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Glucocorticoid modulation of the immune response: Studies in zebrafish

Xie, Y

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Author: Xie, Y.

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Chapter 1

Introduction

Glucocorticoids and the Glucocorticoid Receptor

Glucocorticoids (GCs) are a class of steroid hormones secreted by the adrenal gland. The main endogenous GC hormone in humans is cortisol. Under basal conditions, the level of cortisol keeps a circadian rhythm, peaking in the morning when the diurnal activity phase starts [1, 2]. However, in response to stress, the secretion of cortisol can increase rapidly and this secretion is mainly regulated by the hypothalamic-pituitary-adrenal (HPA) axis [3]. Upon stress, corticotropin releasing hormone (CRH) is secreted by the hypothalamus and promotes the secretion of adrenocorticotrophic hormone (ACTH) in the pituitary, while ACTH in turn stimulates the secretion of cortisol from the adrenal gland [3, 4].

In most vertebrate organisms, GCs such as cortisol exert their function through an intracellular receptor, the Glucocorticoid Receptor (GR) [5]. Like all other steroid receptors, GR belongs the nuclear receptor family and acts as a ligand-activated transcription factor. A schematic overview of the molecular mechanism of GR action is presented in Figure 1. GR usually forms a heterocomplex with a variety of chaperone proteins, including heat shock proteins (hsps) and immunophilins. Its affinity to GCs is dependent on the conformation of the receptor which is induced by the complex in an ATP-dependent manner [6, 7]. Upon ligand binding, the GR heterocomplex translocates to the nucleus, and alterations in the chaperone protein composition of the complex play a fundamental role in GR nuclear import. The recognition of the nuclear location signal (NLS) by importins mediates the translocation across the nuclear pore complex (NPC) with microtubules supporting the movement of the complex [8]. In the nucleus, GR can, as a dimer, bind directly to glucocorticoid response elements (GREs) in the DNA and recruit coregulators, mainly leading to transcriptional activation of various genes, which is the most typical mechanism of transactivation [9-11]. In addition, GR can repress gene transcription when it binds to negative GREs (nGREs) [12]. Moreover, monomeric GRs interact with other transcription factors (like STAT, AP-1) by binding to “composite” response elements, resulting in a positive or negative transcriptional regulation, or tether to other transcription factors (like AP-1, NF- κ B and STAT) and interfere with their activity, thereby positively or negatively modulating the transcription of the genes regulated by these transcription factors [13]. Apart from these genomic effects, GR can also exert immediate and reversible nongenomic effects, regulating signal transduction cascades and cell function through cytosolic GR and membrane-bound GR, by directly influencing the fluidity and composition of membrane, or by interacting with membrane receptors and kinases [14, 15]. As a result, through activation of GR, GCs regulates a wide variety of systems in our body, such as the immune, metabolic, reproductive, cardiovascular and central nervous system, helping the body to cope with stress and maintain homeostasis [16, 17].

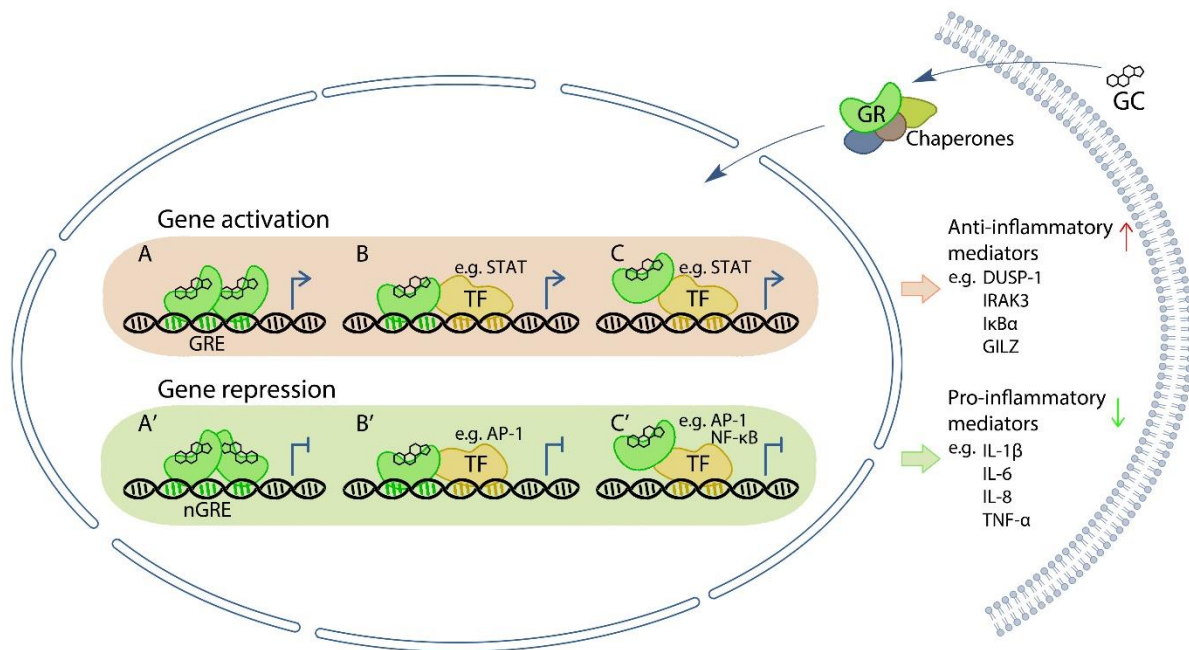


Figure 1. Molecular mechanisms underlying the anti-inflammatory action of glucocorticoids (GCs). GCs diffuse through the membrane freely and bind to the cytoplasmic glucocorticoid receptor (GR) complex, leading to a conformational change of the receptor and an alteration in the composition of the complex, allowing nuclear translocation. GR can activate and repress gene transcription through several mechanisms, including binding to glucocorticoid response elements (GREs) (A) or negative glucocorticoid response elements (nGREs) (A'), or by interaction with other TFs (B, B'), which may involve binding to composite elements or tethering (C, C'). As a result, the transcription of genes encoding anti-inflammatory proteins is upregulated, whereas the transcription of pro-inflammatory genes is downregulated.

Effects of GCs on the immune system

The vertebrate immune system consists of two major components. The first component is the innate immune system, which is composed of the surface barriers, innate leukocytes (such as phagocytes, mast cells, natural killer cells) and the complement system. The second component is the adaptive immune system, which is dependent on the recognition of specific antigens by T-cells and B-cells, and is able to generate memory cells [18]. When the body encounters harmful stimuli, such as invading pathogens, wounding or damage to cells, the immune system will be activated and an inflammatory response is triggered [19, 20]. This response is mainly induced by Pattern Recognition Receptors (PRRs) of the innate immune system, such as Toll-Like Receptors (TLRs), which recognize patterns in molecules frequently found in microbes (Pathogen-Associated Molecular Patterns (PAMPs)), or molecules released by damaged cells (Damage-Associated Molecular Patterns (DAMPs)). Subsequently, immune cells release pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor α (TNF- α), which in turn stimulate the synthesis and release of other inflammatory mediators, including chemokines and prostaglandins [19, 21]. Directed by the chemokine gradients, leukocytes migrate towards the inflamed site to deal with the damaged tissue or invading microbes [22, 23]. These

changes at the molecular level will lead to the five classical symptoms of inflammation: heat, pain, redness, swelling and loss of function. Normally, the inflammatory processes are actively terminated through functional reprogramming of the involved cells, which results in a restoration of homeostasis [19].

Generally, GCs exert anti-inflammatory effects on the immune system. Transcription factors downstream of the TLR signaling pathway, such as NF- κ B and AP-1, which are critical for the initiation of inflammation, can be inhibited by GCs, mainly through tethering mechanisms [13]. Moreover, GCs can upregulate the expression of inhibitors of TLR signaling, such as dual-specificity protein phosphatase 1 (DUSP1) [24], IL-1 receptor-associated kinase 3 (IRAK3) [25], and NF- κ B inhibitors [26, 27]. As a result, GCs inhibit the transcription of downstream pro-inflammatory cytokines and chemokines, which are important for the propagation of inflammation, like IL-1, IL-6, IL-8, TNF and CCL2 (also known as MCP-1) [28, 29]. Due to the decreased level of pro-inflammatory mediators, the extravasation and migration of leukocytes towards the inflamed site is reduced by GCs [30, 31]. In addition, GCs inhibit the antigen presentation by dendritic cells (DCs), T-cell activation, immunoglobulin production by B-cells, and suppress vascular permeability and dilation by repressing the expression of lipid mediators such as eicosanoids and prostaglandins [29]. In the resolution phase of inflammation, GCs induce the expression of Annexin-1, directing neutrophil apoptosis [32], and promote the differentiation of macrophages to an anti-inflammatory (M2) phenotype with high expression of scavenger receptors, which is essential for the clearance of apoptotic cells and debris [33-35]. These effects of GCs enhance the elimination of inflammation and restoration of homeostasis.

Although GCs are renowned for their repressive effects on the immune system, it has become clear that their effects are much more complicated than traditionally thought. Under specific conditions, GCs have been shown to play pro-inflammatory roles rather than the classical anti-inflammatory roles, which is thought to be dependent on the specific cell types, the phase of the immune response and the dose of GCs [36, 37]. For example, GCs can increase the level of TLR2, leading to an increased secretion of critical cytokines in HeLa cells (IL-1 β , IL-8, TNF- α) [38] and in a lung epithelial cell line (IL-6, IL-8) [39]. The expression of a member of the NOD-like receptor family (NLRP3) was also reported to be upregulated by GCs in macrophages, enhancing the secretion of IL-1 β , IL-6 and TNF- α [40]. GCs have also been shown to induce the expression of the purinergic receptor P2Y2R in a microvascular endothelial cell line, resulting in increased IL-6 secretion [41]. Apart from the cell-specificity of the effects, the timing of GC treatment also plays a role in determining the outcome. Prior exposure to GCs enhances the pro-inflammatory response to lipopolysaccharide (LPS) challenge, while post-exposure to GCs suppresses this response [42]. In addition, the dose of GCs can influence their effect since low-dose GC treatment was found to enhance inflammation, whereas a high dose of GCs resulted in

inhibition of inflammation in macrophages [43]. This GC-induced increase in inflammatory signaling may represent a sensitization of cells to inflammatory mediators to establish , a rapid inflammatory activation. In conclusion, the physiological and therapeutic outcomes of GCs result from the complex signaling mechanisms and the treatment conditions.

GCs as immunosuppressive drugs

In 1949, the GC 'Compound E' was used in the Mayo Clinic to treat rheumatoid arthritis (RA) patients and the symptoms of patients were found to be alleviated after treatment [44]. A year later, the Nobel Prize in Physiology or Medicine was awarded to Hench, Kendall and Reichstein for their research on 'hormones of the adrenal cortex' [45]. Since this 'Compound E' (which is generally referred to as cortisone nowadays) was described to alleviate RA, a lot of efforts have been made to synthesize and modify GCs for pharmaceutical purposes [44, 46]. Currently, a variety of synthetic GCs are used clinically, including prednisolone, dexamethasone and beclomethasone, which differ in their solubility, biological half-life and affinity to receptors, and can be administrated via different routes [47, 48]. Due to their well-established immunosuppressive effects, GCs are widely prescribed to treat various immune-related diseases, including asthma, dermatitis, several autoimmune diseases (e.g. multiple sclerosis, RA) and even some cancers (e.g. leukemia) [47, 49]. They have also been applied to treat inflammatory complications of infectious diseases, for example tuberculosis [50-52]. Recently, GCs, in particular dexamethasone, were adopted for treating patients with coronavirus disease 2019 (COVID-19), and were shown to decrease the mortality of patients with severe respiratory complications [53, 54].

Resistance and side effects of GCs

As effective anti-inflammatory drugs, the clinical use of GCs is largely limited by their side effects. Due to their intricate effects on various systems in the whole body, prolonged treatment with GCs may evoke osteoporosis, muscle weakness, hypertension, hyperglycemia and diabetes [55, 56]. The therapeutic immunosuppressive effect of GCs can also lead to infectious complications because of the inhibited function and lower number of immune cells [56-58]. In addition, patients under long-term GC therapy are at risk of developing adrenal insufficiency, which is mediated by the negative feedback loop in the HPA axis [47].

In order to improve the benefit/risk ratio of GC therapy, methods for delivering GCs locally have been adopted to reduce the systemic effects. For example, inhaled GCs may be used in asthma patients to achieve a maximal response in the lungs [59]. Similarly, intra-articular injection for RA, topical creams applied on the skin for dermatological problems and ocular drops for eye conditions induce mainly

local effects [47, 60]. However, side effects are not fully eliminated in these situations, since systemic distribution upon absorption into the bloodstream is often observed [47]. Another method to increase the efficacy of drug delivery at the target site can be achieved by encapsulation of GCs in nanoparticles like liposomes, which can accumulate specifically in the inflamed tissue, probably dependent on the enhanced permeability and retention (EPR) effect, or the phagocytosis by macrophages and their migration towards inflammatory sites [61-67]. A liposome-encapsulated prednisolone phosphate (PLP) formulation containing phospholipids linked to a polymer polyethylene glycol (PEG) chain has shown anti-inflammatory effects in a rabbit model of atherosclerosis [68]. However, when it was tested in a clinical trial, it failed to inhibit inflammation in atherosclerosis patients [69].

Traditionally, it is believed that the side effects of GCs are related to the transactivation activity of GCs, while therapeutic effects mainly result from transrepression [70]. Therefore, novel selective GR agonists or modulators (SEGRAMs) that favor transrepression over transactivation were developed, such as Compound A (CpdA), RU24858, mapracorat and fosdagrocorat [70-73]. It was reported that CpdA effectively suppresses inflammation in mouse models of arthritis and inflammatory polyneuropathies and does not induce hyperinsulinemia and hyperglycemia, which could be related to the CpdA-induced GR conformation that is different from classical GC-bound GR and does not allow receptor dimerization [74, 75]. Indeed, strong evidence exists that GR dimerization is indispensable for the treatment of acute inflammation [76, 77]. However, GR dimerization appears to be essential for the therapeutic effects in some conditions such as septic shock [78], contact allergy [79] and TNF-induced lethal inflammation [80].

Another possible way to establish a better therapeutic ratio for GCs is to modify the structure of existing GCs. For example, anti-CD163-dexamethasone conjugate, designed to target activated macrophages, showed a more potent effect in inhibiting LPS-induced acute phase response in rats compared to non-conjugated dexamethasone, and did not cause any systemic side effects [81]. Other modifications include the conjugation of prednisolone with hydrolysable polyethylene glycol (PEG) which increased the retention time in the lungs of rats [82]. Finally, the addition of γ -lactones and cyclic carbonates can make GCs easily inactivatable by specific enzymes once they enter the blood stream [83].

Besides the side effects of GC therapies, another issue limiting the usage of GCs is the occurrence of resistance to GCs, reflected by a decreased sensitivity and a reduced maximal response, which may be evoked in patients with different diseases, including chronic obstructive pulmonary disease (COPD), asthma and RA [84-86]. Multiple molecular mechanisms have been elucidated to account for this GC resistance. In GC-resistant asthma patients, defective GR ligand binding and nuclear translocation were

observed, which could have resulted from GR phosphorylation by mitogen-activated protein kinase (MAPK) or GR nitrosylation and could be reversed by using kinase inhibitors or nitric oxide (NO) synthase inhibitors [87-89]. Another mechanism of GC resistance is related to the increased expression of the alternative splice variant of GR, GR β , which acts as a dominant-negative inhibitor of the canonical GR α -isoform [90-92]. Increased activation or expression of some pro-inflammatory transcription factors, including AP-1, NF- κ B and STAT5, have also been reported to play a role in GR resistance [93-95]. Other possible mechanisms underlying GC resistance include decreased histone deacetylase 2 (HDAC2) activity which influences the repression of inflammatory gene expression by GCs, and increased expression of the efflux pump P-glycoprotein which transports foreign substances out of the cell [86, 96].

To overcome these problems regarding GC therapy and develop novel GC drugs, more research is required into the molecular and cellular mechanisms of the anti-inflammatory action, the side effects and the occurrence of resistance.

The zebrafish as an animal model for studying GC action

Over the last decades, the zebrafish has emerged as a useful animal model in diverse areas of biomedical research, including immunology, toxicology, cancer, and behavioral studies, adding to its traditional application in research on embryonic development [97, 98]. The zebrafish has a strong reproductive ability and can easily be maintained and bred under laboratory conditions. Moreover, the small size and the optically transparent embryonic and larval stages of the zebrafish make them suitable for microscopic imaging, and the successful sequencing of the zebrafish genome has enabled rapid screening of gene function. In recent years, more genetic tools and other experimental methods have been developed, which has results in the generation of numerous transgenic and mutant fish lines, and applications in drug screening [99, 100].

Like in humans, and in all other teleost fish species, the secretion of GCs in the zebrafish occurs upon stress and in a circadian rhythm. This process is regulated by the hypothalamus-pituitary-interrenal (HPI) axis, the fish equivalent of the HPA axis. The main GC in the zebrafish is also cortisol which acts through the zebrafish orthologue of the GR [101]. Most teleost fish contain two genes encoding a Gr due to a genome duplication that happened during their evolution, which has resulted in the presence of two different Gr proteins. However, in zebrafish only one *gr* gene has been identified [102-104]. The gene organization and protein structure of the zebrafish and human GR share a high level of similarity [104, 105]. Interestingly, both the human and zebrafish gene encode two GR splice variants, the α -isoform and the β -isoform. The canonical GR α -isoform of humans and zebrafish share an overall

similarity of 59.3 % at the amino acid level. The alternative splice variant of the zebrafish Gr, Gr β , which contains a different amino acid sequence at its C-terminal end than Gr α , also highly resembles the human GR β -isoform [104]. Like its human equivalent, the zebrafish Gr β was shown to act as a dominant-negative inhibitor of Gr α in cultured cells and is expressed at a significantly lower level compared to Gr α [104, 106]. However, the dominant-negative activity of Gr β could not be confirmed *in vivo* [106, 107]. All these advantages of the zebrafish model and similarities between the human and zebrafish GC signaling pathway make the zebrafish an excellent *in vivo* model system for GC-related research.

In recent years, many studies on GCs and GR have been performed in zebrafish, advancing our knowledge about their mode of action. Gene knockdown could be achieved in zebrafish by injecting morpholino (MO) antisense oligomers at the 1-cell stage, which inhibits translation or mRNA splicing of target genes [108]. Using a *gr* splice-blocking MO, it was demonstrated that the GC-induced inhibitory effect on zebrafish caudal fin regeneration was dependent on Gr activation [109] and that the induction of the *cripto-1* gene by Gr is involved in this process [110]. Interestingly, using transcriptome analysis it was observed in our laboratory that knockdown of the *gr* gene by a splice-blocking MO altered the expression of a distinct cluster of genes than treatment with the synthetic GC dexamethasone, suggesting that Gr regulates different sets of genes under basal conditions than upon increased activation, e.g. after stress [106]. Upon knockdown of *gr* with a translation blocking MO, multiple developmental defects were observed, demonstrating the crucial role of maternal *gr* transcripts [111], which is supported by the programming function of cortisol in the development of multiple organs including muscle, heart, bone and nervous system [112, 113]. Similarly, cortisol treatment during embryogenesis influences the cardiac performance [114] and the inflammatory responses [115], which mimics maternal stress.

The first *gr* mutant zebrafish line, *gr*^{s357}, was identified from a forward genetic screen based on behavioral assays [116]. This *gr*^{s357} mutant is characterized by a point mutation in the DNA binding domain, leading to defective GRE binding activity, and high cortisol levels [117, 118]. It has been used to study HPI hyperactivation related to depressive behavior and may provide a model to screen for potential anti-depressive drugs [118]. Furthermore, using this line, it has been established that Gr signaling increases the embryonic hematopoietic stem and progenitor cell production [119], affects the white skeletal muscle transcriptome [120], regulates the visual function of the retina [121] and increases anxiety-related behavior in adults [122], emphasizing the essential role of GC/Gr signaling during development. A zebrafish *gr*^{ia30} null mutant was produced using CRISPR/Cas9-mediated gene editing, which has a 5-nucleotide insertion in the *gr* gene, resulting in a frameshift that leads to a premature stop codon truncating the receptor upstream of its DNA binding domain. DBD. Larvae of

this mutant line show unresponsiveness to GCs, and have high cortisol levels, similar to the observations in the *gr^{s357}* line. Interestingly, *gr^{ia30}* larvae do not elicit an inflammatory response upon treatment with dextran sodium sulphate (DSS), whereas the *gr^{s357}* does show this response [123]. Using this mutant, it was revealed that GC regulates the amplitude of the circadian rhythm, the feeding behavior and the synchronization of feeding to circadian rhythm [124-126]. Recently, several mutant zebrafish lines targeting different positions of *gr* or blocking Gr synthesis have also been generated, such as *gr^{ca401}* [127], *gr^{sh543}* [128], *gr³⁶⁹⁻* [129] and *fdx1b^{uob205}* [130]. The generation of these different *gr* mutants helps to dissect the role of GC/Gr on the development, behavior, circadian rhythm, metabolism and inflammatory response. In addition, the generation of reporter zebrafish lines, such as the *Tg(GRE:Luciferase^{sb6})* and *Tg(GRE:GFP^{ia20})* lines, allows for *in vivo* visualization and monitoring of the transcriptional activity of the zebrafish Gr [131, 132].

Since the immune system and the response to inflammation are highly similar between zebrafish and humans, the zebrafish model is also extensively used for studies on the immune system [133-136]. The adaptive immune system matures after three to four weeks [137], which means that the innate immune system can be studied separately during early embryonic and larval stages. Various zebrafish models for human inflammatory diseases have been developed. In the embryo/larval tail amputation-induced inflammation model, GC treatment inhibits the migration of neutrophils towards the wounded site in a Gr-dependent manner, but does not affect macrophage migration [109, 138-140]. This inhibitory effect of GCs on inflammation is associated with a broad attenuation of the transcriptional response [138]. In addition, certain anti-inflammatory genes are upregulated. For example, Gr-induced MAPK phosphatase-1 (Mkp-1) gene expression was demonstrated to be involved, which inactivates JNK, resulting in reduced AP-1-induced transcriptional activation of pro-inflammatory genes [139]. In adult zebrafish, although GCs do not influence tail wounding-induced neutrophil recruitment [141], they were shown to inhibit the expression of pro-inflammatory genes like *il8*, *tnfa* and *il1b* and they do reduce the recruitment of leukocytes towards the wounded area upon brain and heart injuries [142, 143]. Inhibitory effects of GCs on the inflammatory response were also observed in embryonic and larval models for LPS-induced inflammation [144-147], CuSO₄-induced inflammation [148] and DSS-induced enterocolitis [149]. Furthermore, it has been shown that chronic stress-related increases in GC levels during early-life stages can cause a pro-inflammatory adult phenotype which is unable to exert appropriate regulation upon injury or immunological challenge [150]. In summary, zebrafish models for research on GC action are a valuable addition to help understanding the molecular and cellular mechanisms of their signaling pathway, which may accelerate the development of novel improved GC drugs.

Outline of the thesis

The side effects of GC therapy and the occurrence of resistance to this class of drugs are still major limitations for the clinical use of GCs. In addition, significant gaps remain in our understanding of the molecular and cellular mechanisms underlying the immunosuppressive and side effects of GCs. Therefore, more research into the mechanisms of GC action and the development of novel GC therapies is needed. In this thesis, we aim to study the mechanisms underlying the immune-suppressive effects of GCs in the context of wounding-induced inflammation and infection in the zebrafish model. In addition, we exploit this model to study liposome-mediated GC delivery as a therapeutic refinement.

This introductory chapter, **Chapter 1**, provides background information on the cellular and molecular mechanisms of GCs, specifically the actions of GCs in the immune system. As an anti-inflammatory drug, the application of GCs is limited by side effects and drug resistance, of which the mechanisms are discussed. Furthermore, this chapter highlights the recent contribution of zebrafish models to this field, which have been used to investigate the effects of GCs on development, metabolism and the inflammatory response.

Chapter 2 presents a comprehensive overview of the different inflammation models that have been established in zebrafish, including wounding-induced inflammation, chemical-induced inflammation, and mutation-induced inflammation models. The models are increasingly used to investigate the molecular mechanisms underlying the inflammatory response, contributing to our understanding of inflammation and inflammatory diseases. This review chapter also highlights the use of zebrafish inflammation models for screening and exploring novel anti-inflammatory drugs, including GC drugs.

In **Chapter 3**, a detailed analysis is performed on macrophage and neutrophil migration in the zebrafish larval tail amputation model, building further on our previous observation that GCs inhibit neutrophil migration but do not affect the migration of macrophages. Using quantitative PCR, we have dissected differential effects of GCs on chemokines that specifically attract neutrophil or macrophage migration. Based on RNA sequencing data of isolated macrophages, we determined the effect of GCs on wounding-induced transcriptional changes. By in vivo imaging, we have further substantiated the anti-inflammatory effects of GCs based on the morphology and differentiation status of macrophages, using a fluorescent reporter line for *tnfa*, a pro-inflammatory marker.

In **Chapter 4**, we have used the zebrafish *Mycobacterium marinum* (*Mm*) infection model for tuberculosis to study the functional consequences of the effects of GCs on the macrophage phenotype in relation to the defense response of the host. To this end, we studied the effects of GCs on the

severity of *Mm* infection (including the bacterial burden and dissemination in the zebrafish host) and the phagocytic and microbicidal capacity of macrophages. Having identified an inhibitory effect of GCs on phagocytosis, we further investigated this phenotype by assessing the intracellular/extracellular distribution of bacteria, the consequences for macrophage cell death, and the expression levels of genes involved in phagocytosis in macrophages.

The two previous chapters mainly focus on mechanisms of the immune-suppressive effects of GCs, while in **Chapter 5** we aim to set up a screening model for novel GC therapies, in particular the liposome delivery approach of GCs. Through confocal microscopy imaging, we studied the biodistribution of liposomes with different formulations, especially a new macrophage-targeting formulation. Using a laser wounding model in zebrafish larvae, we assessed the anti-inflammatory effect by comparing the effect of a liposome-encapsulated GC, prednisolone phosphate, on wounding-induced neutrophil migration to that of the free drug. Moreover, the drug effects on tail fin regeneration and GRE activation were studied as indications for the severity of side effects. The studies demonstrate the potential of liposome encapsulation of GCs to improve their therapeutic ratio.

Chapter 6 summarizes the results from the research chapters and discusses the findings in the context of current scientific literature.

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