INVESTIGATING THE ROLE OF ARHGAP25 IN INFLAMMATORY DISEASES USING MURINE MODELS

Ph.D. thesis

Domonkos Czárán

Molecular Medicine Doctoral School
Semmelweis University

Supervisor: Roland Csépányi-Kömi, Ph.D

Official reviewers: László Cervenak, Ph.D
Andrea Papp-Balogh, Ph.D

Head of the Complex Examination Committee:
Sára Tóth, Ph.D

Members of the Final Examination Committee:
Éva Ruisanchez, Ph.D
Dorottya Kövesdi, Ph.D

Budapest, 2025

I. Introduction

Rheumatoid arthritis (RA) is a prevalent autoimmune inflammatory disease primarily affecting synovial joints. It is especially common in women over 50 years, and is characterized by immune-mediated cartilage and bone destruction. The etiology of RA involves both genetic predispositions, particularly HLA gene variants, and environmental triggers such as smoking and microbial exposure. The early development of autoantibodies such as rheumatoid factors (RF), and anti-citrullinated protein antibodies (ACPA) precedes clinical symptoms and contributes to disease progression through epitope spreading. Inflammatory processes are driven by complex cellular interactions between infiltrating immune cells—especially neutrophils, monocytes/macrophages, and T cells—and resident joint cells, most importantly fibroblast-like synoviocytes (FLS). These FLS become hyperproliferative and invasive, leading to pannus formation, synovial inflammation, and bone erosion through RANKL-mediated activation of osteoclasts.

Allergic contact dermatitis (ACD) is a type IV delayed-type hypersensitivity reaction triggered by haptens such as low molecular weight chemicals, e.g., poison ivy, rubber mixes, or metal ions. It consists of two phases: in the sensitization phase,

antigen presentation by dendritic cells to T helper cells happens, forming a memory T helper cell population. In the elicitation phase, the hapten re-exposure leads to T-cell-mediated inflammation. At this stage, T cells, macrophages, neutrophils, and natural killer cells coordinate cytokine release, cell recruitment, and subsequent tissue damage, emphasizing the importance of both innate and adaptive immunity in ACD.

Murine disease models are indispensable for understanding human pathologies. The K/BxN serum transfer arthritis model is a useful tool for studying the effector phase of RA. The passive transfer of anti-G6PI autoantibody containing K/BxN serum leads to immune complex deposition. This in turn triggers the activation and cytokine production of neutrophils and macrophages, independent of adaptive immunity. In contrast, the TNCB-induced contact hypersensitivity model captures both the sensitization and elicitation phases of ACD and involves a coordinated Th1/Th2 cytokine response, leading to leukocyte infiltration and subsequent tissue damage.

ARHGAP25 is a RAC-specific GTPase-activating protein (GAP). Our research group characterized the full-length protein for the first time. In these early studies, our group demonstrated its role in neutrophil functions, such as migration, phagocytosis, and reactive oxygen species production, through the regulation

of actin rearrangement. Additionally, ARHGAP25 modulates B cell differentiation and germinal center reactions. Although initially ARHGAP25 was considered leukocyte-specific, recently it has been implicated in various tumors as a tumor-suppressive factor via various mechanisms, such as the Wnt/β-catenin or AKT/mTOR signaling. Our recent interactome study revealed 76 potential binding partners, suggesting that ARHGAP25 functions through diverse protein networks. Collectively, these findings highlight ARHGAP25 as a key regulator in immune and pathological processes.

II. Objectives

During my PhD studies, I aimed to investigate the role of ARHGAP25 in murine models of two mechanistically different diseases: The K/BxN serum transfer arthritis model and the TNCB-induced allergic contact hypersensitivity model. Our main questions were the following:

1. Does ARHGAP25 influence the symptoms of autoantibody-induced arthritis and allergic contact hypersensitivity?

2. If it does, which immune cells are responsible for these changes? What other cells besides leukocytes, if any, are involved?

3. Does ARHGAP25 alter the cytokine composition of inflamed tissues? If so, which cytokines might be significant?

III. Methods

1. Experimental animals

Age-matched male wild-type (WT) and Arhgap25 knock-out (*Arhgap25*$^{-/-}$ or KO) mice were kept and bred under specific pathogen-free conditions. Bone marrow chimeras were generated via lethal irradiation of WT or *Arhgap25*$^{-/-}$ recipients, followed by transplantation of unfractionated bone marrow from donors. After one month, the success of the transplantation was verified by flow cytometry. Only the mice with >95% neutrophils of donor origin were used.

2. Disease models

Autoimmune arthritis was induced via intraperitoneal injection of 150 μL K/BxN serum. Contact hypersensitivity (CHS) was initiated using topical TNCB treatment on the abdominal skin (sensitization), and 5 days later on the ears (elicitation). Arthritis severity was evaluated daily via clinical scoring, ankle thickness measurement, and plethysmometry, while CHS was assessed by ear thickness measurements before and 24 hours after allergen treatment of the ears.

3. Functional assessments

Joint function was examined using horizontal grid test 3, 6, and 8 days after treatment. Mechanonociceptive threshold was measured by dynamic plantar aesthesiometry to assess pain sensitivity to mechanical stimulus.

4. Histology

On day 8 post-serum injection, ankles were harvested and sections were prepared and stained with hematoxylin and eosin (HE), or Safranin O. Scoring was performed in a blinded setup for synovial hyperplasia, collagen deposition and cartilage destruction. Ears were excised 24 hours after TNCB treatment and processed similarly for HE staining.

5. In vivo and in vitro neutrophil function measurements

Myeloperoxidase (MPO) activity was visualized using in vivo bioluminescence imaging after luminol injection. In vitro superoxide production was assessed via ferricytochrome c reduction assay. Bone marrow-derived neutrophils were stimulated on immune-complex or fibrinogen-TNFα-coated surfaces, or with PMA, and OD was measured. Neutrophil migration toward ear tissue lysate supernatants was assessed using a Transwell assay.

6. Analysis of the inflammatory infiltrate

Flow cytometry was used to quantify and analyze the ratio of distinct types of infiltrating leukocytes in joint and ear tissues. T cell subpopulations and activation markers from the draining lymph nodes were assessed by flow cytometry as well. Cytokine levels were measured via cytokine array and ELISA kits.

7 Expression analyses

Immunoblotting was used to measure signaling protein levels and phosphorylation in total ankle lysates. Expression of ARHGAP25 was determined by RT-qPCR and western blot in murine and human skin.

8 Lymph nodal cell transfer experiments

Lymph node-derived cells from TNCB-sensitized mice were intraocularly injected into WT or Arhgap25$^{-/-}$ recipients. Afterward, ear thickness was measured, ears were coated with TNCB, and 24 hours later, ear thickness was measured again.

9 Statistical Analysis

Data were analyzed using GraphPad Prism 10.0.1. In the case of horizontal grid test, comparison of experimental groups was performed by Kaplan-Meier survival analysis, followed by log-rank test. Analysis of histology was performed with the Mann-

Whitney test. Two-way ANOVA with Tukey's multiple comparison tests was used in all other cases. All p values <.05 were considered statistically significant.

IV. Results

1. ARHGAP25 deficiency alleviates arthritis-related inflammation

In the K/BxN serum transfer arthritis (STA) model, ARHGAP25-deficient (KO) mice exhibited significantly milder clinical symptoms than wild-type (WT) animals. While WT animals displayed extensive hind paw swelling, redness, and disfigurement after serum injection, KO animals showed only moderate changes. More objective methods, such as ankle thickness and paw edema measurements, confirmed a ~50% reduction in inflammation in KO mice.

2. Improved joint function and reduced pain sensitivity in the absence of ARHGAP25

Using horizontal grid test, longer gripping times revealed preserved articular function in KO compared to WT animals after arthritogen serum treatment. Dynamic plantar aesthesiometry showed that KO mice had significantly higher mechanonociceptive thresholds at all measured time points, suggesting reduced arthritis-induced pain.

3. KO mice show partial protection against joint damage

Histological analyses showed severe synovial hyperplasia and cartilage damage in WT mice 8 days after serum treatment. In contrast, KO mice displayed attenuated synovial hyperplasia and reduced collagen deposition. Notably, cartilage destruction was absent in KO animals.

4. Neutrophil effector functions remain intact in KO animals in the K/BxN STA model

Despite the milder disease phenotype, neutrophil effector mechanisms were not impaired. Myeloperoxidase (MPO) activity in vivo and in vitro superoxide production were comparable between WT and KO animals, suggesting that ARHGAP25 deletion does not hinder basic neutrophil function in this model.

5. ARHGAP25 deficiency reduces leukocyte recruitment and cytokine production

Flow cytometry revealed reduced neutrophil and macrophage infiltration into inflamed joints in KO mice. Levels of pro-inflammatory cytokines IL-1β and MIP-2 were also significantly lower in KO compared to WT, suggesting ARHGAP25 influences immune cell recruitment by modulating the cytokine milieu.

6. Altered signaling pathways in ARHGAP25-deficient mice

Western blot analyses of paw lysates identified changes in several signaling pathways. In KO mice, arthritic serum treatment failed to induce IκB expression, suggesting altered NF-κB regulation. Additionally, lower levels of MAPK, ERK1/2, and E-cadherin were observed in KO mice post-treatment, implying ARHGAP25 affects inflammation via multiple signaling cascades.

7. Both hematopoietic and non-hematopoietic cells mediate ARHGAP25 effects

Bone marrow chimera experiments revealed moderate but significant reductions in inflammation in both "normal" (KO → WT) and "reverse" (WT → KO) chimeras. These results suggest ARHGAP25 exerts its pro-inflammatory effects through both immune and non-immune cell types.

8. High ARHGAP25 expression in fibroblast-like synoviocytes

Western blot analysis confirmed that fibroblast-like synoviocytes express ARHGAP25 at levels comparable to neutrophils. This expression was specific, as mouse embryonic

fibroblasts showed minimal protein levels. These findings suggest synoviocytes as important mediators of ARHGAP25-driven inflammation.

9. ARHGAP25 is upregulated in inflammatory skin conditions

Human samples from allergic contact dermatitis (ACD) patients revealed elevated ARHGAP25 expression in inflamed skin. Similarly, in a murine contact hypersensitivity (CHS) model, both mRNA and protein levels of ARHGAP25 were upregulated during the elicitation phase. Epidermal keratinocytes, however, did not express ARHGAP25.

10. ARHGAP25 deficiency reduces CHS-induced inflammation

In the case of the TNCB-induced CHS model, KO animals exhibited reduced ear swelling following elicitation compared to WT. The experiments with “normal” bone marrow chimeras mirrored these findings, showing that in the case of CHS, regulation through the hematopoietic compartment results in the attenuated inflammatory response in the absence of ARHGAP25.

11. Reduced leukocyte infiltration in elicited ears of KO animals

Flow cytometry revealed significantly reduced numbers of leukocytes in KO ears compared to WT after elicitation with the allergen. While neutrophil infiltration only showed a decreasing trend, macrophage and cytotoxic T cell recruitment were significantly impaired in KO mice.

12. Altered cytokine profile and neutrophil transmigration in KO mice

Cytokine array from ear lysates revealed that in the sensitization phase, several interleukins, but also IFN-γ and MCSF expression, were decreased in KO animals. On the other hand, in the elicitation phase, several CXCL and CCL chemokines and also IL-1β levels were reduced in the absence of ARHGAP25. Quantitative, ELISA measurements of IL-1β and MIP-2 confirmed these findings. In vitro transwell assays demonstrated that neutrophils—regardless of genotype—migrated less towards supernatant from KO elicited ears than from WT elicited ones, confirming that ARHGAP25 influences inflammatory cell recruitment indirectly via cytokine milieu modulation.

13. T cell activation and lymph node homing are unaffected by ARHGAP25

Flow cytometry of draining lymph nodes indicated that T cell numbers and activation markers (CD25, CD69) were similarly elevated in both WT and KO mice after sensitization. Transfer of lymph node-derived cells demonstrated that KO recipients receiving WT cells, but not KO-derived T cell-receiving WT animals, displayed decreased ear swelling, after elicitation. This supports our hypothesis that ARHGAP25 influences the elicitation rather than sensitization phase of CHS.

V. Conclusions

According to our results described above, our conclusions are the following:

1: ARHGAP25 is involved in the development of both autoantibody-induced arthritis and allergic contact hypersensitivity.

2: This protein is critical in the regulation of the cytokine environment and, as a result, leukocyte recruitment in inflammation.

4: In arthritis, the effect of ARHGAP25 on neutrophils and macrophages, and non-immune cells, particularly FLS, are both crucial for disease development.

5: In contact hypersensitivity, ARHGAP25 affects the pathomechanism through regulating only leukocytes, primarily macrophages and cytotoxic T cells.

Given its involvement in multiple immune mechanisms, ARHGAP25 represents a promising novel target for therapeutic intervention in autoimmune and allergic inflammatory diseases.

VI. Bibliography of the candidate's publications

Publications related to the work discussed in this thesis

Czárán, Domonkos; Sasvári, Péter; Horváth, Ádám István; Ella, Krisztina; Réka Sűdy, Ágnes; Borbely, Eva; Rusznák, Kitti; Czéh, Boldizsár; Mócsai, Attila; Helyes, Zsuzsanna
Lacking ARHGAP25 mitigates the symptoms of autoantibody-induced arthritis in mice
FRONTIERS IN IMMUNOLOGY 14 Paper: 1182278, 13 p. (2023)
IF: 5,7
DOI: 10.3389/fimmu.2023.1182278

Czárán D, Sasvári P, Lőrincz K, Ella K, Gellén V, Csépányi-Kömi R.
ARHGAP25: a novel player in the Pathomechanism of allergic contact hypersensitivity.
FRONTIERS IN IMMUNOLOGY 26; 16:1509713. (2025)
IF: 5,9
DOI: 10.3389/fimmu.2025.1509713.

Publications not related to the work discussed in this thesis

Wisniewski, Éva; **Czárán, Domonkos***; Kovács, Fanni; Bahurek, Enikő; Németh, Afrodité; Sasvári, Péter; Szanda, Gergő; Pettkó-Szandtner, Aladár; Klement, Eva; Ligeti, Erzsébet
A Novel BRET-Based GAP assay reveals phosphorylation-dependent regulation of the RAC-specific GTPase activating protein ARHGAP25
FASEB JOURNAL 36: 11 Paper: e22584, 19 p. (2022)
*Éva Wisniewski and Domonkos Czárán should be considered joint first authors.
IF: 4,8
DOI: 10.1096/fj.202200689R

Szederkényi, G; Kocsis, D; Vághy, M A; **Czárán, D**; Sasvári, P; Lengyel, M; Naszlady, M B; Kreis, F; Antal, I; Csépányi-Kömi, R
Mathematical modeling of transdermal delivery of topical drug formulations in a dynamic microfluidic diffusion chamber in health and disease
PLOS ONE 19: 4 Paper: 0299501 , 17 p. (2024)
IF: 2,6

DOI: 10.1371/journal.pone.0299501

Kovács F, Posvai T, Zsáry E, Kolonics F, Garai R, Herczeg V, **Czárán D**, Takács J, Szabó AJ, Krivácsy P, Csépányi-Kömi R.
Long COVID syndrome in children: neutrophilic granulocyte dysfunction and its correlation with disease severity.
PEDIATRIC RESEARCH Nov 27. 13 p. (2024)
IF: 3,1
DOI: 10.1038/s41390-024-03731-1.