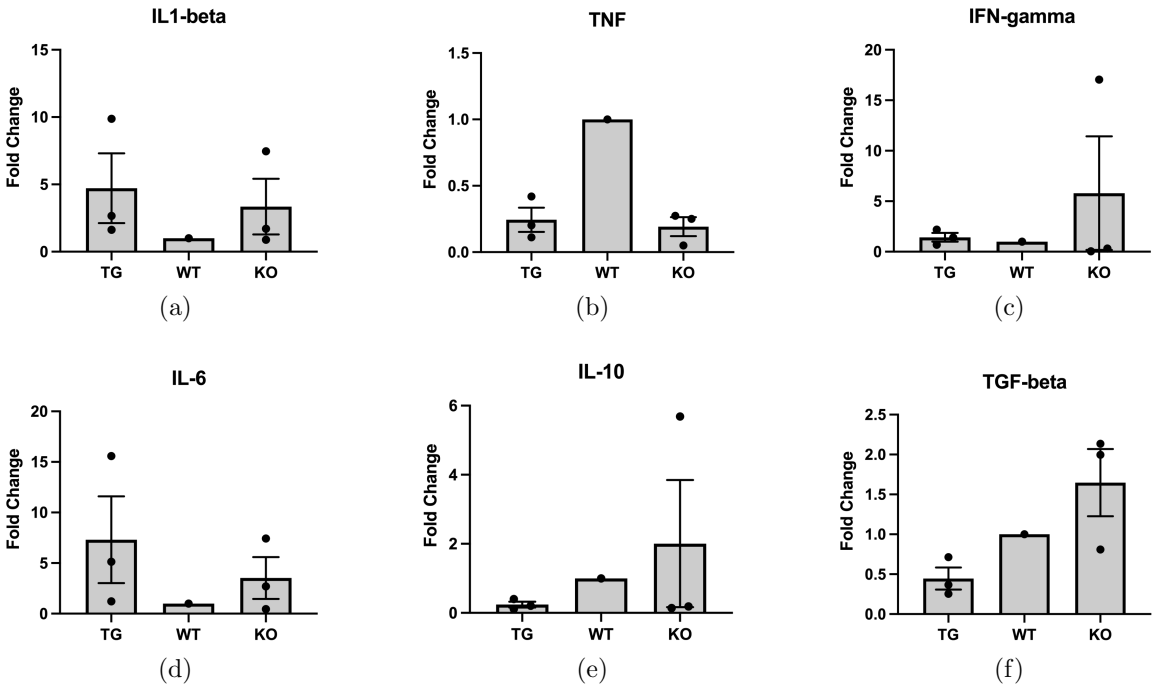
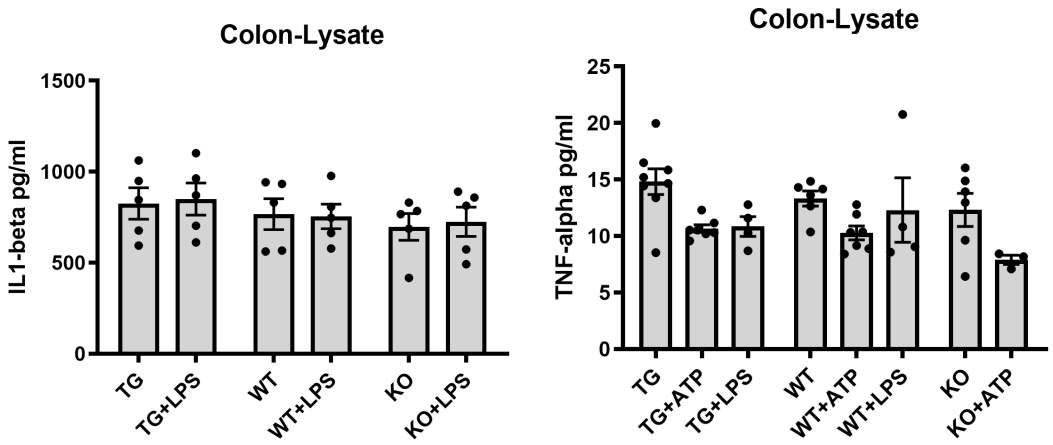


Figure 3.7: **Gene expression of different cytokines in colon.** RNA was obtained from mechanically dissociated murine colon and analysed by qPCR. The fold change compared to WT mice is shown. Significance of the ΔCt-values of TG and KO mice compared to the ΔCt-values of WT mice was analysed by using a two sided Mann Whitney U Test. Data are presented as mean ±SEM from two to three animals per group.

3.2.2 Protein expression of cytokines

Increased levels of mRNA do not necessarily result in increased protein expression since there are different mechanisms which may enhance or repress the synthesis of proteins from mRNA [29]. Therefore, we studied the protein levels of the pro-inflammatory cytokines IL-1β and TNF-α by using ELISA in addition to their mRNA expression (3.8a, 3.8b). We did this analysis comparing two different groups for IL-1β, one unstimulated and one stimulated with $1\frac{\mu g}{ml}$ LPS and 3mM ATP, and three different groups for TNF-α: one was unstimulated, one stimulated with 3mM ATP and one stimulated with $1\frac{\mu g}{ml}$ LPS and 3mM ATP. We could not detect any significant differences in IL-1β or TNF-α levels in different genotypes and different stimulation groups.



(a) IL-1β protein expression in colon. (b) TNF-α protein expression in colon.

Figure 3.8: **Protein expression of IL-1β and TNF-α in colon.** Protein was obtained by mechanical and chemical dissociation of murine colon and analysed by ELISA. Significant differences compared to WT mice or unstimulated groups were analysed by using a two sided Mann Whitney U Test. We analysed five to eight animals per group. Data are presented as mean ±SEM.

3.2.3 Western blot of IL-1β

P2X7 is known to be involved in the procession of Pro-IL-1β to IL-1β by activation of caspase-1 [46]. Since ELISA can not differentiate between IL-1β and its precursor, we used western blot in order to distinguish between these two forms by their weight (17,5 kDa vs. 30,75 kDa). We tested two different detergents, RIPA and NP-40, in order to extract IL-1β. NP-40 showed to be more successful. Furthermore, we stimulated the P2X7 receptor by applying ATP and compared these stimulated samples to unstimulated samples.

We could not detect IL-1β in any mouse line, but only Pro-IL-1β (3.9). Unspecific bands at 25kDa and 50kDa were detected in all our experiments.

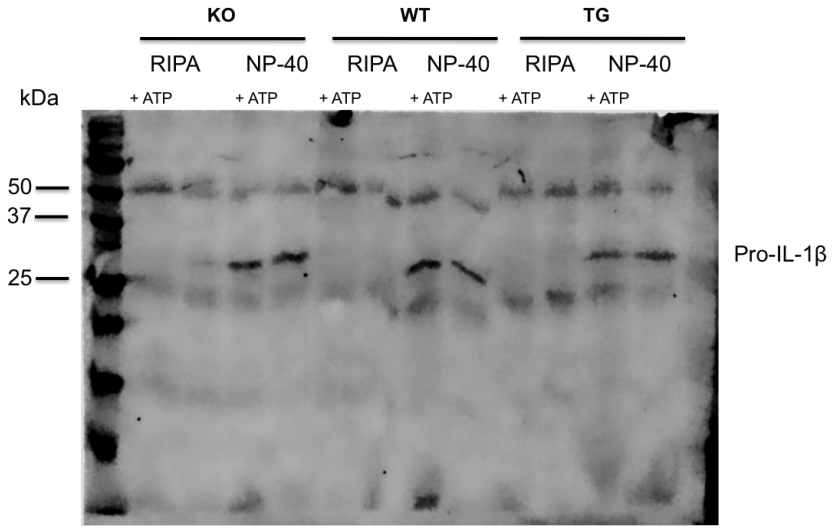
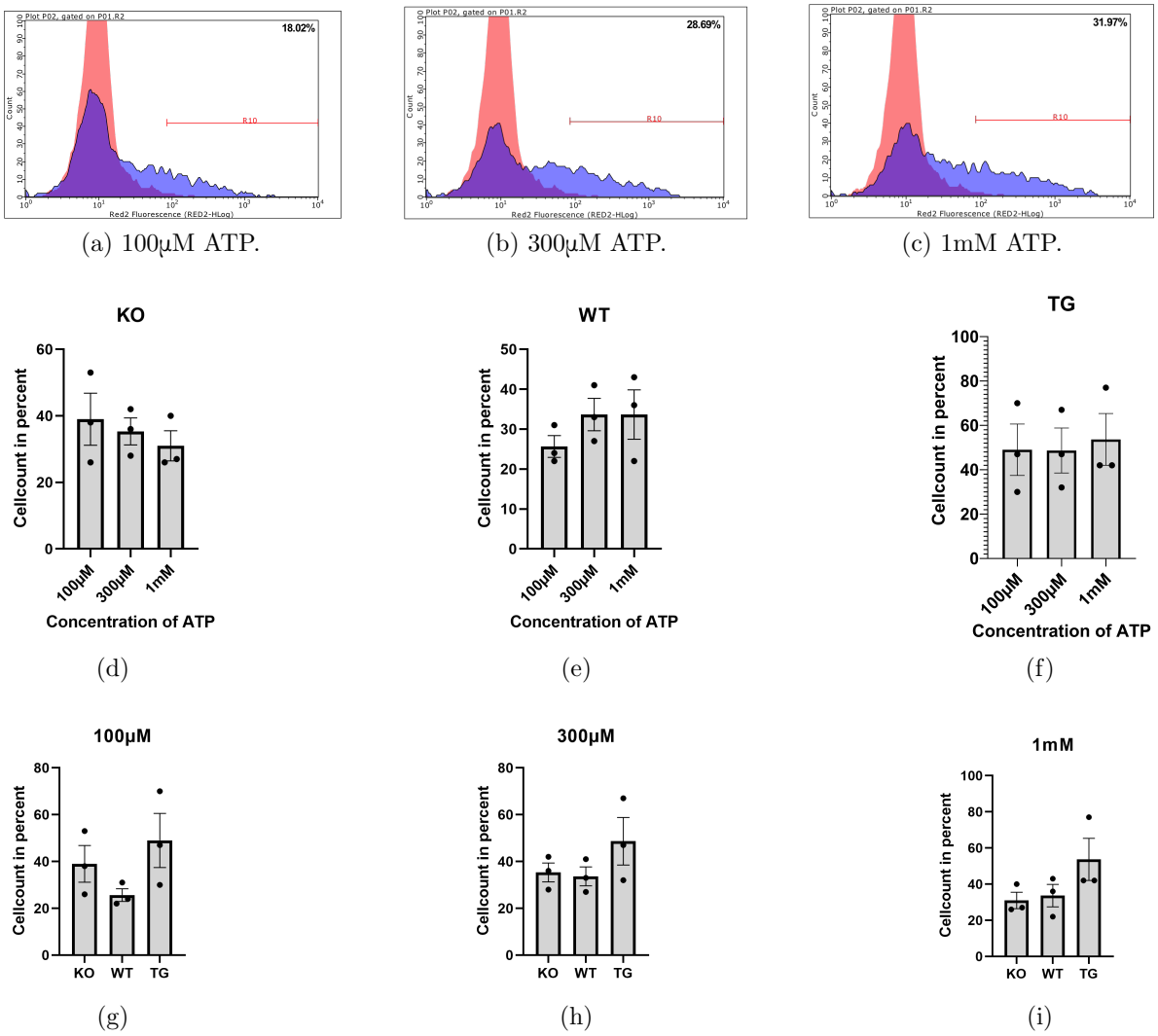


Figure 3.9: **Detection of Pro-IL-1β and IL-1β in murine colon with and without previous stimulation. A representative western blot with samples of the indicated mouse models is shown.** Protein was obtained by mechanical and chemical dissociation of murine colon. The samples were extracted with RIPA buffer or 10% NP40. The tissue was stimulated with 3mM ATP as indicated.

3.3 Functional analysis

3.3.1 Apoptosis assay

Besides its role in inflammation the P2X7 receptor can induce apoptosis [23]. We aimed to determine the effect of P2X7 expression on ATP associated cell death. For this purpose we used Annexin V. This protein detects phosphatidylserine which is a marker of apoptosis since it is expressed on the outer leaflet of the plasma membrane upon apoptosis. The effect was measured using FACS in order to count Annexin V positive and therefore apoptotic cells as shown in 3.10a, 3.10b and 3.10c. On the one hand we compared the dependence of dosage on apoptosis by using 100µM, 300µM and 1mM ATP, on the other hand we compared the different effects in our mouse models and therefore the effect of the P2X7 receptor. Despite our expectations we could not detect any significant changes in the apoptosis rate between different P2X7 expressions or different ATP concentrations (3.10d-3.10i).



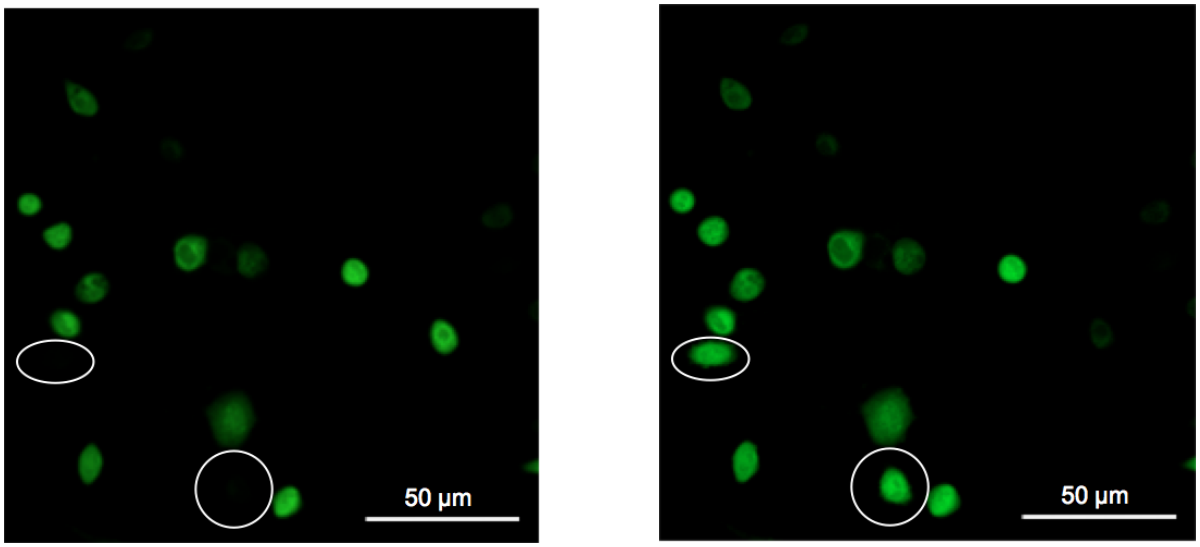
(a) 100µM ATP. (b) 300µM ATP. (c) 1mM ATP.

(d) (e) (f)

(g) (h) (i)

Figure 3.10: **Comparison of apoptosis in different genotypes by the indicated concentrations of ATP.** Cells were obtained by peritoneal lavage and incubated with the indicated concentrations of ATP in order to induce apoptosis. Afterwards cells were stained with Annexin V (blue) as a marker for apoptosis and compared to an isotype control (red). (a-c) Representative images of Annexin V stainings of mononuclear phagocytes in WT mice after stimulation with different concentrations of ATP. (d-f) Effect of the different concentrations of ATP on apoptosis. (g-i) Effect of the different genotypes on apoptosis. Significance compared to our WT mice was analysed by using an ANOVA. We used three animals per group in three independent experiments.

3.3.2 ROS assay



(a) ROS activity at start point.

(b) ROS activity during stimulation.

Figure 3.11: **Visualization of ROS activity of peritoneal macrophages over time after stimulation.** Cells were obtained by peritoneal lavage and stained with tomato lectin (not shown) for differentiation of macrophages and a ROS marker (green). Afterwards cells were stimulated by ATP. Pictures were obtained over a time course by confocal microscopy. (a) Some cells are already ROS positive before stimulation due to commotion. (b) Macrophages start to produce ROS after stimulation.

Macrophages have different mechanisms for eliminating pathogens as for instance by the production of reactive oxygen species (ROS) [34]. However, ROS are not only a harmful byproduct of metabolism, but they are also physiological mediators of cell signalling and play an important role in migration and macrophage polarisation promoting a pro-inflammatory phenotype [47]. ROS production can be induced by the activation of P2X7 [25]. P2X7 associated ROS production of microglia is described to be a mechanism of neuronal damage in the central nervous system [44]. Therefore, we hypothesised that the increased neuronal loss in the MP upon ATP stimulation in TG mice, described by our working group, could be associated with an elevated production of ROS due to the abundance of P2X7. In order to study this question, we performed a ROS assay on isolated peritoneal macrophages that we stimulated with the specific P2X7 activator BzATP [25]. Since ROS production of macrophages is already induced by commotion, there were already ROS positive cells at the beginning of the measurements without stimulation (3.11a). In

order to measure the effect of BzATP on ROS production, we only measured the activity of cells that were negative at the beginning (3.11b). Unfortunately early results of our experiments were not reproducible. Thus we could not draw any conclusions concerning the effects of P2X7 expression on ROS production.

3.3.3 Calcium imaging

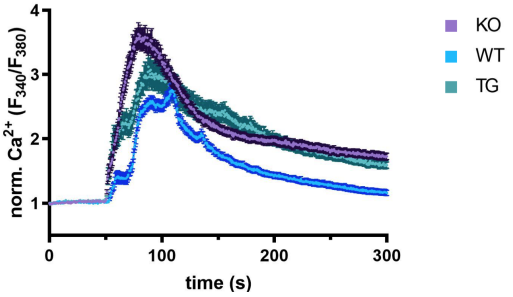
In order to examine the functionality of the P2X7-receptor of P2X7-EGFP-expressing macrophages, we used calcium imaging. We stimulated isolated peritoneal macrophages of different mouse lines with the specific agonist BzATP (300µM) and measured the provoked signal. At first we tried to measure the signal by using a plate reader. But the signal of stained (FURA-2) and stimulated cells was not higher than that of unstained cells which represented the background signal. This may be due to the low amount and the small transversal section of the cells. Thus the bottom of the well is stimulated which increases the background signal. Since it was not possible to gain more cells by peritoneal lavage, we turned to fluorescent microscopy as this method is more specific and allows the measurement of single cells.

The cells of all genotypes showed a very reliable signal by stimulation with BzATP (3.12a). As expected, the TG mice showed a higher signal than WT mice since they have more P2X7 receptors per cell. However, the KO mice showed an even higher signal than TG mice, although they are supposed to have no P2X7 receptor expression (3.12d). In order to exclude that this effect is generated by overestimating the peak of the KO mice due to a lower baseline, we measured the baselines of each genotype. Indeed the baseline of KO mice was lower than that of WT mice and TG mice (3.12c). Therefore we calculated the delta instead of the ratio of the calcium signal (3.12b). This showed that the TG mice reacted more strongly to stimulation than the KO mice. Curiously the signal of the KO mice was still stronger than that of the WT mice (3.12e).

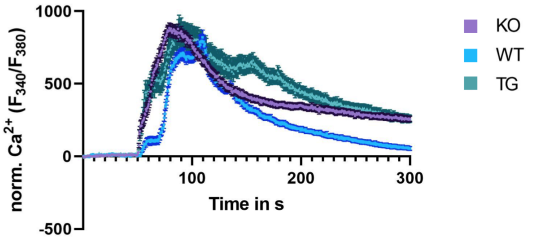
3.3.4 Live cell imaging

In previous IHC experiments we could see that macrophages tended to be located near or around the ganglions of the MP, especially when neurons underwent apoptosis. Therefore we hypothesised that macrophages are involved in the neuronal loss under ATP stimulation. We aimed to find out if there is any alteration of the movements of macrophages in the MP, especially towards the ganglions, by ATP stimulation. Therefore we tried to

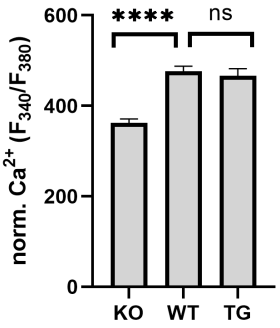
establish a method of live cell imaging that keeps the tissue alive by perfusion with buffer and enables a quick switch to perfusion with a stimulant. Since our TG mice express the P2X7-EGFP receptor, macrophages and structures of ganglions can be visualised without staining. However, we failed to establish such a method. We could not detect any movements of macrophages in any of our experiments.



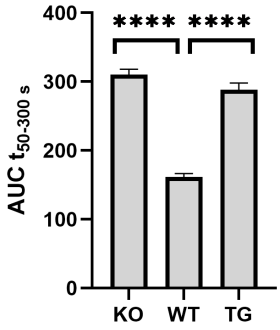
(a) Ratio of the calcium signal after stimulation and baseline.



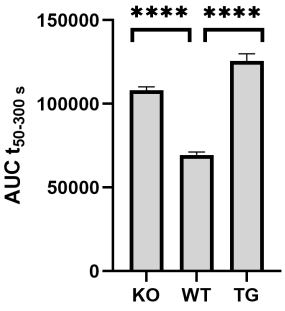
(b) Delta of the calcium signal after stimulation and baseline.



(c) Baseline of calcium imaging of the different genotypes.



(d) Area under the curve of ratio analysis after stimulation with BzATP.



(e) Area under the curve of Delta analysis after stimulation with BzATP.

Figure 3.12: **Measurement of the calcium signal of peritoneal macrophages before (baseline) and after stimulation over time.** Cells were obtained by peritoneal lavage and stained by FURA-2. Macrophages were determined by their morphology in light microscopy. The baseline signal of FURA-2 was measured for 50 seconds before BzATP was added to the solution for stimulation. (a) The calcium signal was normalized by calculating the ratio to the baseline. (b) The calcium signal was normalized by subtraction of the baseline. (c) Strength of the basal activity of FURA-2 in different genotypes. (d) Area under the curve after stimulation, when normalized by calculating the ratio. (e) Area under the curve after stimulation, when normalized by calculating the delta. Significance compared to our WT mice was analysed by using an unpaired Student's t-test. We analysed 108 to 121 cells in 3 different animals per group. Data are presented as mean ±SEM. **** = significant difference in calcium signal ($p<0.0001$).

Chapter 4

Discussion

4.1 The P2X7 receptor - a potential target for IBD treatment

Since its discovery by Burnstock in 1972 and years of controversial debates purinergic signalling has become a huge field of research due to its various effects in all kinds of signalling pathways. While in early studies research mostly focused on short-term signalling like neurotransmission, the interest in long-term signalling like cell differentiation and cell death is constantly rising [7]. By now there is a great deal of evidence concerning the role of purinergic signalling in inflammation. The receptor most involved in inflammatory processes is the P2X7 receptor, expressed by almost all cells of innate and adaptive immunity [13]. It has been shown to be involved in all kinds of inflammatory diseases as for instance in the pathology of IBD.

The analysis of cytokine expression

Neves et al. analysed the expression of P2X7 in IBD patients by means of qPCR and detected elevated levels in inflamed tissue compared to non-inflamed tissue. Furthermore, basal levels of pro-inflammatory cytokines were elevated in colonic samples of IBD patients that could be stimulated even more by adding ATP, while the treatment with a selective P2X7 blocker decreased their levels. Moreover, the levels of anti-inflammatory cytokines were lower in comparison to control groups [1]. According to these findings we expected similar effects in our TG mouse model compared to those of WT mice. However, we could not detect any altered pro- and anti-inflammatory cytokines in qPCR and detected no

relevant changes in ELISA in TG mice.

Various reasons for this effect must be considered. First, we should take into account that there might be no such effect on cytokines as shown by Neves et al. Their study only considered small groups (n=15), mixed sexes and analysed a broad range of ages between 18 and 61 years, meaning a range between teenage age and almost seniority. All of these factors have the potential to influence results and might create the significant effects observed in this study. Hence there might be no effect of P2X7 on inflammatory processes in IBD? After all, that is highly unlikely given the vast amount of evidence of other studies and considering the fact that inflamed tissue reacted differently to non-inflamed tissue within IBD patients. It is more probable that there might have been a problem with the design of our experiment: We analysed cytokine levels in colonic tissue and used a well established method in order to induce the production of pro-inflammatory cytokines, especially IL-1β. But this method, incubation with LPS and ATP, has primarily been used on macrophage cultures and there has proved to be successful. We have to face the fact that the bowel is a very complex organ with extremely complicated regulatory pathways for immunity. The change of the expression of one single receptor in this interaction of numerous signalling pathways may simply be not sufficient in order to change the inflammatory output since reverse regulations could take place at many different levels. A suppression of inflammation could also explain why it was not possible to detect processed IL-1βin western blot. For future research it is necessary to step back and perform these experiments on macrophage cultures to evaluate a possible effect of P2X7 expression on single immune cells.

The role of mononuclear phagocytes

In the study of Neves et al. the overexpression of P2X7 in the colon of IBD patients was mainly induced by mononuclear phagocytes and T cells in the lamina propria. In previous studies of our working group we also found increased numbers of mononuclear phagocytes in myenteric plexus preparations of TG mice. This correlated with an elevated rate of neuronal death after ATP stimulation. Therefore, we aimed to count the number of those cells for the whole bowel wall by means of flow cytometry and to determine the polarisation of mononuclear phagocytes as well. Unfortunately, the preparation led to high rates of apoptosis making these results highly unreliable since the P2X7 receptor is involved in apoptosis. Different expression levels could therefore have an impact on cell counts [37]. Instead we used peritoneal macrophages that can be obtained easily without further stress on cells and can therefore serve as an indicator for the number of mononu-

clear phagocytes in the bowel wall. Despite our previous findings in MP preparations, we could not see any alterations in the number of mononuclear phagocytes (3.3e). Moreover, all mononuclear phagocytes seemed to have an anti-inflammatory polarisation (3.4a) as it is described for macrophages in steady state [31]. Other studies have shown that in experimental colitis in rats infiltrating macrophages have also been CD206 positive and therefore anti-inflammatory [49]. The question if those cells may have pro-inflammatory properties after all needs further investigations. Another explanation for our findings could lay in our experimental design. Overexpression and a short stimulation with ATP may not suffice to drive macrophage polarisation and to change immune cell numbers on a large scale. In previous studies cell populations in experimental colitis changed within days [49]. Moreover, compared to the number of immune cells in the lamina propria, immune cells within the MP are marginal. For further analysis of this issue, an inflammatory environment is necessary as simulated in experimental colitis. Shi et al. already described the migration of immune cells into the different compartments of the bowel wall during the time course of experimental colitis [42]. Considering the possible role of the P2X7 receptor in immune cell migration, experimental colitis could also clarify further questions [26].

The role of P2X7 splice variants

All in all, our findings do not indicate a shift towards inflammation by P2X7 overexpression in the bowel. But why do we see increased neuronal death in our TG mice after stimulation? As described before, mice possess an alternatively spliced version of the P2X7 receptor that is also activated by NAD^+. Since NAD^+ is released during cell preparation, our preparations of the myenteric plexus may have led to an increased activation of P2X7k in TG mice and thereby to an increased inflammatory reaction that is not caused by ATP stimulation of the normal P2X7 receptor. However, NAD^+ signalling does not seem to play an important role in P2X7 activation in macrophages as it does in T cells. T cells themselves are rare in the MP [37] [38]. So they are unlikely to contribute to this effect. Therefore it is more probable that ATP mediated stimulation of the P2X7 receptor is responsible for this neuronal death after all, especially since the inhibition of P2X7 protected against neuronal loss in previous studies [17].

P2X7-expressing cell types

However, the mechanisms for ATP induced neuronal death are still not clear. Gulbransen et al. stated a pathway mediated by neuronal P2X7 supported by glial pannexin-1 that releases ATP into the extracellular compartment. Furthermore, they claimed that no immune cells were observed adjacent to myenteric ganglia. But this does not correspond to our immunohistochemical observations. There has been a controversial debate in the last years about neuronal expression of P2X7. However, the selectivity of commercial antibodies has been questioned and it has been difficult to differentiate between direct effects of neuronal P2X7 activation and indirect effects mediated by glia cells [2] [44]. In order to finally resolve this controversy about the localisation of the receptor, our research group created a BAC transgenic mouse model with a P2X7-EGFP-receptor and used highly specific nano bodies [24]. With this approach we could not detect any neuronal P2X7 expression neither in the brain nor in the ENS [24]. Hence, the model proposed by Gulbransen et al. can not be completely correct. We could indeed verify the colocalisation of P2X7 with glia cells of the ENS. But we could not determine an absence of immune cells. On the contrary, we could detect an abundance of mononuclear phagocytes within the MP that were P2X7 positive in steady state in all genotypes. This result stands in contrast to other studies like Vieira et al. that did not detect CD11b positive cells and therefore macrophages in the MP of control groups, but only found them in the MP of mice with colitis [49]. This may look surprising at first sight, but on closer analysis Vieira et. al. may have misjudged mononuclear phagocytes in their MP preparations as P2X7 positive neuromuscular layers and other cells. While in our stainings neuromuscular cells display as long drawn-out cells (anoctamin-1 positive, data not shown), we could detect between myenteric ganglia an abundance of dendritic shaped cells that were positive for macrophage markers like iba1 or CD68 (data of CD68 not shown) and had an endogenous GFP signal in our TG mice. This indicates that these dendritic shaped cells are P2X7 positive. We verified that by antibody staining against P2X7 and GFP [24]. Our vast evidence for the existence of P2X7 positive mononuclear cells stands against Vieira et al.'s CD11b staining that is indeed supposed to detect those cells in the MP as they are described to be CD11b positive [31]. The answer may lie in the fact that this staining method is established for mice while Vieira et al. performed their stainings on rats. It is described for humans to possess only a minority of CD11b positive mononuclear cells in the bowel [28]. If this also applies to rats is not verified. Gulbransen et al. only used a hematoxylin and eosin staining in order to rule out the presence of immune cells without further immunostaining. This method may not

suffice.

The apoptotic properties of the P2X7 receptor

Finally we concentrated on the apoptotic properties of the P2X7 receptor. There is a vast amount of evidence for its role in apoptosis [23] [13]. We wanted to evaluate the dependence of P2X7 mediated apoptosis on ATP dosage and on P2X7 expression. In contrast to our expectations, we could not detect any significant changes in our apoptosis rates, but only a trend towards an elevated rate of apoptosis in TG mice. Since these experiments were preliminary, the size of our groups of mice was rather small (n=3). This is very likely the cause for this effect. More experiments are necessary to evaluate these findings.

4.2 The use of knock-out and transgenic mouse models

As described above, the interest in the P2X7 receptor has risen tremendously during the past years. However, new results were always accompanied by controversies some of which lasting for more than a decade. These even concern putatively simple questions such as the localisation of the receptor, which is still not completely clarified. But what is the reason for this problem of determining the exact localisation of the receptor? The reason for this is many factors some of which have already been mentioned above. Firstly a functional splice variant of the P2X7 receptor has been found in rodents that may account for P2X7 pseudo immunoreactivity and high variability of P2X7 stimulation induced responses [44]. Furthermore, the issue has been discussed if the expression of the P2X7 receptor depends on its activity. The receptor is supposed to be upregulated or induced by stress such as ischaemia and surprisingly also by mild stimuli like saline injections [44]. Another aspect is that the selectivity of commercial antibodies has been reported to be very poor [2]. For these reasons, various tools have been developed in order to examine the biochemical features, the localisation and the functional properties of the P2X7 receptor. Hence, a number of P2X7 deficient mouse models has been generated as well as a BAC transgenic reporter mouse line and a humanized mouse model. However, those models either failed to dismiss all variants of the receptor or did not reveal consistent data for the localisation of the P2X7 receptor [24]. In order to finally clarify those important questions, our working group generated a transgenic mouse line that overexpresses EGFP-tagged P2X7 receptors

that are under control of a BAC-derived mouse P2X7 gene promoter. Its reliability has extensively been analysed [24] [36].

The functionality of the P2X7 receptor in different genotypes

In this study we used a P2X7-deficient KO mouse and the described BAC transgenic mouse in order to study physiological effects of the P2X7 expression on the immunity of the bowel. The findings concerning the localisation on cells have been discussed above. Surprisingly, we could not see any significant or meaningful alterations in immunity or in inflammatory processes. Therefore, we reevaluated the functionality and the expression of our P2X7-EGFP-receptor. For this purpose we performed a calcium imaging on peritoneal macrophages in order to compare the intensity of the calcium signal after stimulation with the specific agonist BzATP within our different genotypes. The baseline signal before stimulation was decreased in KO mice (3.12c). This is surprising since P2X7 is only activated by high concentrations of extracellular ATP which is not expected to occur during steady state. Therefore, the P2X7 receptor should not have an impact on the basal calcium activity in cells. The stimulation with BzATP led to a reproducible and typical increase of the intensity of the signal in all genotypes (3.12a). Intriguingly, the KO mice seemed to have the strongest signal when the area under the curve of all genotypes was normalized to their level before stimulation (3.12d). Since this may be caused by the lower basal activity of the KO mice and therefore an overestimation of its peak, we substracted the base line and thus calculated a delta adapted area under the curve that simply put genotypes on the same start activity without alterating the peak (3.12b). In this analysis the TG mice had, as expected, the strongest signal. But astonishingly we found the second strongest signal in macrophages from the KO mice (3.12e). The WT-derived macrophages had the weakest signal. While this experiment could confirm the functionality and the increased activity of the P2X7-EGFP-receptor of TG mice on single cells, i.e. macrophages, the results concerning the KO mice are confusing. This effect has not been described for other KO cells such as astrocytes [39] or fibrogenic pancreatic stellate cells [18].

The influence of unspecific stimulation

One source of error could be the fact that BzATP is not as specific as it was thought to be in the past [44]. In fact, it stimulates other P2X receptors with equal activity as the P2X7 [2]. Syed et al. even assigned the highest affinity of BzATP to P2X1 [20]. A possible explanation for the reverse effects of the KO mice could therefore be an upregulation of

other P2X receptors as a result of the P2X7 KO. And indeed there was a trend towards increased P2X4 receptor expression in P2X7 KO mice compared to WT mice in brain (3.5d). Moreover BzATP is described to stimulate P2X4 [20]. However, we could not see any significant differences in P2X4 expression in colon, there was even a reverse trend (3.5b). In order to investigate if other P2X receptors are involved in this effect, further experiments on this matter are necessary. Currently, our working group is investigating a possible connection between P2X7 and P2X4 expression.

The possible effects of TRPM7

Another possible effect for P2X7 independent calcium influx could be an activation of TRPM7. TRPM7 is a channel that conducts calcium and monovalent cations into cells. It is inhibited in steady state by a Mg^{2+}-block. Since ATP is able to complex divalent cations such as magnesium, it could cause P2X7-like currents through TRPM7 by releasing the Mg^{2+}-block [32]. However, there are many reasons that make this explanation unlikely. First, for those electrophysiological experiments performed by Norenberg et al. Mg^{2+}-free medium was used. In contrast, we used 1mM $MgCl_2$ for our external solution (2.3). Mg^{2+} containing mediums by Norenberg et al. showed only weak effects. Moreover, Norenberg et al. used millimolar concentrations of ATP starting with 1mM and assigned the effect only to low millimolar concentrations of ATP. In contrast, we were using only 300µM BzATP. Furthermore, the ATP dependent currents of TRPM7 showed amplitudes of less than $30\frac{pA}{pF}$ and are very low compared to those of P2X7 that showed amplitudes of over $200\frac{pA}{pF}$ [32]. Therefore, the TRPM7 dependent currents are unlikely to have such a strong impact on our calcium measurements that it could explain those high differences between our genotypes. And finally, this effect would concern all three genotypes. Therefore, even if there was a measurable effect, it should not affect the differences between the genotypes.

The role of the P2X7 receptor in colonic immunity

But apart from the fact that our TG mice did not show an increased inflammatory state, although our P2X7-EGFP-receptor is functional, our KO model did not show any alterations compared to wild type mice, too. Since P2X7 deficiency protects mice against experimental colitis, we expected an anti-inflammatory state compared to WT mice for instance by decreased levels of pro-inflammatory cytokines. Taken together and in combination with our results for TG mice, this indicates a minor role of the P2X7 receptor in immunity in steady state in general.

4.3 Outlook

In this study we aimed to illuminate the role of the P2X7 receptor in inflammatory processes of the bowel. We studied the effects of P2X7 on abundance and polarisation of immune cells, especially of mononuclear phagocytes i.e. macrophages, on cytokine production and on inflammatory responses. In contrast to the vast amount of evidence concerning the role of the P2X7 receptor in IBD and all sorts of inflammatory pathways, we could not detect any relevant differences in immunity between our genotypes neither in steady state nor after stimulation. We have already discussed a lot of possible reasons for that. In summary, we see two main problems in our experimental designs. First, we performed most of our experiments on the bowel itself. However, this is a very complex organ that underlies very complicated immunological regulations that are only partly understood. It is therefore impossible for us to survey the regulatory pathways that may be activated by altered P2X7 expression. Second, we only used ATP and BzATP for stimulation. While this is an efficient way to stimulate the P2X7 receptor on cells in cell culture thus inducing various effects, this may not apply to the induction of inflammatory processes in a whole organ. ATP is a DAMP indeed and plays an important role in inflammation, but a vast amount of cytokines and other factors is involved in inflammation itself. ATP alone may simply not be enough to induce inflammation in tissue; in our case it is rather likely that regulatory pathways within tissues prevent the induction of inflammation. A reasonable procedure for future experiments is, in my opinion, the replication of our cytokine measurements on single cells i.e. macrophages gained by peritoneal lavage for instance. And finally, in order to assess the role of the receptor in IBD, a colitis model that provides an inflammatory state is necessary.

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit eigenständig und ohne fremde Hilfe angefertigt habe. Textpassagen, die wörtlich oder dem Sinn nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche kenntlich gemacht.

Die Arbeit wurde bisher keiner anderen Prüfungsbehörde vorgelegt und auch noch nicht veröffentlicht.

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