Tyrosine kinase signaling pathways in experimental autoimmune skin blistering models

Ph.D. thesis

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Introduction

Autoimmune diseases are prevalent conditions with significant impact on the quality of life, creating a serious burden to the medical system and the overall society. Multiple aspects of their complex pathology are still not fully elucidated, which could be useful for the identification of more effective therapeutic options.

Subepidermal autoimmune blistering skin diseases are prototypical autoantibody-mediated disorders. Among those, epidermolysis bullosa acquisita (EBA) is a rare but severe condition where autoantibodies are formed against a key anchoring protein of the dermal-epidermal junction (DEJ), namely the type VII collagen (C7). During the effector phase of the disease, anti-C7 antibodies are deposited along the DEJ, inducing the recruitment of effector cells, mainly neutrophils. Finally, the developing inflammation leads to tissue damage and blister formation. Skin lesions of EBA patients are often severe with fairly limited therapeutic options.

Tyrosine kinase-associated signaling in immune cells are important drivers of inflammation and tissue damage during the effector phase of autoantibody-driven diseases. Immunoreceptors, like Fc receptors are involved in the recognition of deposited autoantibodies by myeloid cells, like neutrophils. They utilize tyrosine kinases such as Src-family kinases and Syk in their signaling mechanism. Our workgroup has shown that Src-family kinases and Syk are essential for the development of antibody-induced arthritis and skin blistering in mice.

Phospholipase Cγ2 (PLCγ2) is expressed in cells of hematopoietic origin. PLCγ2 is an important component of immunoreceptor signaling, activated by tyrosine kinases. Our

laboratory and others previously showed that PLCγ2-deficient mice remained protected in autoantibody-induced arthritis models. Moreover, severe inflammatory skin lesions and epidermolysis bullosa-like phenotype were described in gain-of-function mutations of PLCγ2 in both mice and humans. However, the role of PLCγ2 in autoimmune skin blistering has been not investigated.

In addition, various cytokine receptors signal through tyrosine kinases, namely Janus kinases (Jaks). Inhibition of Jaks has revolutionized disease therapy in rheumatoid arthritis and other inflammatory and autoimmune diseases. However, there are scarcely any information about this pathway regarding autoimmune blistering skin diseases.

During my PhD studies, I aimed to further elucidate the immunoreceptor signaling cascade downstream of tyrosine kinases in a mouse model of autoantibody-induced skin blistering resembling EBA. Namely, I investigated the role of PLCγ2 with a transgenic approach. Moreover, I translated my studies to human pathology using a pharmacological approach in an ex vivo human skin separation assay. I also intended to investigate the role of Janus kinases in the mouse model of EBA.

Objectives

I aimed to analyze the molecular mechanisms during the effector phase of autoimmune skin blistering, utilizing in vitro, ex vivo and in vivo models of EBA according to the following points:

1. General characterization of *Plcg2*$^{-/-}$ mice. Measurement of PLCγ2 expression in circulating leukocyte subsets. Comparison of circulating leukocyte counts and the expression of critical cell surface markers on myeloid cells.

2. Investigating the role of PLCγ2 in the development of an antibody-induced skin blistering in intact mice and bone-marrow chimeras.

3. Checking in vitro functional responses of murine *Plcg2*$^{-/-}$ neutrophils to chemotactic- and immune complex stimuli.

4. Examining the effect of neutrophil-specific PLCγ2 expression during antibody-induced skin blistering.

5. Studying the effect of PLC inhibition in a neutrophil-dependent, autoantibody-induced human ex vivo skin separation assay.

6. Investigating the impact of Jak inhibition in the development of autoimmune skin blistering in mice.

Methods

Mouse strains and bone marrow chimeras

Total *Plcg2* knock out (*Plcg2*$^{-/-}$) were used along with wild-type C57BL/6 mice as controls. To achieve neutrophil-specific *Plcg2*-deletion, we used mice that carried floxed *Plcg2* alleles with neutrophil-specific promoter-driven Cre recombinase (*Plcg2*$^{\Delta PMN}$ mice). All transgenic strains were on C57BL/6 genetic background, expressing the CD45.2 allele.

Bone marrow chimeras were generated by transplanting bone marrow cells of wild-type or *Plcg2*$^{-/-}$ donor mice into previously lethally irradiated wild-type recipients (expressing the CD45.1 allele). The donor origin of circulating leukocytes was checked by measuring the expression of CD45.2 by flow cytometry.

Anti-C7-induced skin blistering model

We used a mouse model mimicking the effector phase of the inflammatory form of EBA. Disease was induced by repeated subcutaneous injections of anti-C7 antibodies. Anti-C7 was purified from rabbits (immunized against the immunogenic epitope of C7) using protein G-based affinity chromatography.

Disease onset and progression was followed every second day for 14 days by assessing the size of total affected skin area, a severity score, the size of erosions and by ear thickness measurements.

In vivo mechanistic studies

Ear samples were obtained on Day 8 and embedded in paraffin for histological analysis or digested with collagenase. Single cell suspension was analyzed by flow cytometry and inflammatory mediators were measured in the supernatant.

To assess the intrinsic migratory capacity of leukocytes, we generated mixed bone marrow chimeras. Namely, bone marrow cells from CD45.2$^+$ wild-type or *Plcg2*$^{-/-}$ mice were mixed with CD45.1$^+$ wild-type bone marrow and transplanted into wild-type recipients. The ratio of CD45.2$^+$ cells in the blood and in the ears was determined 8 days after the induction of skin blistering.

In vivo production of reactive oxygen species was detected by measuring myeloperoxidase activity using a luminol-based chemiluminescence assay on Day 8 at the Szentágothai Research Centre of the University of Pécs.

In vitro experiments using murine neutrophils

Murine neutrophils were isolated from bone marrow by Percoll gradient centrifugation. Neutrophil migration was assessed in a Transwell assay towards 100 ng/ml MIP-2, 50 ng/ml LTB_4. Immobilized immune complex surfaces were made by C7 and anti-C7 antibodies and cytokine release was determined by ELISA from the supernatants after 6 hours.

Ex vivo human skin separation assay

Cryosections of healthy human skin were treated with anti-C7, then incubated with human neutrophils in the presence or absence of the PLC inhibitor U73122.

Flow cytometry measurements of circulating leukocytes

The different leukocyte populations in peripheral blood were identified based on their specific cell surface marker expression.

Presentation of the data and statistical analysis

Statistical analyses were carried out by the STATISTICA software using two-way analysis of variance (ANOVA). Treatment and genotype were the two independent variables. One-way ANOVA was used to evaluate in vivo migration.

Results

General characterization of *Plcg2*$^{-/-}$ mice

PLCγ2 deficiency resulted in a survival defect in embryos and/or newborn pups. PLCγ2 was expressed in every major circulating leukocyte subset except T cells obtained from wild-type mice. We found no difference between cell counts of wild type and *Plcg2*$^{-/-}$ neutrophils, eosinophils, monocytes, B cells or T cells in the blood and PLCγ2 deficiency did not alter the expression of maturation markers, integrins and FcγRs on myeloid cells.

The role of PLCγ2 in antibody-induced skin blistering

Macroscopic and microscopic phenotype

We repeatedly injected wild-type and *Plcg2*$^{-/-}$ mice with anti-C7 or with PBS as control. Wild-type mice developed severe skin disease upon anti-C7 treatment with erosions, crust formation and signs of cutaneous inflammation. *Plcg2*$^{-/-}$ intact mice, as well as bone-marrow chimeras remained completely protected from the development of any clinical signs and quantitative measures of anti-C7-induced skin disease, including the extent and severity of skin lesions, the extent of erosions alone, and ear thickening.

Histologically, neutrophil-rich dermal infiltration and significant dermal-epidermal separation occurred upon anti-C7 treatment in the ears of wild-type mice, which was completely absent in *Plcg2*$^{-/-}$ samples. However, circulating anti-C7 levels and antibody deposition was similar in both genotypes.

Dermal infiltration and intrinsic migratory capacity of leukocytes

We quantified the accumulation of myeloid cells on digested ear samples obtained from Day 8. The absolute number of neutrophils, eosinophils and monocytes/macrophages significantly increased upon anti-C7 treatment in wild-type samples, but this was completely abrogated in *Plcg2*$^{-/-}$ chimeras.

In order to test the mechanism of the infiltration defect upon PLCγ2 deficiency, we investigated the intrinsic migratory capacity of myeloid cells in vivo in mixed bone-marrow chimeras. Surprisingly, *Plcg2*$^{-/-}$ myeloid cells could migrate to the site of antibody deposition as well as wild-type cells when both cell types were present within the same animal.

Development of the proinflammatory microenvironment

An alternative explanation for the defective leukocyte accumulation in *Plcg2*$^{-/-}$ mice might be related to the development of the inflammatory microenvironment upon anti-C7 deposition in the skin. Indeed, the accumulation of several proinflammatory mediators including IL-1β, MIP-2 and LTB_4, as well as the production of reactive oxygen species were completely absent in the ears of *Plcg2*$^{-/-}$ chimeras during anti-C7-induced skin blistering.

In vitro functional responses of PLCγ2-deficient neutrophils

We tested neutrophil migration in a Transwell migration assay. In line with our previous in vivo results, *Plcg2*$^{-/-}$ neutrophils had preserved migration ability towards chemoattractants MIP-2 and LTB_4.

When plated onto C7/anti-C7 immune complex surfaces, wild-type neutrophils produced significant amounts of MIP-2

and LTB_4 measured by ELISA. In sharp contrast, *Plcg2*$^{-/-}$ neutrophils completely failed to release those important inflammatory mediators.

Neutrophil-specific analysis of PLCγ2 in anti-C7-induced skin blistering

Efficacy and specificity of Plcg2 deletion in Plcg2$^{\Delta PMN}$ *mice*

We measured PLCγ2 fluorescence intensity in circulating neutrophils, eosinophils, monocytes, B cells and T cells obtained from wild-type and *Plcg2*$^{\Delta PMN}$ mice. Regarding *Plcg2*$^{\Delta PMN}$ neutrophils, we observed a strong but not complete reduction in PLCγ2 staining compared to wild-type neutrophils. Importantly, there was no substantial difference in other leukocyte subsets.

Anti-C7-induced skin blistering in Plcg2$^{\Delta PMN}$ *mice*

We subjected *Plcg2*$^{\Delta PMN}$ mice to our anti-C7-induced skin blistering model together with wild-type and *Plcg2*$^{-/-}$ animals serving as controls. *Plcg2*$^{\Delta PMN}$ animals were almost completely protected from the disease regarding extent and severity of skin lesions, similar to total PLCγ2-deficient mice. *Plcg2*$^{\Delta PMN}$ mice were also practically free from erosions during the entire investigation period. Neutrophil-specific deletion of PLCγ2 also resulted in the massive reduction of anti-C7-induced dermal-epidermal separation at the microscopic level, in contrast to wild-type mice.

Quantitative analysis of infiltrating leukocytes

We also checked the number of accumulating leukocytes in digested ears after anti-C7 treatment. Compared to wild-type samples, a partial reduction was observed in the absolute number of leukocytes, as well as in the number of neutrophils and eosinophils in ears of *Plcg2*$^{\Delta PMN}$ mice. This might be partly explained by the incomplete deletion of PLCγ2 in neutrophils.

The effect of PLC inhibition on human ex vivo skin separation

To extend our previous findings to human pathology, we set up a human ex vivo dermal-epidermal separation assay. We investigated the effect of PLC inhibition in this model by a pharmacologic approach.

Healthy human cryosections were pretreated with anti-C7 antibodies and then coincubated with freshly isolated human neutrophils in the presence of a PLC inhibitor (10 μM U73122) or vehicle. While we could observe strong neutrophil accumulation and massive dermal-epidermal separation in vehicle-treated skin samples, PLC inhibition significantly reduced dermal-epidermal separation after anti-C7 treatment.

These results suggest that PLC enzymes have an important role in autoantibody-induced blister formation in human pemphigoid diseases, as well.

The effect of tofacitinib treatment on anti-C7-induced skin blistering in vivo

We treated wild-type mice twice daily with 0.4 mg, 1 mg tofacitinib or with vehicle by oral gavage and subjected them to anti-C7-induced skin blistering. Tofacitinib-treatment was able to diminish, in a dose-dependent manner, both the extent and the severity of the lesions induced by anti-C7 injections. The development of erosions also tended to be reduced compared to the vehicle-treated group, however, this difference was not significant. We found no difference between the ear thickening of anti-C7-treated groups over time, either.

This small initial study underlines the relevance for additional experiments to delineate the role of Jaks in the pathogenesis of autoimmune skin blistering.

Conclusions

To conclude my work, I list my findings in the following points:

1. Adult *Plcg2*$^{-/-}$ mice have normal numbers of circulating leukocyte subsets and normal expression of major leukocyte antigens.

2. Intact *Plcg2*$^{-/-}$ mice, as well as *Plcg2*$^{-/-}$ bone-marrow chimeras are completely protected from anti-C7-induced skin blistering. PLCγ2 influences the development of the proinflammatory microenvironment without affecting endogenous migration capacity of myeloid cells.

3. PLCγ2 is essential for the C7/anti-C7-induced release of inflammatory mediators, but not for the migration of neutrophils in vitro.

4. Neutrophil-specific deletion of PLCγ2 is effective and specific, but not complete. *Plcg2*$^{\Delta PMN}$ mice are almost completely protected from anti-C7-induced skin blistering. PLCγ2 expressed by neutrophils has an essential role in the effector phase of experimental EBA.

5. Pharmacological inhibition of PLC diminishes neutrophil-mediated dermal-epidermal separation of anti-C7-treated human skin. This underlines the human relevance of our studies and suggests a potential therapeutic application.

6. Inhibition of Janus kinases by the systemic administration of tofacitinib is able to reduce the extent and severity of anti-C7-induced skin lesions.

Publications

Publications related to thesis

Szilveszter, K. P., Németh, T., and Mócsai, A., *Tyrosine Kinases in Autoimmune and Inflammatory Skin Diseases.* **Front Immunol**, 2019. 10: p. 1862.

IF: 5.085

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IF: 15.483

Szilveszter, K. P., Vikár, S., Horváth, A. I., Helyes, Z., Sárdy, M. and Mócsai, A., *Phospholipase Cγ2 Is Essential for Experimental Models of Epidermolysis Bullosa Acquisita.* J Invest Dermatol, 2022. 142(4): p. 1114-1125.

IF: 7.590

Publications not related to thesis

Németh, T., Futosi, K., **Szilveszter, K**., Vilinovszki, O., Kiss-Pápai, L., and Mócsai, A., *Lineage-Specific Analysis of Syk Function in Autoantibody-Induced Arthritis*. **Front Immunol**, 2018. 9: p. 555.

IF: 4.716