Final Report on ÖGGH Research Funding

Define the role of NR2F6 in ILCs biology during colitis

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Project objective

The project aimed to investigate NR2F6 as a regulatory factor in intestinal innate lymphoid cells. The main objectives were to establish robust methods for analysing murine ILC subsets and to determine whether loss of *Nr2f6* alters their composition, phenotype, function, and development under homeostatic conditions and during experimental colitis.

Work performed and key findings

Establishment of ILC analysis

Protocols for isolating and multiparameter flow-cytometrically analyzing ILCs from mesenteric lymph nodes, as well as from the lamina propria and intraepithelial fractions of the small and large intestine, were established and optimized. These protocols enabled reproducible identification of ILC1, ILC2, ILC3, NKp46-positive ILC3 or ex-ILC3, and LTi subsets. In addition, we established *ex vivo* restimulation assays to measure IFN-γ, IL-17A, IL-17F, and IL-22, along with panels to analyze ILC progenitor populations in the bone marrow.

Collaboration with Charité Berlin

The project was strengthened by a new collaboration with the group of Prof. Chiara Romagnani at Charité - Universitätsmedizin Berlin. Christina Stehle provided additional protocols for intestinal tissue preparation, FACS gating strategies, and antibody panels for the identification of murine ILC subsets, and supported their adaptation to the experimental conditions in Innsbruck through technical and scientific exchange. This collaboration was instrumental in establishing intestinal ILC analysis and provides a foundation for further mechanistic and human-oriented studies.

Evidence that NR2F6 regulates intestinal ILCs

Analyses of germline *Nr2f6*-deficient mice provided initial evidence of tissue-specific changes in ILC composition. The most consistent finding was increased NKp46 expression on ILC1 and NKp46-positive ILC3 or ex-ILC3 in mesenteric lymph nodes and the small intestine. By contrast, after *ex vivo* restimulation, the ILC3 populations analysed under the conditions used showed no major changes in the production of IFN-γ, IL-17A, IL-17F, or IL-22. The functional assays were limited by low cell numbers and inter-individual variability and require confirmation in larger cohorts.

Analysis of early bone marrow populations revealed no robust differences in CLP or ILCP. We observed a possible reduction in CHILP only in a small exploratory cohort, which does not support a definitive conclusion about the affected developmental stage.

Interpretation of the conditional mouse models

To assess the function of NR2F6 in NKp46-expressing cells, an *Nr2f6* flox/flox Ncr1-Cre line was investigated. However, subsequent molecular validation by RNA sequencing and qPCR in FACS-sorted NK cells did not confirm sufficient Cre-mediated deletion of *Nr2f6*. Phenotypic data generated with this line are

therefore not interpreted as biological evidence either for or against a cell-intrinsic function of NR2F6 in NKp46-positive cells.

Promising initial data are now available from the validated haematopoietic *Nr2f6* flox/flox Vav-Cre model. These data show increased NKp46 expression and reduced T-bet expression, supporting a haematopoietically mediated role of NR2F6 in regulating the ILC phenotype. Because Vav-Cre targets all haematopoietic cells, these findings do not yet demonstrate an ILC-specific effect. The cohorts are currently being repeated, and genotyping and deletion efficiency are being re-evaluated.

Colitis experiments

To investigate the chronic inflammatory phase, germline *Nr2f6*-deficient and wild-type mice were analysed in a chronic low-dose DSS model. The animals received two cycles of 2% DSS on days 0-5 and 21-26, with final analysis on day 29. Clinical course and colonic phenotype were assessed together with adaptive and innate lymphoid cell populations in mesenteric lymph nodes and the large intestine.

To date, the analyses indicate a milder chronic disease course and reduced weight loss in *Nr2f6*-deficient mice. This finding differs from the published acute DSS phenotype and supports a context- or phase-dependent function of NR2F6 during intestinal inflammation. In the large intestine, no significant differences were detected on day 29 in CD4 and CD8 T cells, γδ T cells, or the NK and ILC compartments analyzed; ILC2 and ILC3 subsets were also largely unchanged. However, FACS analyses in the mesenteric lymph nodes indicated a shift within the ILC3 population and an increased proportion of NKp46-positive ILC3 or ex-ILC3. Thus, the association between NR2F6 loss and enhanced NKp46 expression observed under homeostatic conditions remains evident in the chronic DSS setting. Quantitative evaluation of additional longitudinal, serum cytokine, and qPCR data will further refine the mechanistic interpretation.

Conclusion

The project established the methodological basis for detailed analysis of intestinal ILCs and provided initial evidence that NR2F6 is particularly associated with regulating NKp46 and the phenotypic characteristics of ILC1 and ILC3. The chronic DSS experiments indicate a phase-dependent function of NR2F6 and a persistent alteration of NKp46-positive ILC3 in the mesenteric lymph nodes. The lack of deletion in the Ncr1-Cre line limits the interpretability of this model; we addressed this limitation methodologically by establishing and initially analyzing the Vav-Cre model. Ongoing confirmation of the Vav-Cre findings and complementary molecular analysis of the colitis experiments will further clarify the functional interpretation.

Scientific outputs and publications

- Woelk J, Hornsteiner F, Aschauer-Wallner S, Stoitzner P, Baier G, Hermann-Kleiter N. Regulation of NK cell development, maturation, and antitumor responses by the nuclear receptor NR2F6. Cell Death & Disease. 2025;16:77. doi:10.1038/s41419-025-07407-4. The ÖGGH research grant is explicitly acknowledged in the funding statement.
- Loidl S. Define the role of NR2F6 in intestinal innate lymphoid cell biology. Master's thesis, University of Innsbruck and Medical University of Innsbruck, 2025.