Validating the genetic alterations in cutaneous T-cell lymphoma: unraveling the role of SOCS1 and HNRNPK through genetically engineered mouse models

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General discussion

General discussion and future perspective

The work presented in this thesis focused on developing advanced genetically engineered mouse models (GEMMs) to enhance research on Cutaneous T-cell Lymphoma (CTCL). By specifically targeting skin-homing CD4+ T cells and incorporating an inflammatory skin reaction, these models replicate the natural progression of CTCL, significantly improving our understanding of its early-stage genesis. They also offer promising avenues for the development of early interventions. This final chapter summarizes and discusses the findings of **Chapters 2, 3, 4, and 5**, culminating in conclusions and future perspectives.

Cd4CreER^{T2} System and Inducible Conditional Knockout Mouse Generation

In **Chapter 1**, we provided an overview of mouse models utilized in CTCL research, with a particular emphasis on the importance of the development of GEMMs. In the realm of CTCL studies, GEMMs are categorized into non-skin targeted and skin-targeted CD4+ T cell GEMMs. The latter category, which is the main content in this thesis and characterized by skin-target CD4+ T cells, offers a more faithful representation of the natural origin of CTCL pathogenesis.

The most commonly used tool for targeting gene knockout in (skin-homing) CD4+ T cells is the *Cd4CreER^{T2}* system. (1) This innovative system combines the versatility of the Cre-loxP recombination strategy with the precision of inducible gene targeting, making it an useful tool in the study of gene function in a temporally controlled manner. (2) Fundamentally, the *Cd4CreER^{T2}* system relies on Cre recombinase, derived from the P1 bacteriophage, renowned for its ability to catalyze site-specific recombination between loxP sites. These loxP sites are short DNA sequences strategically inserted into the genome, flanking the target gene. (3) Cre recombinase efficiently excises or inverts the DNA between these loxP sites, leading to the deletion or modification of the gene of interest. (4)

The unique aspect of the *Cd4CreER^{T2}* variant lies in the fusion of *Cre* recombinase with a mutated estrogen receptor (ERT2), which renders the enzyme inactive at physiological estrogen levels. (5) This inactivity persists until the administration of Tamoxifen, a selective estrogen receptor modulator. Tamoxifen binds to the ERT2 portion, causing a conformational change that activates the *Cre* recombinase. (6) This inducible system provides a high degree of control over the timing of gene recombination, allowing researchers to study gene function in various developmental stages or disease states. In addition, topical administration of tamoxifen (e. g. painting on the skin) offers the possibility to restrict recombination in discerning regions of the body.

This *Cd4CreER*^{T2} system is crucial to our thesis's main experiments. In **Chapters 2** and **Chapter 3**, we crossed *Cd4CreER*^{T2} mice with *Socs1* flox mice, while in **Chapters 4** and **Chapter 5**, the crossbreeding involved *Cd4CreER*^{T2} and *Hnrnpk* flox mice. The *Cre* recombinase in this context is under the control of the *Cd4* promoter, enabling its specific activation in CD4+ T cells. The knockout of *Socs1* in CD4+ T cells of these crossbred mice could be detected using flow cytometry, thanks to a reporter. (7) For *Hnrnpk*, which lacks a reporter, the knockout was verified by extracting DNA from enriched CD4+ T cells and using PCR techniques. These procedures have confirmed the effectiveness of the *Cd4CreER*^{T2} tool.

In the Cre-lox system, there are various methods to activate *Cre* with Tamoxifen, especially in *in vivo* models. These methods include intraperitoneal injection, gavage (oral administration), and intravenous injection, depending on the experimental purpose and the target cells. (1) (8) (9) To verify the effectiveness of the *Cd4CreER*^{T2}-LoxP system in our mouse model, we initially used intraperitoneal injection of Tamoxifen for validation (**Chapter 2**). Our research focuses on primary cutaneous T-cell lymphoma, targeting skin-homing CD4+ T cells. Hence, we employed a topical application method. Additionally, since Tamoxifen needs to be metabolized in the liver to form 4OH Tamoxifen, and topical Tamoxifen cannot undergo this metabolism, we used the active form, 4OH Tamoxifen, directly. There is no standard protocol for the topical application of 4OH Tamoxifen, so we compared the effects of 3 and 5 topical applications on the genes in systemic CD4+ T cells. We determined that three topical applications of 4OH Tamoxifen were sufficient to knock out the target gene in skin-homing CD4+ T cells (**Chapter 2**). In further research, we optimized the use of 4OH Tamoxifen. Our study data show that even a single topical application of Tamoxifen is enough to activate the *Cd4CreER*^{T2}-LoxP system and can do so with minimal systemic disturbance (**Chapters 3, 4, and 5**).

Initial Effects of Genome Alteration in the Pathogenesis of CTCL

Genetic alteration in the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway is a critical chain of interactions between proteins within a cell. Genetic alterations in the JAK-STAT pathway, including deletions and mutations, play a significant role in the pathogenesis of CTCL. (10) Activating mutations in JAK kinases have been reported in CTCL. (11) These mutations frequently occur in the JAK-STAT signaling pathway and may serve as indicators for targeted therapy. (12) For instance, recurrent mutations in genes of the JAK-STAT signaling pathway, including *STAT3*, *JAK1*, and others have been identified. (11) (13) In addition to mutations, deletions of tumor suppressors *HNRNPK* and *SOCS1* were found to be the most frequent genetic alterations in MF after deletion of *CDKN2A*. (14) Notably, *SOCS1* deletion could be detected even in early-stage MF. These

deletions, resulting from genomic rearrangements, lead to up-regulation of the JAK-STAT pathway, among others.

Biallalic knockout and Haploinsufficient of tumor suppressor genes in CTCL

Biallelic knockout, which involves the complete loss of both alleles of a gene, can result in the complete absence of gene function. In the context of CTCL, this can be particularly relevant when studying TSGs (Tumor Suppressor Genes) that are implicated in the regulation of T-cell proliferation and survival. (15)

Understanding the consequences of biallelic knockout of specific TSGs can provide insights into whether their loss contributes to the uncontrolled proliferation of CD4+ T cells seen in CTCL. On the other hand, haploinsufficiency arises when an organism possesses only one functional copy of a gene due to the loss of function of a single allele. (16) In the context of CTCL, haploinsufficiency of certain TSGs may lead to reduced protein levels in CD4+ T cells, potentially impairing their normal regulatory functions. This can create an environment conducive to the development and progression of CTCL. (17)

Exploring the interplay between biallelic knockout, haploinsufficiency, and specific TSGs in the context of CTCL offers a deeper understanding of the molecular mechanisms underlying this lymphoma. (18) By elucidating how genetic alterations in TSGs affect T-cell behavior, researchers can uncover novel therapeutic targets and potential interventions for CTCL.

Specific Insights into Biallalic knockout and Haploinsufficiency of *Socs1* and *Hnrnpk* Genes

Haploinsufficiency of *SOCS1* is a recognized cause of early-onset autoimmune diseases. (19) Human studies align with findings in mice, notably that *Socs1*-/- mice experience neonatal lethality attributed to excessive IFN-γ signaling and extensive inflammatory infiltration. (7) Lineage mutations in *SOCS1* leading to haploinsufficiency result in heightened activation of the JAK-STAT pathway in response to various cytokines. (20) Mice with a haploinsufficient dose of *Socs1* can survive to adulthood, but with age, they develop systemic autoimmune diseases. Given the widespread expression of *SOCS1* and the coordinating role of cytokines in the immune system, multiple cell types may contribute to autoimmunity under conditions of *SOCS1* haploinsufficiency.

While *SOCS1* haploinsufficiency is a recognized cause of early-onset autoimmunity, our study is the first to demonstrate data specifically focused on *Socs1* haploinsufficiency in skin-resident CD4+ T cells. In **Chapter 2**, although the experiments conducted over only

8 weeks showed that *Socs1* monoallelic knockout in skin-homing CD4+ T cells was not sufficient to induce CTCL, we were able to observe differences between experimental mice and wild-type mice, confirming the impact of *Socs1* haploinsufficiency even when restricted to CD4+ T cells.

Additionally, despite insufficient numbers of mice with bi-allelic knockout of *Socs1* for statistical analysis, all these mice exhibited visibly thicker epidermis, increased inflammatory cell presence (CD3, CD4, CD8), and a greater number of cells positive for p-STAT3 in the lymphocytes. Even without quantitative statistical data, the distinctions between mice with homozygous *Socs1* knockout and heterozygous *Socs1* knockout in CD4+ T cells were evident, suggesting that homozygous *Socs1* knockout in CD4+ T cells could lead to more pronounced phenotypic changes.

In **Chapter 3**, with an extended experimental period, the differences in phenotype between mice with monoallelic knockout of *Socs1* in skin-homing CD4+ T cells and wild-type mice were more pronounced, likely indicating systemic pathological lymphocyte proliferation. This further confirmed the effects of *Socs1* haploinsufficiency, highlighting the role of *Socs1* as a tumor suppressor gene.

The knockout of both alleles of *Hnrnpk* (*Hnrnpk*-/-) is generally incompatible with embryonic survival; complete loss of *Hnrnpk* is lethal in early developmental stages. (21) (22) Such loss also severely impairs the function and development of functional CD4+ T cells. (23) This underlines the significance of our development of *Hnrnpk* flox mice in **Chapter 4**, where, through crossing with *Cd4CreER*^{T2} mice, we obtained a model that allows controlled timing of specific *Hnrnpk* knockouts in skin-homing CD4+ T cells.

Hnrnpk functions as a haploinsufficient tumor suppressor. *Hnrnpk* haploinsufficiency can lead to reduced survival, increased tumor formation, genomic instability, and the development of transplantable hematologic malignancies. (24) In **Chapter 4**, we present, for the first time, data on the effects of single-gene knockout of *Hnrnpk* in skin-homing CD4+ T cells combined with chronic skin allergies. Coupled with the homozygous knockout mice of *Hnrnpk* in **Chapter 5**, our research encompasses homozygous and heterozygous *Hnrnpk* knockouts, as well as wild-type *Hnrnpk* mice, allowing for comprehensive comparison. We confirmed that the loss of a single allele of the *Hnrnpk* gene is sufficient to affect the phenotype of the mouse model, and no significant differences were observed between the phenotypes resulting from bi-allelic and mono-allelic knockouts.

The Role of *SOCS1* in the Pathogenesis of Early-Stage CTCL

SOCS1, a key regulator of cytokine signaling, when knocked out in skin-resident CD4+ T

cells, leads to a state of autonomous skin inflammation. (**Chapter 2 and Chapter3**) The *Socs1* knockout mouse model mimics the features of early-stage mycosis fungoides, the most common form of CTCL. The loss of *Socs1* results in uncontrolled JAK-STAT signaling, leading to the initiation of skin inflammation that does not resolve on its own, along with sustained activation of STAT3 within the skin.

Within these *Socs1* knockout mice, we observe a significant increase in T-cell infiltration in the skin, along with heightened and sustained expression of activated STAT3. These changes are indicative of the disrupted immune surveillance and control mechanisms typically seen in CTCL. The prolonged contact-allergic reaction in these mice further demonstrates the potential for chronic inflammation to progress into a malignant state, emphasizing the critical role of *Socs1* in maintaining normal immune homeostasis and preventing the onset of CTCL.

The *Socs1* flox *Cd4CreER*^{T2} mouse model discussed in **Chapter 2** and **Chapter 3** is of importance in CTCL research, especially for understanding the role of *SOCS1* in the progression of the disease. *SOCS1* has been found to be deficient in patients with CTCL, particularly in those with early-stage MF. (14) By selectively deleting the *Socs1* gene in the CD4+ T cells of mice—which is accomplished by the cross breeding of *Cre* transgenic and *Socs1* floxed mice - researchers can observe the effects of the absence of this gene in skin-resident T cells involved in inflammation of the skin (activation of *Cre* by topical application of tamoxifen on the inflamed skin). This model has revealed that isolated loss of the *Socs1* gene does not lead to pronounced malignant changes, even when coupled with short term inflammatory challenges.

However, with protracted challenges - specifically, over a period of 20 weeks— the skin inflammation becomes autonomous and mirrors early-stage MF, offering a more profound understanding of the disease's early development and potential intervention.

***Hnrnpk* Knockout Mouse Model and Its Implications in Early-Stage CTCL**

Hnrnpk flox *Cd4CreER*^{T2} mouse model can be utilized to examine the impact of *Hnrnpk* gene haplodeficiency or its complete absence in CD4+ T cells, aiming to elucidate its role in CTCL pathogenesis. (14, 25) This aproach is informed by prior studies highlighting significant deletions of *Hnrnpk* in the JAK-STAT pathway among CTCL patients. (25) It builds on the foundational research conducted using the *Socs1* flox *Cd4CreER*^{T2} mouse model, which has paved the way for exploring the effects of these genes related to CTCL.

The *Hnrnpk* knockout model has provided significant insights into the pathogenesis of early- stage CTCL. The loss of one copy of *Hnrnpk* in CD4+ T cells leads to a cascade of

molecular events that simulate the early stages of CTCL. (**Chapter 4 and Chapter 5**) This model has shed light on the intricate role of *Hnrnpk* in maintaining genomic stability and regulating gene expression. The *Hnrnpk* knockout mice exhibit characteristics such as increased cellular proliferation, impaired apoptosis, and dysregulated cytokine signaling, all of which are hallmarks of early-stage CTCL.

The induction of chronic skin inflammation in these mice parallels the inflammatory environment seen in CTCL patients. Notably, the persistent activation of the JAK-STAT pathway, a consequence of *Hnrnpk* loss, mirrors the cytokine dysregulation observed in CTCL. These findings underscore the importance of *HNRNPK* as a regulatory factor in T-cell homeostasis and its potential role in the initiation of lymphomagenesis.

Comparing the *Socs1* knockout model with *Hnrnpk* knockout model reveals both overlapping and unique features pertinent to CTCL pathogenesis. Although, the function of *Hnrnpk* emphasizes the role of genetic stability and gene regulation in the disease's progression (26), whereas the function of *Socs1* gene related the impact of disrupted cytokine signaling control. Both models show dysregulated JAK-STAT signaling, an important pathway in CTCL, the similar inflammatory environment seen in CTCL patients and the pathological proliferation of immune cells. (14) Together, these models significantly contribute to our understanding of early-stage CTCL. The insights gained from these models are crucial for the development of targeted therapies and offer a more detailed understanding of the molecular mechanisms underlying CTCL.

Future Perspectives and Concluding Remarks

In this thesis, the work has provided a viable model and practical methods for the targeted knockout of skin-homing CD4+ T cells in the mouse skin, along with an optimized approach applying Tamoxifen topically. Our data indicates that under current experimental conditions, although the mice exhibit early-stage CTCL-like characteristics, they are not yet satisfactory as a fully developed, ready-to-use tumor model.

As a next step from **Chapter 2** and **Chapter 3**, which explored the role of *Socs1* following its knockout in mouse skin-homing CD4+ T cells, our goal is to further validate our results by expanding the sample size of mice with *Socs1* bi-allelic knockout. We aim to assess whether bi-allelic and mono-allelic knockouts of *Socs1* have different impacts on the onset and progression of CTCL.

Our subsequent steps for *Hnrnpk* gene research in **Chapter 4** and **Chapter 5** are similar to those for *Socs1*. Additionally, transcriptome analysis of skin-homing CD4+ T cells isolated from *Hnrnpk* knockout mice can be used to characterize the imbalance of affected target

genes and transcripts. This approach helps identify cellular processes impacted by the loss of the *Hnrnpk* gene, ultimately elucidating the mechanisms behind the exacerbated skin inflammation observed in our transgenic mice. Moreover, this method can be employed to validate the enhanced JAK-STAT signaling pathways observed in our *Hnrnpk* gene heterozygous and homozygous knockout mice treated and challenged with OXA. By extending the experimental period and utilizing skin-homing CD4+ T cell bi-allelic knockout GEMM mice, along with constructing large-sample experiments, we can further determine and optimize early-stage CTCL mouse models.

Continuously refining our model-building approach for gene knockout in skin CD4+ T cells is a key focus. Our next step is to explore genetic alterations relevant to CTCL pathogenesis, using our knockout models to investigate potential gene changes. In future model construction, we plan to cross with other GEMMs harboring genetic alterations, such as loss of *Cdkn2A* often observed in CTCL(10) or, which hold potential for tumorigenesis. These endeavors will enhance our understanding of CTCL's genetic underpinnings and might contribute to more efficient model development.

In conclusion, our research focuses on establishing autochthonous mouse models closely resembling CTCL, allowing selective gene knockouts in skin-homing CD4+ T-cells. While our skin-target GEMMs may not capture all malignant aspects, they closely mimic CTCL's origin and early changes. Future research will leverage emerging technologies like *CRISPR-Cas9* gene editing to enhance model accuracy and relevance, enabling exploration of gene effects and understanding the genesis of early-stage CTCL. This work can contribute to a better understanding of CTCL pathogenesis, validates the driving role of relevant genes in its early stages, and creates research platforms for (precision) medicine in early-stage CTCL treatment.

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