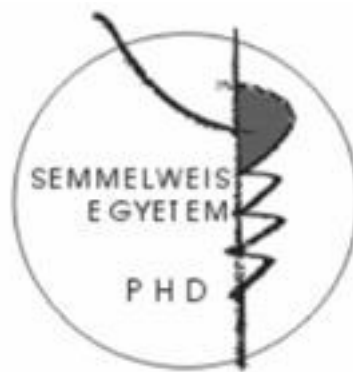


# Investigation of signal transduction in neutrophils and in autoimmune arthritis

Ph.D. thesis

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## SUMMARY

Neutrophils are essential in maintaining health: both their reduced functional capacity (through severe infections) or their overactivation (e.g. in some autoimmune diseases) lead to significant damage to the host. During my Ph.D. work, I investigated neutrophil signaling and signaling molecules in the initiation and progression of autoantibody-induced autoimmune arthritis.

In our first experiments, we characterized a novel p190RhoGAP null mutation in mice and we demonstrated that p190RhoGAP did not play a major indispensable role in the investigated  $\beta_2$  integrin-dependent and -independent in vitro neutrophil functions. The molecule was also dispensable in the effector phase of a neutrophil-dependent autoantibody-induced autoimmune arthritis.

My further experiments revealed an important role for Fc receptor  $\gamma$ -chain (FcR $\gamma$ ) in Fc $\gamma$  receptor-mediated in vitro neutrophil functions. A structure-function analysis pointed out an essential role for the intracellular ITAM tyrosines of FcR $\gamma$  in the above mentioned cell responses and in the development of the effector phase of autoantibody-induced autoimmune arthritis.

Our further results indicate that neutrophil Fc $\gamma$  receptor signaling divides into two distinct signaling routes downstream of Syk: to a CARD9-independent pathway leading to short-term cell responses and to a CARD9-dependent, NF- $\kappa$ B-mediated pathway resulting in long-term responses. The absence of CARD9 resulted in an intermediate phenotype of autoimmune arthritis, behind of those we propose a role for altered neutrophil and macrophage long-term responses.

Finally, I found that the tyrosine kinase inhibitor dasatinib did not significantly influence the inside-out signaling of neutrophil  $\beta_2$  integrins and the bacterial phagytosis by mature human neutrophils.

Our results provide further insights into the signaling of neutrophils and into the pathogenesis of autoimmune arthritis. The identified molecules can contribute to the development of a more efficient therapy in several inflammatory diseases (e.g. in autoimmune inflammation) in the future.

## INTRODUCTION

Neutrophils play a crucial role in innate immunity. Neutrophils circulate in blood vessels until being attracted to tissues by invading microorganisms, where they can be activated through various cell surface receptors. Activated neutrophils are massive effector cells: they can eliminate the invading microorganisms by phagocytosis, superoxide release or degranulation, while they can modify or attract more immune cells to the site of infection by their cytokine/chemokine production. Neutrophil functions are strictly regulated both in space and time: inappropriate activation can lead to tissue destruction.

Autoimmune diseases are chronic, progressive disorders that affect approximately 3% of the Western population and cause significant mortality in the young and in the middle-aged population groups. One of the biggest group of autoimmune diseases consist of autoimmune joint inflammation (e.g. rheumatoid arthritis). In contrast to their frequency, their chronic progression and the increasing disability of the affected patients, the therapy of autoimmune arthritides is relatively unsolved. Therefore there is an urgent need for developing new drugs, for which a better understanding of the pathomechanism is crucial. As there are emerging data that neutrophils can participate in some autoimmune diseases, that raise the possibility that altering neutrophil functions can be a therapeutic aim in the future, we focused our research on this cell type.

Several receptors participate in immune functions, that link trigger signals to effector responses. Antigen receptors and Fc receptors belong to classical immunoreceptors. In general, these receptors use several tyrosine kinases in their pathways that start from the tyrosine-containing ITAM motif of the receptor complex and lead to the effector functions through the activation of different transcription factors (e.g. NF- $\kappa$ B). Neutrophils express a large number of Fc $\gamma$  receptors, the cell surface receptors for immunoglobulin G that show a similar signal transduction to that of classical immunoreceptors. It is presently unclear whether the ITAM-containing adaptor molecule Fc receptor  $\gamma$  chain –

that associates to the ligand-binding chain through a salt bridge – is required for neutrophil Fc $\gamma$  receptor signaling, and if yes, then how. The role of NF- $\kappa$ B and CARD9 has also not been investigated in the process so far.

The heterodimer integrins are important adhesion molecules in cell-cell/cell-extracellular matrix interactions. Signals from inside of the cell result in a conformational change and lead to an upregulation of the molecules on the cell surface and to a change in the localization in the membrane through a process called 'inside-out signaling', which makes ligand binding and further signaling ('outside-in signaling') possible. Integrin signaling in phagocytes is a complex and not fully understood process, in which Src family kinases play a crucial role. Src kinases partially exert their effects through small GTP-ases. Small GTP-ases (e.g. Rho, Rac, Cdc42) have a special activation cycle, in the regulation which GTP-ase activation proteins are essential. As p190RhoGAP has been shown to be an important Src substrate in fibroblasts and in neurons, where it is activated through integrin-mediated processes, we examined if p190RhoGAP has a role in neutrophil  $\beta_2$  integrin signaling.

Tyrosine kinases regulate several cellular events from proliferation to immune functions. While mutations of these proteins can lead to the development of multiple cancers, the excessive function of tyrosine kinases related to innate immune cell overactivation can result in autoimmune tissue damage. The so-called Bcr-Abl fusion protein that leads to the dysregulated overactivation of Abl kinase is an important feature of some hematological malignancies. In the therapy of these diseases, the Abl-specific imatinib and the Abl-Src dual inhibitor dasatinib is widely used. The role of Src kinases in neutrophils raised the possibility that besides inhibiting malignant cells, dasatinib can also alter the functions of mature human neutrophils. This question is important for two reasons: as a side-effect of the drug in its regular clinical dose and, from the other side, as a potential new approach in the control of autoimmune inflammation.

Animal models are useful tools in understanding human autoimmune arthritides. In some animal autoimmune disease models neutrophils, Fc $\gamma$  receptors and  $\beta_2$  integrins are crucial participants, which underlines how important it is to investigate neutrophil signal transduction.

During my Ph.D. work, I investigated the effects of genetic or pharmacological modifications of the above mentioned signal transduction routes on in vitro neutrophil functions and on the development and progression of autoimmune arthritis in mice.

## OBJECTIVES

During my Ph.D. work, I was working on four projects and I was searching for the answers for the following questions:

- How does a GTP-ase activating protein, p190RhoGAP participate in neutrophil  $\beta_2$  integrin-dependent responses and in the initiation of the neutrophil-/ $\beta_2$  integrin-mediated K/BxN serum transfer arthritis?
- Does Fc $\gamma$  receptor signaling require Fc receptor  $\gamma$  chain or is this adapter protein only needed for the cell surface expression of the receptors? How does the replacement of the two ITAM-tyrosines to phenylalanines influence neutrophil Fc $\gamma$  receptor signaling and the development of autoimmune arthritis?
- Does CARD9 have a role in neutrophil Fc $\gamma$  receptor signaling and in experimental autoimmune arthritis?
- By what means can the tyrosine kinase inhibitor dasatinib influence neutrophil  $\beta_2$  integrin inside-out signaling and bacterial phagocytosis?

During my experiments related to the first three projects, I used a genetic approach, while a pharmacological approach was used in the last part.

## METHODS

### Mice and bone marrow chimeras

For investigating the role of p190RhoGAP, we generated p190RhoGAP<sup>-/-</sup> bone marrow chimeras by the help of mice carrying a novel p190RhoGAP null mutant allele (*Grlf1*<sup>tm2JSet</sup>). As an integrin-dependent reference, we used CD18<sup>-/-</sup> chimeras. For investigating the role of the intracellular ITAM tyrosines of Fc receptor  $\gamma$  chain, mice expressing transgenic wild type or ITAM tyrosine mutant Fc receptor  $\gamma$  chain on an FcR  $\gamma$  chain-deficient background were used. We also used CARD9-, Bcl10- and Syk-deficient bone marrow chimeras.

Bone marrow transplantation was carried out by the injection of CD45.2-positive hematopoietic cells into lethally irradiated CD45.1-negative recipients. The efficiency of transplantation was checked four weeks later by flow cytometry.

We used wild type mice or wild type bone marrow chimeras as controls. During our experiments, the genotypes of cells were checked by Western blot.

### Isolation of neutrophils, experimental conditions

Mouse neutrophils were isolated from bone marrow by Percoll gradient centrifugation. Human neutrophils were separated from the peripheral blood of healthy volunteers by Ficoll gradient centrifugation. For the inhibitor studies, human neutrophils were preincubated with dasatinib. Samples containing dimethyl sulfoxide served as controls. Neutrophils were kept in an HBSS medium during their isolation until their functional tests. The solutions were supplemented with calcium chloride and (except for some cases) by magnesium chloride) just before stimulation. The functional tests were done at 37 °C.

### Neutrophil activation and cell response detection methods

For integrin-mediated processes we used immobilized human fibrinogen and a costimulus (e.g. TNF $\alpha$ ). Immobilized immune complexes were made by

human serum albumin and anti-albumin antibodies or by lactoferrin and anti-lactoferrin antibodies.

Superoxide release was measured by a real-time cytochrome c reduction test, degranulation was detected by gelatinase zymography, cell spreading was quantified by phase contrast microscopy. Neutrophil migration was assessed by the Transwell migration assay. Upregulation and activation of CD11b was quantified by flow cytometry. Cytokine release was determined by ELISA from the supernatants after 6 hours of stimulation. Bacterial phagocytosis was assessed by the uptake of GFP-expressing *Staphylococcus aureus*.

We used the Western-blot technique for the identification of intracellular signaling events. Electrophoretic mobility shift assay was performed for the detection of NF- $\kappa$ B activation.

In vivo migration of neutrophils was investigated in mixed bone marrow chimeras by a competitive migration assay.

### **K/BxN serum transfer arthritis experiments**

Arthritis was induced by a single intraperitoneal injection of arthritogenic or control serum, followed by daily assessment of arthritis development. Scoring of visible clinical signs of arthritis, measurement of ankle thickness, and assessment of articular function were performed.

### **Presentation and interpretation of data**

We performed our experiments at least three times. Within one in vitro measurement, we usually used triplicates. The results were analyzed by Student paired two-population test, unpaired two-tailed two-population t test or repetitive two-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Analysis were performed by the STATISTICA programme. The p values <0.05 were considered statistically significant.



## RESULTS

### **The role of p190RhoGAP in neutrophils and in autoimmune arthritis**

#### *The novel null mutation and the analysis of the p190RhoGAP<sup>-/-</sup> animals*

For our experiments, we used mice carrying the novel null mutation of the p190RhoGAP allele (*Grlf1*<sup>tm2JSet</sup>) that was made by an American workgroup. This mutation was characterized and published by our laboratory.

The absence of p190RhoGAP resulted in perinatal lethality. For making our neutrophil studies possible, p190RhoGAP-deficient bone marrow chimeras were generated from the fetal liver of the p190RhoGAP<sup>-/-</sup> (and control) fetuses.

#### *The role of p190RhoGAP in mouse neutrophil $\beta_2$ integrin signaling*

In contrast to CD18-deficient neutrophils, p190RhoGAP<sup>-/-</sup> neutrophils were able to produce superoxide on fibrinogen in the presence of TNF $\alpha$  and showed only a moderate decrease compared to the wild type cells. We could observe the same phenomenon in connection with cell spreading. The absence of p190RhoGAP also did not affect the responses in the presence of other stimuli than TNF $\alpha$  on fibrinogen.  $\beta_2$  integrin-dependent in vitro and in vivo migration also did not require p190RhoGAP.

#### *Is p190RhoGAP required for autoimmune arthritis?*

During our K/BxN serum transfer arthritis experiments arthritogenic serum treated wild type mice showed a severe inflammatory phenotype that was absent in CD18<sup>-/-</sup> mice. In p190RhoGAP<sup>-/-</sup> animals, the arthritis could develop and showed no difference compared to the wild type mice. We could observe a similar phenotype in the functional test.

### **The role of Fc receptor $\gamma$ chain and its ITAM tyrosines in neutrophil Fc $\gamma$ receptor signaling and in autoimmune arthritis**

#### *The role of FcR $\gamma$ in neutrophils*

Compared to wild type cells, FcR  $\gamma$  chain-deficient mice were unable to produce superoxide, to degranulate and to spread on an immobilized immune complex surface. As activating Fc $\gamma$  receptors are not expressed on the cell surface in the absence of FcR  $\gamma$  chain, we raised the question if FcR  $\gamma$  chain is only needed for stabilizing the receptors in the plasma membrane or it is also required for signal transduction through its ITAM tyrosines.

#### *The role of FcR $\gamma$ chain ITAM tyrosines in neutrophils: our approach*

During our structure function analysis, we used mutant FcR  $\gamma$  chain expressing cells that had their intracellular tyrosines replaced by phenylalanines on an FcR  $\gamma$  chain-deficient background (FcR $\gamma$ <sup>-/-</sup> FcR $\gamma$ (YF)Tg animals). As controls, animals expressing wild type FcR  $\gamma$  chain on an FcR  $\gamma$  chain-deficient background were used (FcR $\gamma$ <sup>-/-</sup> FcR $\gamma$ (VT)Tg). The experiments were also carried out with wild type and FcR $\gamma$ <sup>-/-</sup> mice.

#### *The characterization of Fc receptor $\gamma$ chain tyrosine mutant neutrophils and the role of these ITAM tyrosines in neutrophil Fc $\gamma$ receptor signaling*

The above mentioned genetic modifications did not alter neutrophil maturation. The transgenic reexpression of the wild type or the tyrosine mutant FcR  $\gamma$  chain could partially restore the protein expression on Western blots and resulted in activating Fc $\gamma$  receptors on the cell surface.

While the reexpression of the wild type FcR  $\gamma$  chain on an FcR  $\gamma$  chain-deficient background could partially restore the ability of the cells to produce superoxide, the ITAM tyrosine mutant cells were not able to do so. Gelatinase degranulation and cell spreading showed the same phenomenon.

#### *The role of ITAM tyrosines in the effector phase of autoimmune arthritis*

While in FcR $\gamma$ <sup>-/-</sup> FcR $\gamma$ (VT)Tg mice an intermediate arthritis could develop, FcR $\gamma$ <sup>-/-</sup> FcR $\gamma$ (YF)Tg animals were protected against joint inflammation. While in arthritogenic serum treated FcR $\gamma$ <sup>-/-</sup> FcR $\gamma$ (VT)Tg animals

joint dysfunction could appear, the replacement of ITAM tyrosines resulted in a full protection.

## **The role of CARD9 in neutrophils and in autoimmune arthritis**

### *Initial characterization of CARD9-deficient neutrophils*

CARD9 was present in neutrophils and not the cell surface expression of FcγRIII, nor that of FcγRIV was affected by the absence of CARD9.

### *Neutrophil cell responses in the absence of CARD9*

Short-term cell responses appear within 30 minutes after activation (e.g. superoxide release, degranulation, cell spreading). CARD9<sup>-/-</sup> neutrophils produced the same amount of superoxide, degranulated at the same extent and spread at the same ratio as wild type cells on an immobilized immune complex surface.

While Fcγ receptor ligation resulted in a significant phosphorylation/degradation of IκBα and activation of NF-κB, the processes were reduced in CARD9<sup>-/-</sup> neutrophils. CARD9 was also essential for the production of NF-κB-dependent chemokines like CXCL2 or CCL3.

We observed a similar long-term cell response-specific phenomenon in macrophage Fcγ receptor signaling in the absence of CARD9.

### **The role of Syk tyrosine kinase and Bcl10 in neutrophil Fcγ receptor signaling**

During our in vitro studies, we aimed to determine the relative position of CARD9 in neutrophil Fcγ receptor signaling. On the basis of literature, we focused our attention on two molecules: on Syk tyrosine kinase and on Bcl10. While Syk was essential for both the short-term and the long-term cell responses, Bcl10 seemed to be crucial only for the later events.

### *Role of CARD9 in the effector phase of autoimmune arthritis*

Upon the injection of the arthritogenic serum, arthritis could develop in CARD9-deficient mice, but the inflammation showed a partial decrease

compared to wild type animals: the clinical score, the ankle thickness changes and the functional performance showed an intermediate phenotype in the absence of CARD9. A similar phenotype could be observed in an autoimmune blistering skin disease in the absence of CARD9. The CXCL2 levels were reduced in the inflamed synovium of CARD9<sup>-/-</sup> mice, while neutrophils could migrate to the joints in the absence of CARD9.

### **The effect of dasatinib on some neutrophil responses**

#### *The effect of dasatinib on neutrophil $\beta_2$ integrin inside-out signaling*

Dasatinib did not significantly affect the upregulation and activation of CD11b in the presence of TNF $\alpha$ , IL-8 or fMLP.

#### *Intact phagocytosis in the presence of dasatinib*

Dasatinib did not influence the phagocytosis of human serum opsonized, GFP-expressing *Staphylococcus aureus* by human neutrophils.

## CONCLUSIONS

According to our aims, we sum up our conclusions in four points.

1. We characterized a novel null mutation of p190RhoGAP and investigated the effects of the absence of the protein on neutrophil functions. Neutrophils deficient for p190RhoGAP did not show a major reduction in  $\beta_2$  integrin-dependent and -independent in vitro neutrophil functions. According to our results, p190RhoGAP did not play an indispensable role in the initiation and progression of autoimmune arthritis.

2. In the absence of Fc receptor  $\gamma$  chain, neutrophil Fc $\gamma$  receptor mediated cell responses were abolished. Our structure-function analysis revealed a crucial role for the ITAM tyrosines of FcR  $\gamma$  chain in these processes. These tyrosines were also essential in autoantibody-induced autoimmune arthritis.

3. CARD9 was dispensable for short-term cell responses in neutrophils upon Fc $\gamma$  receptor ligation in neutrophils, while it was essential for the long-term events like chemokine production. During this latter process, CARD9 was downstream of Syk and exerted its effects through the activation of NF- $\kappa$ B, probably by interacting with Bcl10. The absence of CARD9 resulted in an intermediate phenotype in autoantibody-induced autoimmune diseases, which was probably mediated by the decreased production of chemokines by neutrophils and macrophages.

4. The small molecular weight tyrosine kinase inhibitor dasatinib did not significantly alter neutrophil  $\beta_2$  integrin inside-out signaling and the bacterial uptake by human neutrophils.

## LIST OF PUBLICATIONS

**The PhD thesis is based on the following publications** (in a chronological order):

Jakus Z, **Németh T**, Verbeek JS, Mócsai A.  
Critical but overlapping role of FcγRIII and FcγRIV in activation of murine neutrophils by immobilized immune complexes.  
*J Immunol* 2008;180:618-629.  
Impact factor: 6,000

**Németh T**, Futosi K, Hably C, Brouns MR, Jakob SM, Kovács M, Kertész Z, Walzog B, Settleman J, Mócsai A.  
Neutrophil functions and autoimmune arthritis in the absence of p190RhoGAP: generation and analysis of a novel null mutation in mice.  
*J Immunol* 2010;185:3064-3075.  
Impact faktor: 5,745

**Németh T**, Mócsai A.  
The role of neutrophils in autoimmune diseases.  
*Immunol Lett* 2012;143:9-19., Review  
Impact factor: 2,511

Futosi K, **Németh T**, Pick R, Vántus T, Walzog B, Mócsai A  
Dasatinib inhibits pro-inflammatory functions of mature human neutrophils.  
*Blood* 2012; 119(21):4981-91.  
Impact factor: 10,558

### **Other publications:**

Csölle C, Andó RD, Kittel A, Gölöncser F, Baranyi M, Soproni K, Zelena D, Haller J, **Németh T**, Mócsai A, Sperlágh B.  
The absence of P2X7 receptors (P2rx7) on non-haematopoietic cells leads to selective alteration in mood-related behaviour with dysregulated gene expression and stress reactivity in mice.  
*Int J Neuropsychopharmacol* 2012.  
(In press, DOI: 10.1017/S1461145711001933)  
Impact factor: 4,669