As planned, we focussed on the biological role and regulation of GTPase activating proteins (GAPs) in phagocytes. The central target of interest was ARHGAP25, the protein that has been cloned earlier in our laboratory. In addition, we did a couple of pilot studies on neutrophil-derived extracellular vesicles (EVs), a new topic that emerged after submission of the research plan.

The detailed results are as follows.

Characterization of the physiological role of ARHGAP25 in phagocytes

We started to investigate the physiological role of ARHGAP25 in different neutrophil functions by comparing bone marrow PMN isolated from wild type or ARHGAP25 deficient animals. First we determined the rate of phagocytosis of yeast particles opsonized with full murine serum. In this function we did not detect any significant difference. These results differ from our earlier data obtained in model cell lines. The difference may be due to the unprimed nature of bone marrow neutrophils or to compensation by other RacGAPs.

Next we investigated the superoxide production of ARHGAP25 deficient neutrophils. We applied chemotactic stimulus and PMA, a pharmacological agent activating protein kinase C directly. However, we did not see any reproducible difference between cells from knock-out or wild-type animals.

In order to be able to evaluate the role of ARHGAP25 in the bacterial killing function of neutrophils, we elaborated a new bacterial killing assay based on flow cytometric detection of bacteria. We carried out detailed comparison of the new technique with previously applied techniques based on optical density changes or spreading on plates. Our proposed method proved to be superior to all other technique applied hitherto, the main advantage being the short time (3 hours) required for the entire determination. The data describing the new killing method has been published in the Journal of Leukocyte Biology.

We evaluated the bacterial killing function of wild-type and ARHGAP25KO murine neutrophils both by the newly developed and by the previously applied techniques, but did not find any difference.

Next we investigated the motility of different leukocyte subsets in ARHGAP25 deficient animals by intravital microscopy. Part of these experiments were carried out in collaboration with Prof. Markus Sperandio at the Ludwig Maximilian University in Munich. We investigated in detail all phases of leukocyte transendothelial migration, i.e. rolling, spreading, crawling and transmigration in the TNF-alpha activated cremaster model. In addition, we controlled blood cell count, blood flow velocity, wall shear rate, microvessel diameter, surface expression and avidity of adhesion proteins. We observed the following alterations in the ARHGAP25 deficient animals as compared to their wild type littermates:

- mean rolling velocity of leukocytes was markedly decreased
- crawling velocity of leukocytes was increased
- mean Euclidean distance of crawling was increased
- tranendothelial migration was significantly increased
- neutrophilic granulocytes represent the major transmigrated leukocyte population

Thus, the detailed in vivo analysis revealed that lack of ARHGAP25 influenced all phases of leukocyte migration.
In flow chamber assays, under ex vivo conditions, using coated artificial surfaces, we were able to reproduce the altered migration pattern observed in the animal experiments.

We repeated the above experiments in bone marrow chimeric animals, where only blood cells are deficient in ARHGAP25 but endothelial cells are not altered. We observed similar increase in transmigration as in complete knock-out animals. Thus, the altered migration properties are the consequence of ARHGAP25 deficiency of leukocytes but not of the endothelial cells.

Searching for the potential mechanism of the observed alteration in leukocyte migration, we determined the amount and activity of major surface proteins involved in direction of leukocyte migration, such as beta-2 integrins, selectins and chemokine receptors. We did not find any difference between ARHGAP25 deficient and wild type cells. In contrast, the amount of active, GTP-bound Rac was significantly increased in ARHGAP25 deficient, TNF-alfa induced neutrophils and it was accompanied by an increase in the amount of filamentous actin.

Our investigations revealed ARHGAP25 as a new regulator of leukocyte migration and a potent anti-inflammatory molecule. The results were summarized and the manuscript has been submitted to the Journal of Immunology. We obtained a favourable Reviewers' report that however required further experimentation.

We were requested to investigate under in vitro conditions the altered migration of ARHGAP25 deficient neutrophils in a chemotactic assay. After trying several coating methods and chemotactic agents, we could demonstrate in a Transwell assay higher transmigration capacity of ARHGAP25 deficient than wild type neutrophils to a gradient of the chemotactic agent CXCL12. Interestingly, the difference was significant only if the cells have been pretreated with TNF.

The second request consisted of demonstration of the migration difference between wild type and ARHGAP25 deficient cells in another animal model, such as the induced peritonitis. We carried out this assay with different stimulants and at different time-points. Thioglycollate stimulation induced very bad general condition of the animals without difference in the number of migrated neutrophils. In case of TNF stimulation we did obtain difference between wild-type and ARHGAP25 deficient animals, but the results were not fully consistent. Therefore we decided to carry out a histological study which resulted in clearly visible difference between wild-type and modified animals. The convincing histological pictures have been incorporated in the revised manuscript.

The third request related to the surface expression of adhesive molecules and receptors. We have shown in the original manuscript that ARHGAP25 deficiency did not alter the expression pattern. The reviewer requested to verify that this condition prevailed also after transmigration of the cells. Therefore we collected the transmigrated cells and determined the quantity of CD18, CD11a, CD11b, L-selectine, PSGL-1, CXCR2 and CD44 and demonstrated the lack of alteration.

The revised manuscript was accepted and selected as "Pick of the week" by the editor of Journal of Immunology.

In order to characterize the involvement of ARHGAP25 in further physiological or pathological processes, we started to apply another widely used animal model of serum-induced arthritis. In a well-controlled study carried out on a large animal population we observed significant diminution of the clinical symptoms such as ankle swelling in the knock-out animals as compared to the wild type controls. These findings reveal ARHGAP25 as potential pharmacological target. In order to decide whether the lack of ARHGAP25 in neutrophils or in other cell types is responsible for the remarkable phenotype, we repeated the experiments on bone marrow chimeric animals, which lack the protein only in the hematopoetic cell population. We obtained similar results indicating that the altered phenotype is due to a primary change in the neutrophilic compartment. These results have been presented at the 2018 meeting of the European Society for Clinical Investigation and of the Hungarian Immunological Society.

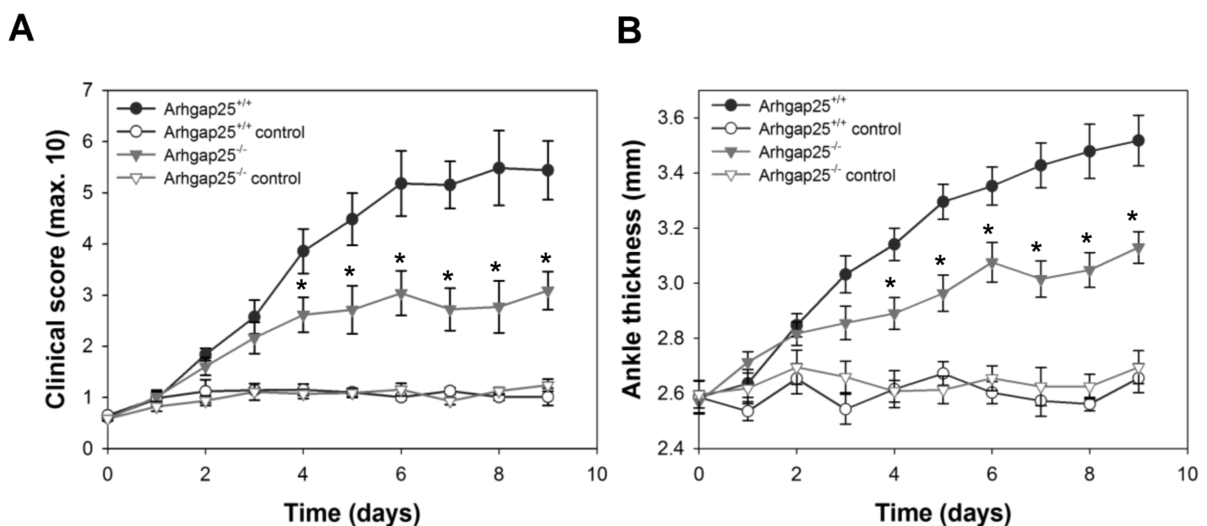

Fig 2. Clinical score (**A**) and ankle thickness (**B**) of Arhgap25$^{-/-}$ and wild type mice after treatment with arthritic (filled) or control serum (empty). Mean ± S.E.M of 14 (arthritic serum treated, 7-7 animal / genotype)) or 8 (control serum treated, 4-4 mice / genotype) separate experiments are shown. *$p < 0.05$ [Csépányi-Kömi et al., unpublished result].

Taken together all the data obtained hitherto, it seems that ARHGAP25 may be involved in very specific functions and/or be active only under specific conditions. This view fits with the large number of Rac/RhoGAPs expressed in different cells and accentuates the need to study the regulation of the protein in details.

Investigation of specific or overlapping functions of RacGAPs in neutrophils

In order to investigate the specific or overlapping functions of different RacGAPs expressed in neutrophilic granulocytes, we carried out experiments where we either added or subtracted specific RacGAPs to or from the NADPH oxidase complex. Addition of 4 different RacGAPs resulted in similar decrease of the RacGTP-dependent superoxide production when the GAPs were added before assembly of the active enzyme complex. In contrast, none of them had any significant effect if added after assembly of the complex, indicating that cytosolic GAPs probably have no access to Rac embedded in the active enzyme contrast.

In depletion experiments we succeeded to selectively immunoprecipitate individual RacGAPs. For two GAPs we used highly active polyclonal antibodies prepared by ourselves. Testing the enzyme activity in selectively depleted membranes we observed that reduction of the amount of ARHGAP1 and ARHGAP25 resulted in significant increase of superoxide production and combined depletion of these two GAPs resulted in additive effects. In contrast to this, depletion of ARHGAP35 had no effect upon superoxide production. Thus, we could demonstrate selective effect of RacGAPs on the Rac-dependent functioning of NADPH oxidase.

In collaboration with Prof. Susan Smith we carried out molecular modeling studies where we have revealed that simultaneous interaction of RacGTP with its target protein p67phox and regulator RacGAP is sterically possible. Thus, membrane-associated RacGAPs may have a regulatory action upon the assembled oxidase complex helping the down-regulation and termination of superoxide production.
These results have been published in the journal Free Radical Biology and Medicine.

Regulation of Rac/RhoGAP activity

We started a systematic investigation of the role of phosphorylation in the regulation and protein interactions of ARHGAP25. Sequencing revealed that the GST fusion protein that we used earlier, contained a couple of alterations as compared to sequences available in databases. First we have mutated these bases in order to have a clealy defined starting material. A pilot study investigating the potential phosphorylation sites by proteomic analysis revealed over 30 sites.

In order to make an inteligent approach, we made use of the information learned from a collaborating group. They showed that the phosphorylation state of ARHGAP25 changed significantly upon mobilization of neutrophils from the bone marrow. Phosphoproteomic analysis revealed the largest increase of phosphorylation on S363, therefore we concentrated our first efforts on this amino acid. A non-phosphorylatable single amino acid mutant (S-A) was created and expressed as GST-fusion protein. Phosphorylation of the wild-type protein with neutrophilic cytosol resulted in 38% inhibition of its RacGAP activity whereas similar treatment of the mutant (S363A) protein did not affect significantly the RacGAP activity of the protein. Interestingly, effect of cytosol treatment was only evident if cytosol was obtained from TNF-preptreated neutrophils. Neither cytosol from non-treated neutrophils nor cytosol

from other cell types was effective.
These results have been published in Blood.

Phosphoproteomic analysis revealed two other amino acid (S379 and S488) where phosphorylation is altered upon egress from the bone marrow. Both fall in the interdomain region where earlier, with radioactive approach we have detected the most abundant phosphorylation. Thus, we prepared single, double and triple mutants of these amino acids. Testing of the GAP activity began, but here we ran into difficulties.
Production of P32-labelled nucleotides in Hungary has been stopped by January 2015. The imported nucleotides are significantly more expensive and we were not able to fully reproduce our previous data.

Hence the detailed in vitro analysis of the effect of these phosphorylations on RacGAP activity required the establishment of a new, bioluminescense-based detection assay. We succeeded with the preparation of the suitably tagged constructs (GST-Venus-Rac and GST-CRIB-Rluc), verified the correct sequence and expressed the proteins. We used dominantly active and inactive mutants of Rac to set the expected end-points of the assay. We determined the optimal conditions and necessary controls for single-turnover RacGAP assay. Furthermore we verified that phosphorylation with neutrophil cytosol did diminish the GAP-activity of the protein. With this result we suceeded to reproduce our earlier observation made with the previously applied radioactive assay. The systematic investigation of all three single-mutant, the three double-mutant and the triple-mutant proteins is being carried out. The first results were presented at the Annual meeting of the Physiological Society and more extensive data were submitted as an abstract to the 2019 meeting of the European Society for Clinical Investigation.

In order to investigate the role of phosphorylation on the intracellular distribution and effects of ARHGAP25, we constructed fusion proteins of the wild type and all the above mutants of ARHGAP25 and CFP attached either on the N- or the C-terminal end. First we expressed these proteins in easily transfectable cells (e.g. COS7), in which we have characterized earlier the distribution and effects of ARHGAP on shape changes. We'll determine whether the lack of one or more of the phosphorylatable amino acids has any influence on the localization and cytoskeletal effects of ARHGAP25.

In neutrophilic granulocytes we have investigated the regulation of ARHGAP25 also at the expression level. We could show that stimulation with opsonized particles resulted in significant reduction of the mRNA of ARHGAP25, whereas in the control cells there was no significant change. Parallel to diminution of the mRNA, the detectable amount of the protein also significantly decreased. This is the first demonstration of regulation of a Rho/Rac family GAP at expression level in a short-lived cell such as the neutrophil. These results were published in the European Journal of Clinical Investigation in 2018.

Investigation of neutrophil-derived EVs

We also investigated the production of extracellular vesicles from neutrophilic granulocytes. We carried out a systematic study of the conditions of successful storage of the vesicles. We determined that the physical properties are not changed when storage is carried out at -20C, but the functional properties are maintained only if storage occurs at -80C preferably not longer than for 1 or 2 weeks. These results have been published in the Journal of Extracellular Vesicles.

Finally, we carried out a detailed characterization of spontaneously produced vesicles, stimulation-induced vesicles, and vesicles released from apoptotic cells. These data have been published in the Journal of Leukocyte Biology.