Lymphatic- and neutrophil-dependent tissue damage in contact hypersensitivity and rheumatoid arthritis

Ph.D. thesis

Petra Aradi

Molecular Medicine Doctoral School
Semmelweis University

Supervisor: Zoltán Jakus, MD, Ph.D., Habil.

Official reviewers: Győző Szolnoky, MD, Ph.D., Habil.
Norbert Kiss, MD, Ph.D.

Head of the Complex Examination Committee:
Edit Buzás, MD, Ph.D., D.Sc.

Members of the Final Examination Committee:
Anna Erdei, Ph.D., D.Sc.
Miklós Sárdy, MD, Ph.D., D.Sc.

Budapest, 2024

1. Introduction

Inflammation is a crucial part of body's defence process, by which the immune system recognizes and eliminates the harmful stimuli followed by the healing process. Inflammation involves a coordinated interaction between immune cells, blood and lymphatic vessels, and molecular mediators to restore tissue homeostasis. However, shifts in the inflammatory response from short- to long-lived can disrupt immune tolerance and result in significant changes across all tissues and organs elevating the susceptibility to various non-communicable diseases. Nowadays, chronic inflammatory conditions, such as diabetes, autoimmune diseases, or allergy represent the most substantial risk to human health according to the World Health Organization.

Allergic contact dermatitis (ACD), characterized by T cell-mediated immune responses to allergens, involves distinct sensitization and elicitation phases. During sensitization, allergens penetrate the skin barrier, activating keratinocytes and dendritic cells to prime antigen-specific T cells in lymph nodes. The subsequent elicitation phase involves rapid activation of memory T cells upon re-exposure to the same allergen, leading to inflammatory responses mediated by cytokines and immune cell infiltration. However, due to one of the most frequently used

animal models for this condition, the murine contact hypersensitivity (CHS) model, our knowledge about the immunological mechanisms of ACD has increased significantly in the past decade, the precise role of lymphatic vessels in modulating these phases remains unclear, highlighting a gap in current research that could inform novel therapeutic strategies for ACD.

The other very common inflammatory disease related to tissue damage is rheumatoid arthritis (RA) manifests through synovial inflammation and tissue damage mediated by dysregulated innate and adaptive immune responses causing a prevalent autoimmune joint disease. Neutrophils, traditionally viewed as short-lived bystanders, play pivotal roles in RA pathogenesis by releasing proteases and activating immune responses via Fcγ receptors, particularly FcRγ-associated ITAM signaling. While the absence of FcRγ has shown protective effects in autoimmune arthritis, the exact mechanisms by which FcRγ ITAM tyrosines influences neutrophil activation and receptor signaling pathways *in vivo* remain unexplored.

2. Objectives

In our experiments we aimed to characterize the lymphatic- and neutrophil-dependent tissue damage in mouse models of allergic contact dermatitis and rheumatoid arthritis.

We investigated the following questions:

1. Finding and characterizing mouse models to investigate the role of the lymphatics separately in the two phases of CHS.
2. Investigating the effect of presence or absence of lymphatic vessels in the sensitization and elicitation phase of CHS model.
3. Specifying the immune cells which contribute to the development of CHS.
4. Identifying the exact role of FcRγ ITAM tyrosines in inflammatory arthritis mouse model *in vivo*.

3. Methods

Experimental animals: For the experiments, wild type and *Vegfr3* kinase-dead point mutant mice on NMRI genetic background were used. To eliminate lymphatics locally in the skin, a transgenic inducible lymphatic vessel elimination model, Flt4-CreER^{T2}, *iDTR$^{fl/fl}$* on C57Bl/6 background was employed followed by diphtheria toxin injection. Furthermore, animals expressing the wild type and the ITAM tyrosine mutant FcRγ were crossed with FcRγ KO mice (referred to as $\gamma^{-/-}$ WT γ Tg and $\gamma^{-/-}$ YF γ Tg animals, respectively) besides the control animals.

Histology procedures and immunostaining of histology slides: Tissue samples were embedded in paraffin after fixation and dehydration. Tissue sections were then processed for hematoxylin-eosin and fluorescent immunohistochemistry staining.

Contact hypersensitivity mouse model: The mice were treated with vehicle or allergen on the site of the sensitization (abdominal skin or hind paw). After 5 days, the initial ear thickness of mice was measured, using a caliper. For elicitation, mice were treated by epicutaneous application of the same allergen on ear skin. 24 hours after the challenge the ear swelling was measured again.

Neutrophil depletion: Three days after the sensitization, mice were intraperitoneally injected with murinized anti-Ly6G antibody to deplete neutrophil granulocytes, or with functional grade IgG2a kappa isotype antibody serving as control.

Characterization of inflammation: The infiltrated immune cells were measured by flow cytometry after digestion of ear skin. Furthermore, the produced cytokines and chemokines were detected by using Mouse Cytokine Array Panel A.

Passive CHS model: The restimulation of regional lymph node cells after sensitization was accomplished with the water-soluble form of the same antigen *in vitro* followed by measurement of IFNγ production.

K/BxN Serum-Transfer Arthitis: We collected blood from the transgene-positive (arthritic) K/BxN and transgene-negative (control) BxN mice retroorbital, and we used 400 µl sera intraperitoneal to induce inflammatory arthritis in the $\gamma^{-/-}$ WT γ Tg, $\gamma^{-/-}$ YF γ Tg and control animals. The histological analysis of the inflamed ankles after 8 days has been investigated.

4. Results

We used various mouse strains to study lymphatics' role in contact hypersensitivity. The *Vegfr3*$^{kd/+}$ mice showed no skin lymphatics but unaffected other tissues and blood vessels. Using Flt4-CreER^{T2}; *iDTR*$^{fl/+}$ mice, we selectively deleted lymphatics in specific tissues, confirming their absence and showing moderate immune cell infiltration and increased ear thickness upon injection. Both mouse models demonstrated robust inflammation with repeated antigen treatments, making them reliable for studying lymphatic involvement in CHS phases.

We examined the skin's response to single antigen exposure in mice lacking lymphatics in both CHS phases and only the elicitation phase. These mice showed significant ear thickness increase compared to those with intact lymphatics, without notable immune cell infiltration, confirmed by histological and cytokine analysis.

Using *Vegfr3*$^{kd/+}$ mice lacking lymphatics in both the sensitization and elicitation phases of CHS, we observed a less pronounced increase in ear thickness and significantly less immune cell infiltration after repeated antigen exposure compared to mice with intact lymphatics. Flow cytometry confirmed reduced infiltration of CD45-positive leukocytes and neutrophils in these lymphatic-deficient mice. Additionally,

proinflammatory cytokine and chemokine levels were significantly lower in the absence of lymphatics, indicating that lymphatic deficiency leads to a reduced immune response after repeated antigen exposure.

We investigated the role of lymphatics during the sensitization phase of CHS by inducing local lymphatic deletion in the hind paw of Flt4-CreER^{T2}; *iDTR$^{fl/fl}$* mice using diphtheria toxin. Lymphatic ablation was confirmed, and compared to controls, mice lacking lymphatics showed less increase in ear thickness and cell infiltration after repeated antigen exposure, with lower CD45^{+} and Gr1^{+} immune cell infiltration, similar to the previously showed mouse strain. Flow cytometry confirmed reduced infiltration of CD45+ and Ly6G^{+} cells, while CD3^{+} and B220^{+} populations were unaffected. The inguinal lymph nodes of lymphatic-deficient mice were smaller, indicating a diminished immune response. These findings suggest that skin lymphatics are crucial for immunization against antigens during the sensitization phase of CHS.

Antigen-presenting cells are crucial for T cell activation in the sensitization phase of CHS, which is linked to increased IFN-γ secretion. We found that lymphatic deficiency during this phase significantly reduced IFN-γ production by naive T cells in

the regional lymph nodes, suggesting lymphatics are vital for both immunization and T cell activation in CHS.

We investigated the role of lymphatics during the elicitation phase of CHS inducing elicitation in ears with either intact or locally deficient lymphatics. Surprisingly, mice lacking lymphatics during the elicitation phase exhibited increased ear swelling, higher immune cell infiltration, and elevated pro-inflammatory cytokine production compared to those with intact lymphatics. These findings suggest that skin lymphatics play an anti-inflammatory role during the elicitation phase of CHS.

During the elicitation phase, mouse ears lacking lymphatics exhibited exacerbated inflammation. To investigate the role of neutrophils in this condition, we used an anti-Ly6G antibody to deplete them, successfully reducing their numbers without affecting other immune cells. Neutrophil absence markedly attenuated ear thickness and immune cell infiltration in lymphatic-deficient ears after repeated exposure, highlighting neutrophils' significant role in exacerbating inflammation. Additionally, neutrophil depletion affected regulatory T cell numbers, suggesting that neutrophils influence their recruitment during inflammation.

The study highlighted neutrophils' critical role in exacerbating inflammation in the absence of lymphatics during

inflammatory skin disease. However, the exact molecular mechanisms underlying this process were not fully elucidated. To explore further, a genetic approach was employed to investigate the involvement of FcRγ ITAM tyrosines in neutrophils and autoimmune arthritis *in vivo*. Results indicated that while wild type FcRγ ($\gamma^{-/-}$ WT γ Tg) mice showed modest inflammation in response to arthritis serum, mice expressing the ITAM tyrosine mutant ($\gamma^{-/-}$ YF γ Tg) did not exhibit ankle thickness changes or joint damage, suggesting a protective role of ITAM tyrosine mutation in autoimmune arthritis.

5. Conclusions

Inflammation is a complex response to local tissue alteration involved in various physiological and pathological processes. Chronic inflammatory diseases are now the leading cause of death worldwide. Our aim was to investigate lymphatics- and neutrophil-dependent processes in inflammation using models like allergic skin disease and autoimmune arthritis.

In conclusion, our findings are:

1. We characterized reliable mouse models in which the two phases of CHS could be investigated separately.
2. Skin lymphatics have distinct but important roles in the two phases of contact hypersensitivity: during the sensitization phase, they aid in the sensitization process of immune cells.
3. In contrast, during the elicitation phase, they regulate the inflammatory response and the infiltration of immune cells.
4. Neutrophils are crucial to create exaggerated inflammation in the absence of lymphatics in elicitation phase influencing the regulatory T cell number.

5. FcRγ ITAM tyrosines play a critical role in the induction of neutrophil effector responses.

Our studies underscore the importance of lymphatic vessels in immune responses during cutaneous inflammation and highlight neutrophils' FcRγ ITAM tyrosines role in autoimmune conditions, offering insights for future therapeutic strategies.

6. List of Publications

Publications Related to the Thesis

I. ***Aradi, P.**, Kovács, G., Kemecsei, É., Molnár, K., Sági, S.M., Horváth, Z., Mehrara, B.J., Kataru, R.P., Jakus, Z.* Lymphatics-dependent modulation of the sensitization and elicitation phases of contact hypersensitivity.
J Invest Dermatol 2024; 26:S0022-202X(24)00261-6. Doi: 10.1016/j.jid.2024.03.021
IF: 5.7

II. *Németh, T., Futosi, K., Szabó, M., **Aradi, P.**, Saito, T., Mócsai, A., Jakus, Z.* Importance of Fc Receptor γ-Chain ITAM Tyrosines in Neutrophil Activation and in vivo Autoimmune Arthritis.
Front Immunol 2019, 10:252. Doi: 10.3389/fimmu.2019.00252
IF: 5.085

Publications not related to the thesis:

III. *Szőke, D., Kovács, G., Kemecsei, É., Bálint, L., Szoták-Ajtay, K., **Aradi, P.**, Styevkóné Dinnyés, A., Mui, B.L., Tam, Y.K., Madden, T.D., Karikó, K., Kataru, R.P., Hope, M.J., Weissman, D., Mehrara, B.J., Pardi, N., Jakus, Z.*
Nucleoside-modified VEGFC mRNA induces organ-specific lymphatic growth and reverses experimental lymphedema.
Nat Commun 2021, 12(1):3460. Doi: 10.1038/s41467-021-23546-6
IF: 17.694

IV. *Bálint, L., Ocskay, Z., Deák, B.A.,* ***Aradi, P.****, Jakus, Z.*
Lymph Flow Induces the Postnatal Formation of Mature and Functional Meningeal Lymphatic Vessels.
Front Immunol 2020, 10:3043. Doi: 10.3389/fimmu.2019.03043
IF: 7.561

V. *Csete, D., Simon, E., Alatshan, A.,* ***Aradi, P.****, Dobó-Nagy, C., Jakus, Z., Benkő, S., Győri, D.S., Mócsai, A.*
Hematopoietic or Osteoclast-Specific Deletion of Syk Leads to Increased Bone Mass in Experimental Mice.
Front Immunol 2019, 10:937, Doi: 10.3389/fimmu.2019.00937
IF: 5.085

VI. *Reed, H.O., Wang, L., Sonett, J., Chen, M., Yang, J., Li, L.,* ***Aradi, P.****, Jakus, Z., D'Armiento, J., Hancock, W.W., Kahn, M.L.*
Lymphatic impairment leads to pulmonary tertiary lymphoid organ formation and alveolar damage.
J Clin Invest 2019, 129(6):2514-2526. Doi: 10.1172/JCI125044
IF: 11.864

VII. *Szarka, E., **Aradi, P.**, Huber, K., Pozsgay, J., Végh, L., Magyar, A., Gyulai, G., Nagy, G., Rojkovich, B., Kiss, É., Hudecz, F., Sármay, G.*
Affinity Purification and Comparative Biosensor Analysis of Citrulline-Peptide-Specific Antibodies in Rheumatoid Arthritis.
Int J Mol Sci 2018, 19(1):326. Doi: 10.3390/ijms19010326.
IF: 4.183

∑IF: 57.162