Final scientific report

Genetic analysis of neutrophil function

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The aim of the project was to delineate the molecular mechanisms of neutrophil function using innovative genetic tools including lineage-specific deletion of target genes in experimental mice and a novel system allowing the in vitro culture and manipulation of near-primary myeloid progenitors which can later be differentiated toward neutrophils both in vitro and in vivo.

We have made very substantial progress towards the above goals of the project. We have identified a number of novel signaling pathway components in various autoimmune and autoinflammatory disease models and characterized the molecular mechanisms involved. Our results have been published in a large number of primary research papers where both the first and last authors were from our research group. A number of additional collaborational papers and a major review article have also been published. We also generated a very substantial amount of yet unpublished results which will provide the basis for additional publications, further experimentation and exciting novel future research directions.

The most significant achievements of the project are described below.

1) Lineage-specific analysis of PLCγ2 in autoantibody-induced arthritis

We have previously shown that the PLCγ2 phospholipase plays a critical role in the development of autoantibody-induced arthritis in experimental mice. We have now extended those studies to the identification of the lineage(s) in which PLCγ2 is required for arthritis development. To this end PLCγ2 has been deleted from various hematopoietic lineages using Cre-lox-mediated conditional deletion. PLCγ2 deletion from all hematopoietic lineages, from the entire myeloid compartment or from neutrophils abrogated arthritis development, whereas PLCγ2 deletion from mast cells or platelets had no effect. Further analysis of the effect of neutrophil-specific PLCγ2 deletion indicated that PLCγ2-deficient neutrophils are intrinsically capable of migrating to the site of inflammation in a CD18-dependent manner but neutrophil PLCγ2 is required for the generation of the proper inflammatory microenvironment. We have also characterized various in vitro functions of PLCγ2-deficient neutrophils.
The above findings have been published in Arthritis and Rheumatology (Scimago D1; IF: 11.0).

2) In vitro and in vivo analysis of Hoxb8 progenitor-derived neutrophils

One of the major aims of the project was to establish and characterize a novel assay system allowing the in vitro culture of transiently immortalized myeloid progenitors (so-called HoxB8 progenitors) which can later be allowed to differentiate towards neutrophil-like cells (so-called HoxB8 neutrophil-like cells). We have set up this system in our lab and established the in vitro culture conditions of HoxB8 progenitors and their differentiation towards neutrophils. HoxB8 neutrophil-like cells but not HoxB8 progenitors expressed neutrophils-specific cell surface markers and were able to perform characteristic neutrophil functions such as respiratory burst,

chemotaxis or phagocytosis of bacterial or fungal pathogens. We have also established the methodology of in vivo differentiation of HoxB8 progenitors towards HoxB8 neutrophil-like cells and tested the in vivo functions of the cells. Injection of HoxB8 progenitors into lethally irradiated mice resulted in the emergence of a single peak of donor-derived HoxB8 neutrophil-like cells approx. 5 days after HoxB8 progenitor transfer. HoxB8 neutrophil-like cells expressed characteristic neutrophil markers, migrated to the inflamed peritoneum and were able to mediate the reverse passive Arthus reaction. Repeated transfer of HoxB8 progenitors allowed the establishment of a stable circulating HoxB8 neutrophil-like cell pool for several days. This stable HoxB8 neutrophil-like cell pool was able to mediate autoantibody-induced arthritis in the K/B×N serum-transfer model. Additional experiments indicated that HoxB8 neutrophil-like cells derived from injected HoxB8 progenitors in vivo were practically indistinguishable from normal mouse neutrophils under identical conditions.
The above findings have been published in the Journal of Immunology (Scimago Q1; IF: 5.2).

3) The role of PLCγ2 in autoantibody-induced skin blistering disease

We have made substantial efforts to extend our prior studies on signaling in neutrophils and arthritis to other autoimmune disease models such as autoantibody-induced skin blistering. We have set up a system whereby antibodies against the dermo-epidermal junctional protein collagen type VII (Col7) triggers inflammation and dermo-epidermal separation in mice. We have found that PLCγ2-deficient mice are completely protected from both inflammation and skin separation in this model. Further studies indicated an intact migratory capacity of PLCγ2-deficient myeloid cells but a defective immune complex-induced activation of PLCγ2-deficient neutrophils. We have also set up an ex vivo human dermo-epidermal skin separation assay and showed an inhibitory effect of the PLC inhibitor U73122.
These findings have been published in the Journal of Investigative Dermatology (Scimago D1; IF: 7.6).

4) Neutrophil Syk in autoantibody-induced skin blistering disease

We have previously shown that the Syk tyrosine kinase plays a critical role in skin inflammation in the above-mentioned anti-Col7 autoantibody-induced skin blistering disease model. During the current project, we have performed lineage-specific analysis on the role of Syk in the same model. We found that lineage-specific deletion of Syk in the entire hematopoietic system or in neutrophils strongly reduced erosion, inflammation and dermo-epidermal separation triggered by systemic injection of autoantibodies against Type VII collagen (Col7). In addition, entospletinib, a second-generation Syk inhibitor, strongly inhibited dermo-epidermal separation of frozen human skin sections triggered by human neutrophils in the presence of anti-Col7 antibodies.
These findings have been published in the Journal of Investigative Dermatology (Scimago D1; IF: 6.5).

5) Src-family kinases in experimental gout and autoinflammation

Besides our prior experiments on the role of Src-family kinases in autoantibody-induced inflammation models, we also aimed to test the role of these signaling molecules in an experimental model of gout, an autoinflammatory (non-autoimmune) disease. To this end, we have set up an in vivo assay of gouty arthritis triggered by the injection of monosodium urate (MSU) crystals in the hind paws of experimental mice. Inflammation in this model has been

tested by clinical assessment, as well as analysis of the inflammatory environment and in vivo imaging for reactive oxygen species production. We have also performed additional in vitro experiments on the responses of human and mouse neutrophils to MSU crystals. We found that genetic deletion of three myeloid-specific Src-family kinases (Hck, Fgr and Lyn) strongly reduced in vivo gouty arthritis and abrogated MSU crystal-induced in vitro responses of neutrophils. Analysis of single and double knockouts revealed substantial functional overlap between Hck, Fgr and Lyn. Dasatinib, a clinically used Src-family kinase inhibitor, reduced gouty arthritis in both prophylactic and therapeutic settings, and abrogated MSU crystal-induced responses of human neutrophils. Together with Clifford Lowell at UCSF, we have also shown that the triple deficiency of Hck, Fgr and Lyn protected mice from severe autoinflammation caused by a loss-of-function mutation of SHP-1, a negative regulator of tyrosine kinase signaling in myeloid cells (so-called motheaten model).
These findings have been published in the Journal of Experimental Medicine (Scimago D1; IF: 15.3).

6) The effect of Syk inhibition on human dermo-epidermal separation

To explore the translational potential of our prior findings on the role of Syk in autoimmune skin diseases, we have set up a fully human assay for the analysis of dermo-epidermal separation of human frozen skin sections by human neutrophils in the presence of serum samples from patients suffering from bullous pemphigoid, an autoantibody-mediated blistering skin disease. We have also shown that entospletinib, a second-generation Syk inhibitor, abrogated dermo-epidermal separation in this fully human assay system. Further mechanistic studies revealed that entospletinib inhibited immune complex-induced ROS production and granule release but did not affect the survival or migratory capacity of neutrophils.
These findings have been published in the Journal of Investigative Dermatology (Scimago D1; IF: 5.7).

7) Rapid analysis of the in vivo effects of tyrosine kinase inhibitors

To extend our findings to potential applications in the pharmaceutical industry, we decided to set up a novel in vivo assay system for the rapid analysis of the in vivo effects of orally administered tyrosine kinase inhibitors in experimental mice. To this end we have set up an intracellular flow cytometry-based assay for the analysis of the basal tyrosine phosphorylation in circulating neutrophils. We have used various approaches to confirm the specificity of this assay and showed that the genetic deficiency of the Src-family kinases Hck, Fgr and Lyn abrogated the neutrophil tyrosine phosphorylation response. Finally, we have shown that oral administration of dasatinib, a clinically used Src-family kinase inhibitor, also inhibited this tyrosine phosphorylation response, with a well-defined time course and dose-response relationship.
These findings have been published in Frontiers in Pharmacology (Scimago Q1; IF: 5.6).

8) Src-family kinases in experimental glomerulonephritis

We have also tested some of our various knockout mutants in an autoantibody-induced glomerulonephritis model. To this end, we have set up an autoantibody-induced glomerulonephritis (so-called nephrotoxic nephritis) model, triggered by pre-immunization of mice with normal sheep IgG, followed by systemic injection of sheep antibodies against mouse glomerular antigen preparations. This treatment triggered robust glomerulonephritis and

kidney injury, indicated by massive albuminuria, increased plasma creatinine concentration, reduced plasma albumin levels, as well as signs of kidney inflammation and injury such as crescent formation and leukocyte infiltration. Genetic deficiency of the Src-family kinases Hck, Fgr and Lyn strongly reduced all signs of kidney dysfunction and glomerular injury in this model. Bone marrow transplantation experiments indicated a major role for Src-family kinases within cells of hematopoietic origin. Human database searches also indicated upregulated Hck, Fgr and Lyn expression in human lupus nephritis. Additional experiments revealed that Src-family kinases are critical for leukocyte accumulation in the inflamed kidneys, but this is likely due to an indirect, non-cell-autonomous role of Src-family kinases. Biochemical experiments also indicated that the role of Src-family kinases is mediated by activation of the Syk tyrosine kinase. Finally, collaborative experiments with Gábor Szénási at our university revealed that myeloid Src-family kinases are also likely involved in ischemia-reperfusion injury.
The manuscript describing these findings has been submitted to Kidney International but has then been rejected by the journal. After discussions with the editorial team, we are now performing additional experiments and aim for a resubmission to the same journal in early 2025.

9) Neutrophil PLCγ2 in autoantibody-induced skin blistering disease

To extend our recent experiments on the role of PLCγ2 in skin blistering, we have performed lineage-specific experiments on the role of PLCγ2 in anti-Col7 antibody-induced skin disease. Neutrophil-specific deletion of PLCγ2 strongly reduced autoantibody-induced skin erosion, inflammation and dermo-epidermal separation. Neutrophil-specific PLCγ2 deficiency also reduced accumulation of various inflammatory mediators in the ear tissue.
These findings have been presented at numerous national and international meetings. A corresponding manuscript is under preparation and is planned for submission during 2025.

10) Genetic modification and analysis of Hoxb8-transduced progenitors and neutrophils

We have invested a very large amount of time, efforts and resources in the genetic modification and functional analysis of myeloid progenitors conditionally immortalized by retroviral transduction with an ER-HoxB8 fusion protein (so-called HoxB8 progenitors). We have established HoxB8 progenitors constitutively expressing the Cas9 endonuclease. We have set up the lentiviral transduction of Cas9-transgenic HoxB8 progenitor cells with CRISPR guide RNAs targeting CD18, FcRγ, gp91phox and Syk and were able to very effectively delete all four target proteins. We have optimized the protocol for practically full target deletion in as little as 5-6 days. We have also set up assays for the in vivo repopulation of mouse hematopoietic system with the HoxB8 progenitors and found that both the non-targeted and targeted HoxB8 progenitors were able to differentiate towards apparently normal circulating neutrophils. We have set up several in vivo inflammation models (including the reverse passive Arthus (RPA) reaction and K/B×N serum-transfer arthritis (STA)) for the analysis of the functional responsiveness of HoxB8 progenitor-derived neutrophils and showed that the CRISPR/Cas9-mediated deletion of CD18, FcRγ and Syk strongly reduced the RPA and K/B×N STA response. We have also begun testing our mutants in in vitro phagocytosis and in vivo bacterial infection models. We have also made substantial progress in better characterizing the genetic changes in our model, including analysis of single-cell clones, various gRNA sequences and characterization of the target site mutations.

Parts of these experiments have been presented at national and international meetings. After additional experiments, we plan to put together a manuscript describing these findings in mid-2025.

11) The role of complement in a bullous pemphigoid model

We have recently tested the role of complement activation in a fully human ex vivo bullous pemphigoid model, recently set up in our group. We have found that antibody preparations of diverse sources against various complement components strongly inhibited dermo-epidermal separation of human skin sections triggered by human neutrophils in the presence of bullous pemphigoid patients' serum and fresh human plasma. In particular, the therapeutic anti-C5 complement antibody eculizumab dramatically reduced dermo-epidermal separation. By using plasma samples from eculizumab-treated patients, we found that the effect of eculizumab was achieved at therapeutically relevant concentrations. Eculizumab also strongly reduced the migration of neutrophils towards the dermo-epidermal junction in real-time in vitro imaging experiments. Together with additional experiments using other C5-targeting compounds, our results suggest a critical role for neutrophil migration to autoantibody deposition sites via complement-derived C5a generation in the dermo-epidermal response. Those results also suggest complement inhibition as a potential novel therapeutic strategy of bullous pemphigoid.
These results have been presented at national and international meetings. We plan to put together a manuscript describing these findings in late 2025.

12) Collaborative projects

We have also made substantial contribution to a number of collaborative projects on the role of neutrophils in kidney ischemia/reperfusion injury, the molecular mechanisms of K/B×N serum-transfer arthritis, the role of Syk and CARD9 in fungal infection, and the participation of Siglec-H in viral infection.
The results of these experiments have been published in several middle-author publications in the International Journal of Molecular Sciences (Scimago Top 11%; IF: 5.9), Frontiers in Pharmacology (Scimago Top 15%; IF: 5.8), Pharmaceuticals (Scimago Q1; IF: 5.2), Frontiers in Immunology (Scimago Q1; IF: 8.8) and mBio (Scimago D1; IF: 7.8).

13) Review article on neutrophils as therapeutic targets

We have been invited to submit a review article on the potential therapeutic targeting of neutrophils in diverse diseases including infection and inflammatory diseases, as well as in cancer.
The article appeared in Nature Reviews Drug Discovery (Scimago D1; IF: 64.8).