Glucocorticoid modulation of the immune response: Studies in zebrafish

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Chapter 6

Summary and Discussion

Zebrafish as a model to dissect the effect of glucocorticoids in the immune system

Glucocorticoids (GCs) regulate, through the activation of the glucocorticoid receptor (GR), a wide variety of systems, like the metabolic, reproductive, cardiovascular, nervous and immune system [1-3]. Due to their well-established immunosuppressive effects, GCs are widely prescribed as anti-inflammatory drugs. However, their utilization is severely limited by the occurrence of side effects and drug resistance [4, 5]. Therefore, there is still a major need to investigate the molecular and cellular mechanisms underlying the effects of GCs. These mechanisms appear to be highly complex, since the effects of GCs are cell type- and context-specific and their transcriptional regulatory effects on pro-inflammatory genes are not strictly suppressive [6, 7].

Zebrafish are increasingly used as an *in vivo* model system for studying the immune system, in particular the inflammatory response, for research aimed at the discovery of novel drug targets and for screening of drug libraries [8-10]. The advantages of this model system include its evolutionally conserved immune system, the accessibility of embryos for genetic manipulation and non-invasive live imaging and the cost-effective maintenance [10-13]. In **Chapter 2,** an overview is presented of how the zebrafish is used as an animal model for inflammatory diseases. Different models are described, and how they are used for research on the mechanisms underlying the inflammatory response and for testing of potential novel anti-inflammatory drugs, in particular GCs. In addition, the structure and function of the zebrafish Gr and the regulation of the secretion of the endogenous GC cortisol are highly similar to the human system [14-17]. This makes the zebrafish a valuable tool to study the complex modulatory effects of GCs on the immune system. In this thesis, we have used this model system to study molecular and cellular mechanisms of GC action on the immune system and to develop a model for *in vivo* screening of the anti-inflammatory effects as well as possible adverse effects of novel GC therapies. For this purpose, we have studied the effect of GCs on leukocyte migration and differentiation during an inflammatory response **(Chapter 3)**, how GCs modulate the immune response to a mycobacterial infection **(Chapter 4)**, and we have investigated targeting of GCs to inflamed tissue by liposomal delivery (**Chapter 5**). In figure 1, a graphical overview of the experimental chapters is presented.

Glucocorticoids inhibit the differentiation of macrophages towards a pro-inflammatory phenotype through transcriptional regulation

In **Chapter 3**, studies are described in which we have used the zebrafish tail fin amputation model as a model for inflammation. In this model, neutrophils and macrophages migrate towards the area that is

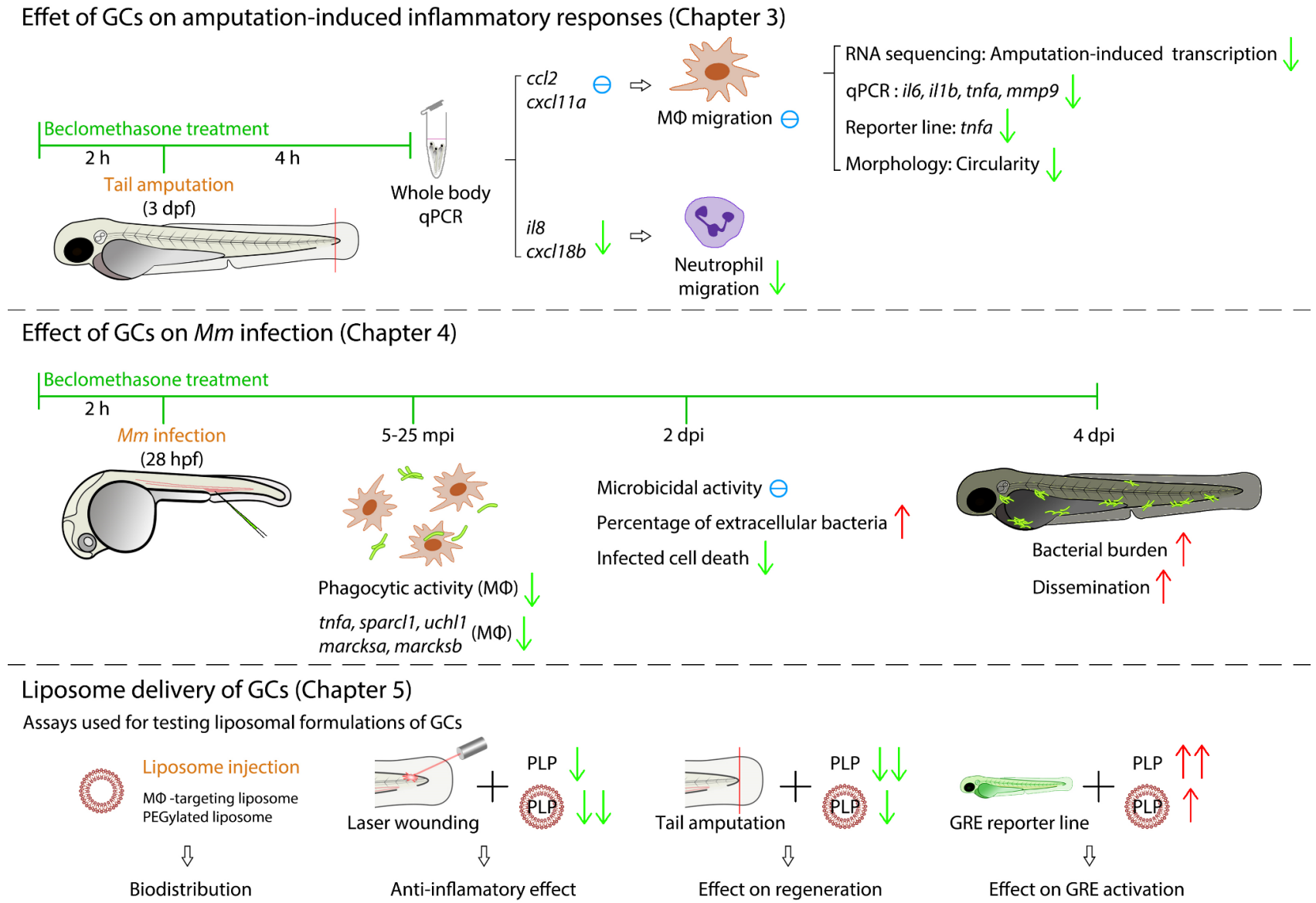

Figure 1. Graphical overview of the experimental chapters

wounded by the amputation. Treatment with the GC beclomethasone inhibits the migration of neutrophils towards the wound. However, the amputation-induced macrophage migration is not decreased by GCs, similar to earlier observations in other studies[18-20]. Our work demonstrated that this difference in the response to GCs is related to the GC-induced decrease in the expression of genes encoding chemoattractants which are involved in neutrophil recruitment such as Il8 and Cxcl18b, whereas the expression of genes encoding chemoattractants involved in macrophage recruitment, such as Ccl2 and Cxcl11aa, is unaffected by the GC treatment. Similar to our results, in a human breast cancer cell line (T47D) GC treatment has no effect on the IL-1-stimulated CCL2 production [21], and no effect of GCs was seen on the IFN-γ-induced CXCL11 production in human lung epithelial cells (A549) [22]. However, in most studies different observations have been made, as GCs have been shown to inhibit inflammation-induced CCL2 levels in humans and rats [23-25]. Moreover, CXCL11 upregulation was inhibited by GCs in isolated human peripheral blood monocytes, IFN-γ- or LPS-stimulated RAW 264.7 macrophages and multiple tissues of endotoxemia mice [26, 27]. These differences between studies suggest that the GC resistance of the *ccl2* and *cxcl11a* transcription observed in our study, which causes the GC insensitivity of the macrophage migration, requires a specific context. The interaction with GR with other transcription factors and various coregulator proteins has been shown to be highly complex and it is often unclear how this results in positive or negative gene regulation [28-32]. In future studies it will be interesting to unravel the molecular factors determining the resistance of the transcription of genes encoding macrophage-specific chemoattractants in our model.

GCs have been shown to strongly inhibit the immune response by inhibiting the transcriptional activity of pro-inflammatory genes and inducing the expression of anti-inflammatory genes [7]. For example, in cultured macrophages, it has been reported that the expression of pro-inflammatory regulators are efficiently suppressed by GC treatment [28, 29, 32, 33]. Similarly, GC treatment attenuates the vast majority of genes induced by tail wounding in zebrafish [19]. To study transcriptional regulation by GCs in macrophages in our model, an RNA sequencing analysis was performed on macrophages isolated from zebrafish larvae. We observed that GC treatment suppresses virtually all amputation-induced changes in gene expression, among which the induction of pro-inflammatory genes. In addition, using the *Tg(tnfa:eGFP-F)* reporter line we showed that the number of macrophages expressing *tnfa* was significantly reduced by GC treatment, and we observed that GCs decreased the percentage of macrophages displaying the typical more rounded morphology that is observed in response to an inflammatory stimulus.

Macrophages display a continuum of phenotypes but two opposite functional phenotypes are often distinguished: a classically activated (M1) phenotype which promotes the inflammatory response, and an alternatively activated (M2) phenotype which is involved in the resolution of inflammation and

wound healing [34, 35]. Our data suggest an inhibitory effect of GC administration on the differentiation of macrophages towards an M1 phenotype. Apparently, GCs allow macrophages to migrate towards a site of inflammation, but prevent them from becoming pro-inflammatory.

It is still unclear whether GCs, in addition to the inhibition of the differentiation to an M1 phenotype, induce an M2 phenotype. A possible approach to study this would be to establish a reporter zebrafish line for the expression of M2 macrophage markers, like *arg2, cxcr4b, tgfb1, ccr2, vegf, irf4,* or *ccl22* [36, 37]. Several such lines are under being generated in laboratories of our collaborators, but not yet available. Therefore, we have analyzed the expression level of several M2 markers in macrophages, but results remained inconclusive because the induction of *arg2* by wounding was not sensitive to GC treatment and the expression of *cxcr4b, tgfb1* and *ccr2* was not induced by wounding. However, this analysis was based on the whole population of macrophages so specific effects in M2 macrophages may have been hidden. In order to further study the effect of GCs on the macrophage phenotype, an expression analysis of *tnfa*-positive (M1) versus -negative (M2) populations could be performed, or single cell RNA sequencing to discriminate all different subpopulations.

Glucocorticoid treatment exacerbates mycobacterial infection by decreasing macrophage phagocytosis

To study the functional consequences of the observed GC effects on immune cells, in **Chapter 4** we have performed research on how GCs modulate an infection with *Mycobacterium marinum* (*Mm*), which is a species closely related to *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB) in humans. Infectious complications are one of the side effects of GC therapy resulting from the compromised immune system [38-40]. Although treatment with GCs is associated with a higher risk of developing TB [41, 42], adjunctive GC therapy has been shown to be beneficial for patients suffering from certain types of TB that are associated with inflammatory complications [43-45].

Mm causes a TB-like infection in zebrafish and other cold-blooded animals naturally [46] and zebrafish larvae are widely used to study host-pathogen interactions underlying TB and to investigate potential host-directed therapeutic strategies [47-49]. We found that GC treatment increased the infection level in zebrafish larvae. This increased *Mm* infection upon GC treatment is related to an inhibited phagocytic activity resulting from adecreased transcription level of phagocytosis-related genes in macrophages. When using another intracellular pathogen, *Salmonella* Typhimurium, the GC-inhibited phagocytic activity of macrophages was also observed. Similarly, it has been reported that GCs inhibit the phagocytosis of several *Escherichia coli* strains by PMA-stimulated human monocyte-derived (THP-

1) macrophages and murine bone marrow-derived macrophages (BMDMs) [50]. In macrophages from rats and rheumatoid arthritis patients, GC treatment also inhibits phagocytosis of carbon particles [51, 52]. However, in some studies GC exposure has been reported to enhance the phagocytosis of apoptotic neutrophils by human blood monocyte-derived macrophages, PMA-stimulated THP-1 macrophages [53-55] and mouse alveolar macrophages [56]. These results suggest that the effects of GCs on macrophage phagocytic capacity could mainly be dependent on the particles they encounter and the tissue environment.

The phagocytic activity of macrophages is essential for eliminating harmful components and maintaining homeostasis during inflammation and infection. In our study, the decreased phagocytic activity of macrophages also resulted in a lower level of macrophage cell death due to the *Mm* infection and exacerbated growth of the extracellular fraction of bacteria. We propose that the increased numbers of extracellular bacteria could traverse endothelial barriers directly and grow more rapidly in a less restrictive environment outside macrophages. These results may explain the increased susceptibility to mycobacterial infections induced by GC treatment. As an adjuvant therapy for severe TB, the beneficial effect of GCs was reported to be observed in a subpopulation of patients with excessive inflammation resulting from specific polymorphisms in the *LTA4H* gene, which was modeled in zebrafish by *lta4h* knockdown or overexpression [57, 58]. To further explore the interplay between these effects, we may study the effect of GCs in relation to the *lta4h* polymorphism and the involvement of *lta4h* expression in changes in phagocytosis and bacterial burden induced by GCs. Investigating the effect of GCs at later stages of *Mm* infection may also help to understand the role of GCs in exacerbation of TB.

Encapsulation of glucocorticoids in liposomes enhances their anti-inflammatory effects and reduces their side effects

Chapter 5 describes studies on the targeting of GCs to inflamed tissue by encapsulating them in liposomes. Targeted delivery of drugs using nanoparticles like liposomes is a promising approach to improve the therapeutic ratio of these drugs, through optimization of their pharmacokinetics [59, 60]. Encapsulation of prednisolone phosphate (PLP) in PEGylated liposomes (which contain phospholipids linked to a polymer polyethylene glycol (PEG) chain) has been shown to increase the therapeutic effects of PLP in several animal models for inflammation-related diseases, such as rheumatoid arthritis [61-64], atherosclerosis [65], multiple sclerosis [66] and cancer [67]. However, the occurrence of side effects [68, 69], unwanted off-target accumulation [62, 70] and unexpected lack of anti-inflammatory effect when applied clinically [71] are still obstacles for the development of liposomal GC drugs. In our study, the targeting of PLP encapsulated in liposomes was visualized in zebrafish larvae in which an

inflammatory response was triggered by laser wounding. In addition, both the anti-inflammatory effects and effects on tissue regeneration after tail fin amputation and the systemic activation of a Gr-responsive reporter gene (as a proxy for possible adverse effects) were determined.

We tested two types of liposomes, a PEGylated liposome which was relatively resistant to scavenging, and a macrophage-targeting liposome. In the zebrafish laser wounding model, we observed liposome accumulation near the wounded area for both liposomes. Our results showed that both liposomes enhanced the inhibitory effect of PLP on wounding-induced neutrophilic migration, and that encapsulation using the macrophage-targeting liposome was even more effective than encapsulation in the PEGylated liposome, probably due an increased accumulation of the liposomes near the wound upon delivery by macrophages. The effect of PLP on tissue regeneration was alleviated by encapsulation in both liposomes, and the activation of a Gr-responsive reporter gene throughout the body of the embryo was only reduced by encapsulation in the PEGylated liposome. This could probably be explained by a slower release of PLP from the PEGylated liposomes, which is protected from scavenging and degradation by the PEG chain, leading to lower concentration of ligands available for Gr activation.

Our results suggest that the zebrafish is a useful model for screening different liposomal formulations, since the (dynamics of the) bio-distribution of the liposomes can be assessed, as well as their therapeutic anti-inflammatory effects and effects on processes such as tissue regeneration, exemplifying the side effects of GCs. Using this model, we showed, as a proof-of-principle, that encapsulation in both PEGylated and macrophage-targeting liposomes increases the therapeutic ratio of PLP treatment. The advantage of using zebrafish includes direct observation on the biodistribution of liposomes and the possibility of high-throughput screening, which may promote solving the problem of unwanted off-target accumulation [62, 70]. In addition, the side effects such as repressed corticosterone level and hyperglycemia [68, 69] could also be assessed using the zebrafish model. In future studies, we therefore recommend using this model for optimization of liposomal formulations, as a first screening model to be used for pre-selection of liposomes before they are tested in rodent models and/or clinical studies.

Conclusions

A broad knowledge of the modulatory effects of GCs in the immune system is necessary for the improvement of anti-inflammatory GC therapeutics. Our work demonstrates a general inhibitory effect of GCs on the pro-inflammatory phenotype and the phagocytic activity of macrophages and illustrates an important role for macrophages as a target for anti-inflammatory GC therapy (**Chapter 3, 4**). Indeed,

specific targeting of GCs to macrophages by encapsulation in liposomes increased the therapeutic efficacy of these drugs, although encapsulation in liposomes that are not scavenged had a similar effect (**Chapter 5**). The work in this thesis has added to our understanding of how GCs modulate the innate immune response upon inflammation and may contribute to the improvement of anti-inflammatory therapies by using different zebrafish models for assays to be used in pre-clinical research.

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