Glucocorticoid-induced attenuation of the inflammatory response in zebrafish

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Abstract

Glucocorticoids are steroid hormones that are secreted upon stress. Their effects are mediated by the glucocorticoid receptor (GR) which acts as a transcription factor. Since the anti-inflammatory activity of glucocorticoids has been well established, they are widely used clinically to treat many inflammatory and immune-related diseases. However, the exact specificity, mechanisms and level of regulation of different inflammatory pathways have not been fully elucidated. In the present study, a tail fin amputation assay was employed in 3-day-old zebrafish larvae to study the immunomodulatory effects of the synthetic glucocorticoid beclomethasone. First, a transcriptome analysis was performed, which showed that upon amputation mainly immune-related genes are regulated. This regulation was inhibited by beclomethasone for 86% of regulated genes. For two immune-related genes, *tlr4bb* and *alox5ap*, the amputation-induced increase was not attenuated by beclomethasone. Alox5ap is involved in eicosanoid biosynthesis, but the increase in LTB4 concentration upon amputation was abolished, and LXA4 levels were unaffected by beclomethasone. Furthermore, we studied the migration of neutrophils and macrophages towards the wound site. Our results show that amputation induced migration of both types of leukocytes, and that this migration was dependent on *de novo* protein synthesis. Beclomethasone treatment attenuated the migratory behavior of neutrophils in a GR-dependent manner, but left the migration of macrophages unaffected. In conclusion, beclomethasone has a dramatic inhibitory effect on the amputation-induced pro-inflammatory gene regulation, and this is reflected in an inhibition of the neutrophil migration, but not the migration of macrophages, which are likely to be involved in inflammation resolution.

Introduction

Glucocorticoids (GCs) regulate a wide range of biological processes, such as our immune response, metabolism, growth, reproduction, vascular tone, bone formation, and brain function (1-6). Because of their anti-inflammatory effects, they are widely used clinically for the treatment of many immune-related diseases, like asthma, rheumatoid arthritis and leukemia (7,8). These effects are mediated by the glucocorticoid receptor (GR), which acts as a ligand-activated transcription factor. In its inactive state, the GR resides within the cytoplasm, and upon GC binding it translocates to the nucleus, where it acts as a transcription factor and orchestrates gene expression (9). GRs may occupy glucocorticoid response elements (GREs) and recruit transcriptional coregulators, which results in a positive or negative regulation of the transcription rate of nearby target genes. GRs may also interact with other transcription factors, e.g. NF-κB or AP-1, and repress their activity (1,2,4,10-12). This mode of action has long been considered the main mechanism by which GCs exert their anti-inflammatory effects, since it results in a downregulation of the expression of a large number of inflammatory mediators (1,2,9-13). However, recent evidence shows that the picture appears to be more complex (14,15). For example, repression of genes is commonly a result of GRE occupancy as well, and GR interaction with transcription factors like NF-κB or AP-1 appears to enhance gene transcription in about half of all cases where this interaction was observed (14).

Many *in vitro* and *in vivo* studies have been performed to elucidate the cellular and molecular pathways within the immune system that are affected by GR signaling (16,17). From these studies it appeared that GCs suppress inflammation by downregulating the expression of a wide variety of genes for pro-inflammatory cytokines (e.g. IL1β, IL6, TNFα), chemokines (e.g.

CCL1, CXCL8), enzymes (e.g. iNOS, COX-2) and adhesion molecules (e.g. ICAM-1), while the gene expression of several anti-inflammatory mediators is upregulated (e.g. DUSP1, IκB, IL10, TGFβ, ANXA1, GILZ) (8,18-20). Furthermore, the synthesis of pro-inflammatory agents like prostaglandins, proteolytic enzymes, free oxygen radicals, and nitric oxide is also inhibited by GCs (18). However, several studies have revealed immunoenhancing effects of GCs, like the induction of Toll-like Receptor (TLR)2 and TLR4, the secretion of MIF (Macrophage Inhibitory Factor) and the upregulation of IL7Ra and serpinA3 (18,21,22).

The aim of the present study is to establish and exploit a robust *in vivo* model to investigate in detail the molecular mechanism of the anti-inflammatory action of GCs. A better understanding of the complex interplay of GR with the different components of the immune response would be of great importance to improve GC therapies, since the clinical use of GCs is currently limited by the deleterious side effects and the occurrence of resistance to GC treatment (23,24).

Over the last decade, the zebrafish has emerged in biomedical research as an important model system for a variety of human diseases (25-27). The zebrafish immune system remarkably resembles that of mammals (28), thus providing an excellent system for modeling various molecular and cellular elements of inflammation such as host-pathogen interactions during infectious diseases and immune cell migration to wound sites (29,30). In the present study, zebrafish larvae are used at three days post fertilization (dpf). At this stage, two types of leukocytes are present which constitute the innate immune system, macrophages and neutrophils (31-35). Cells representing the adaptive immune system, like lymphocytes, do not mature before the second week of zebrafish development (36-38). Furthermore, the zebrafish is used as a model organism for GC research (39-44). Zebrafish have a single GR gene which encodes a GR protein

that upon activation mediates gene transcription in a similar way as its human equivalent (39,42,45-48). Local inflammation can be modeled in zebrafish by amputation of the tail fin of zebrafish larvae (49). Amputation induces the expression of many pro-inflammatory mediators at the wound site and migration of neutrophils and macrophages, towards the site of amputation (46,49-53). Interestingly, it has been demonstrated that this migration is inhibited by glucocorticoid treatment and therefore this model system enables studying of the anti-inflammatory action of glucocorticoids in an *in vivo* situation (46,51).

In the present study we have used the zebrafish tail fin amputation model to study glucocorticoid effects on changes in gene expression at the whole transcriptome level and associated leukocyte migration. Our results demonstrate that tail fin amputation affects the expression of a wide variety of genes, among which many inflammation-related ones, and that glucocorticoid treatment attenuates the vast majority of these changes. In contrast, glucocorticoid treatment specifically inhibits the migration of neutrophils towards the wounded area, but leaves macrophage migration unaffected.

Materials & Methods

Zebrafish, husbandry & egg collection

Zebrafish were maintained and handled according to the guidelines from the Zebrafish Model Organism Database (ZFIN, http://zfin.org) and in compliance with the directives of the local animal welfare committee of Leiden University. Fertilization was performed by natural spawning at the beginning of the light period and eggs were raised at 28.5°C in egg water (60µg/ml Instant Ocean sea salts supplemented with 0.0025% methylene blue (GUUR)). The gr^{s357} mutant line (previously described by Ziv et al. (54) was provided by Dr. H. Baier (Max Planck Institute of Neurobiology, Martinsried, Germany).

Tail amputation &chemical treatments

Three-day-old embryos were anesthetized in egg water containing 0.02% buffered aminobenzoic acid ethyl ester (tricaine, Sigma) and aligned in Petri dishes coated with 2% agarose for subsequent partial amputation of the tail fin as shown in Fig.1A. Amputation was performed using a 1mm sapphire blade (World Precision Instruments) using a Leica M165C stereo-microscope and a micromanipulator. Amputated and non-amputated embryos were pretreated for 2h with either 25µM beclomethasone (Sigma) or vehicle (0.05% DMSO) prior to amputation and again for a specified period of time after amputation. The relatively high dose of beclomethasone was chosen based on studies by Mathew et al. (51), who showed this dose to be maximally effective in zebrafish. Cycloheximide treatment (50 µg/ml, Sigma) was performed similarly. For gene expression analysis samples were collected in TRIzol® reagent (Invitrogen), for ELISA

samples were snap frozen in liquid nitrogen, and for migration studies samples were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and stored at 4°C.

RNA isolation & cDNA synthesis

Total RNA was extracted using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions (Invitrogen). RNA was dissolved in water and denatured for 5min at 60°C. Samples were treated with DNAse using the DNA-*free*™ kit (Ambion). For microarray analysis, RNA was further purified using the RNeasy MinElute™ Cleanup kit from Qiagen and its integrity was checked with a lab-on-chip analysis using the 2100 Bioanalyzer (Agilent Technologies). For subsequent cDNA synthesis, 1µg of total RNA was added as a template for reverse transcription using the iSCRIPT™ cDNA Synthesis Kit (Biorad).

Microarray design

A 4x180k microarray chip platform (customized by Agilent Technologies, (Design ID:028233)) was used in this study. This array consists of all probes already present in an earlier 45.219 custom-made array (55), and another 126.632 newly designed zebrafish probes had been added as described in (56). A total of 16 samples (4 experimental groups from 4 replicate experiments) were processed for transcriptome analysis and were hybridized against a common reference sample, consisting of a mixture of all samples used in this study.

Microarray amplification & labeling

Amplification and labeling of RNA was performed at the MicroArray Department (MAD) of the University of Amsterdam (Amsterdam, The Netherlands). Per sample, 0.5µg total RNA was

amplified and combined with Spike A according to the Agilent Two-Color Microarray-Based Gene Expression Analysis kit (Agilent technologies). As a common reference sample an equimolar pool of all test samples was made and 0.5µg samples were amplified similarly as the test samples with the exception that Spike B was used. Amino-allyl modified nucleotides were incorporated during the aRNA synthesis (2.5mM of each GTP, ATP, UTP (GE Healthcare), 0.75mM CTP (GE Healthcare), 0.3mM AA-CTP (TriLink Biotechnologies)). Synthesized aRNA was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit (Omega Bio-Tek). The quality was inspected on the BioAnalyzer (Agilent Technologies) with the Agilent RNA 6000 kit (Agilent Technologies). Test samples were labeled with Cy3 and the reference sample was labeled with Cy5. Five µg of aRNA was dried out and dissolved in 50mM carbonate buffer pH 8.5. Individual vials of Cy3/Cy5 from the mono-reactive dye packs (GE Healthcare) were dissolved in 200µl DMSO. To each sample, 10µl of the appropriate CyDye dissolved in DMSO was added and the mixture was incubated for 1h. Reactions were quenched with the addition of 5µl 4M hydroxylamine (Sigma-Aldrich). The labeled aRNA was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit. Yields of aRNA and CyDye incorporation were measured on the NanoDrop ND-1000.

Microarray hybridization, scanning & data processing

Each hybridization mixture was made up from 825ng Test (Cy3-labeled) and 825ng Reference (Cy5-labeled) material. Hybridization mixtures were using the Agilent Two-Color Microarray-Based Gene Expression Analysis kit according to the manufacturer's instructions (Agilent technologies). The samples were loaded onto the microarray chips and hybridized for 17h at 65°C. Afterwards the slides were washed and scanned (20 bit, 3µm resolution) in an ozone-free

room with the Agilent G2505C scanner. Data was extracted with Feature Extraction (v10.7.3.1, Agilent Technologies) with the GE2_107_Sep09 protocol for two-color Agilent microarrays. The Agilent output from the 16 hybridizations was then imported into the Rosetta Resolver 7.2 software (Rosetta Biosoftware, Seattle, Washington) and subjected to a factorial design with a re-ratio with common reference application. Data analysis was performed setting cutoff for the p-value of $<10^{-10}$ and for fold change of either >2 or <-2. The raw data were submitted to the Gene Expression Omnibus (GEO) database under accession number GSE69444.

Gene Ontology analysis

Gene ontology analysis of the microarray results was performed as described previously (44). As a starting point, clusters of genes were analyzed using the online functional classification tool DAVID (http://david.abcc.ncifcrf.gov/summary.jsp). In addition, for genes not classified by DAVID, information was gathered on their function (using the websites GeneCards (http://www.genecards.org/), NCBI (http://www.ncbi.nlm.nih.gov/gene), Genetics Home Reference (http://www.ncbi.nlm.nih.gov/gene) and Wikipedia (http://en.wikipedia.org/wiki/). Using this information, all genes were classified in one of the categories assigned by DAVID, or in a new category.

Quantitative Polymerase Chain Reaction (qPCR)

QPCR analysis was performed using the MyiQ Single-Color Real-Time PCR Detection System (Biorad). PCR reactions were performed in a total volume of 25µl containing 6.5µl diluted cDNA, 1µl forward and reverse primer (10µM) and 12.5µl of 2x iQ™ SYBR® Green Supermix (Biorad). Cycling conditions were 95°C for 3min, followed by 40 cycles of 15sec at 95°C, 30sec

at 60°C and 30sec at 72°C. Ct values (cycle number at which a threshold value of the fluorescence intensity was reached) were determined for each sample. A dissociation protocol was added, determining dissociation of the PCR products from 65°C to 95°C, allowing discrimination of specific products. In all qPCR experiments, a water-control was included. Data shown are means (± s.e.m.) of four individual experiments. In each experiment, cDNA samples were assayed in duplicate. Sequences of all primers used for qPCR analysis are presented in Suppl. Table 1, and a phylogenetic tree showing all zebrafish *arachidonate lipoxygenase (alox)* genes is shown in Suppl.Fig.1.

LTB4 and LXA4 ELISA

For each data point, six samples (20 larvae each) were collected. All liquid was removed and samples were snap frozen in liquid nitrogen. For ELISA, 250µl 1x PBS and 0.2 SSB02 stainless steel beads (Next advance) were added to each sample. Larvae were homogenized using the Bullet blender® (Next advance) for 3min on speed 8. The samples were then centrifuged at 3500 rpm for 5min. The supernatant was collected and centrifuged again at 5000 rpm for 5 min after which the supernatant was collected again. An LTB4 ELISA kit (Enzo Life Sciences), and LXA4 ELISA kit (Cloud-Clone) were used according to the manufacturer's instructions. All samples were measured in duplicate (100 µl used per measurement), and the data from the duplicates was averaged. Data shown are the averages (± s.e.m.) from six replicates.

Myeloperoxidase staining and whole mount immunohistochemistry for visualization of macrophages and neutrophils

Embryos were fixed in 4% PFA overnight at 4°C and following washes with PBS containing 0.1% Tween 20 (PBST), the Myeloperoxidase (mpx) activity was detected using the Leukocyte Peroxidase kit (Sigma) according to the manufacturer's instructions. Mpx staining was always performed prior to L-plastin immunohistochemistry. For this purpose, embryos were washed in PBST, gradually dehydrated with methanol in PBS and stored in 100% methanol overnight at 4°C. The next day embryos were rehydrated with graded series of methanol in PBS containing 0.8% Triton X-100 (PBS-TX) and incubated with 10μg/ml Proteinase K (Roche) for 10min at 37°C. Embryos were then incubated in PBS-TX blocking buffer (containing 1% BSA) for 2h at RT and subsequently in blocking buffer containing a rabbit anti-L-plastin polyclonal antibody (provided by Dr. A. Huttenlocher (57), 1:500 dilution) overnight at 4°C. Following washes with PBS-TX, embryos were incubated again in blocking buffer for 1h at RT prior to incubation with goat anti-rabbit Alexa Fluor® 568 dye–labeled secondary antibody (Invitrogen) for 2h at RT (1:200 dilution in blocking buffer).

Imaging of the embryos was performed using a Leica MZ16FA fluorescence stereo-microscope supported by the LAS version 3.7 software. Macrophages were detected based on the red fluorescent labeling by the immunohistochemistry and neutrophils were detected based on their dark brown appearance as a result of the Mpx assay (although they are stained by both methods, the L-plastin immunolabeling is hard to detect in these cells due to the dark staining of the Mpx assay). To determine the number of cells that had migrated to the wounded area, the cells posterior to the caudal vein were counted (see also Suppl.Fig.6). Data shown are means (±

s.e.m.) of three individual experiments. In each experiment, treatment groups consisted of at least 20 larvae.

Statistical analysis

Statistical analyses (one- or two-way ANOVAs with Bonferroni post-hoc tests) were performed using the GraphPad Prism version 4.00 (GraphPad Software, La Jolla, USA).

Results

Analysis of GC effects on the transcriptional response to wounding using the zebrafish tail fin amputation assay

In order to study the anti-inflammatory action of GCs in zebrafish, we set up a tail fin amputation assay using 3 day post fertilization (dpf) larvae that were exposed to either vehicle or the synthetic GC beclomethasone (25μM) for 2h. Tail fins were amputated and vehicle or beclomethasone treatment was continued. Total RNA samples were collected at 4 h post amputation (hpa). This way, four experimental groups were generated: control treated with vehicle (con/vehicle), amputated treated with vehicle (4hpa/vehicle), control treated with beclomethasone (con/beclo), and amputated treated with beclomethasone (4hpa/beclo). The samples were used in a microarray experiment to analyze the transcriptional response to wounding as well as how this response was affected by beclomethasone treatment.

The effects of amputation on gene transcription

First, we identified 380 probes to be significantly regulated due to amputation (comparison con/vehicle vs. 4hpa/vehicle). Gene annotation demonstrated that these probes corresponded to 279 genes, of which 201 were upregulated and 78 downregulated due to amputation. Gene ontology analysis revealed that 31 genes in this cluster were involved in the immune system. Of these 31 genes, 3 encoded anti-inflammatory proteins, 9 were involved in chemokine or cytokine signaling, and 4 were involved in prostaglandin or leukotriene signaling. Furthermore, 29 genes encoding transcription factors (or other proteins involved in transcriptional regulation) were present in this amputation-regulated cluster. The two most strongly upregulated transcription factor genes (*fos* and *atf3*) are both members of the AP-1 transcription factor family, and another

member of this family (*mafk*) was upregulated as well. Several other genes encoding transcription factors known to activate immune-related genes, like *irf9* and *stat3* were also upregulated. Genes involved in metabolic processes also formed a large gene ontology group within this cluster, and were represented by 25 genes. Of these genes, 8 were involved in carbohydrate metabolism, 14 in protein metabolism and 2 in lipid metabolism. An overview of the gene ontology analysis is presented in Fig.1B, and detailed information is presented in Suppl.Table2.

The effects of beclomethasone on gene transcription

Subsequently, we investigated which genes responded to beclomethasone treatment in non-amputated larvae. A cluster of 927 probes was identified to be significantly regulated due to beclomethasone treatment (comparison con/vehicle vs. con/beclo). Gene annotation demonstrated that these probes corresponded to 506 genes (Fig.1B), of which 420 were upregulated and 86 downregulated due to beclomethasone. Gene ontology analysis showed that 90 genes in this cluster were involved in metabolic processes, of which 19 in the metabolism of carbohydrates, 28 in protein metabolism, and 13 in lipid metabolism. Other gene ontology groups overrepresented in this cluster were those containing genes involved in membrane transport (37 genes), cell cycle and apoptosis (30), and genes encoding transcription factors (30). An overview of the gene ontology analysis of this cluster is presented in Suppl.Fig.2A and B, and detailed information is presented in Suppl.Table3. A number of 32 genes were present in both the amputation- and the beclomethasone-regulated cluster of genes (Fig.1C and Suppl.Table3). This cluster may represent the genes that are regulated upon amputation due to increased cortisol levels.

The effects of amputation and beclomethasone on gene transcription

Next, we were interested in genes that were significantly changed due to the combination of amputation and beclomethasone treatment (comparison con/vehicle vs. 4hpa/beclo). We identified 1075 probes to be significantly regulated and gene annotation revealed that these probes corresponded to 594 genes, of which 459 were upregulated and 135 were downregulated. Gene ontology analysis demonstrated that this cluster very much resembles the beclomethasone-regulated gene cluster. For example, the largest gene ontology group were the genes involved in metabolism (Suppl.Fig.2A and B and Suppl.Table4), and 315 genes from the cluster of 506 beclomethasone-regulated genes were present in this cluster as well (Fig.1C). In contrast, only 61 genes from the cluster of 279 amputation-regulated genes were present in this cluster (Fig.1C). Apparently, gene regulation by amputation is attenuated by beclomethasone treatment.

To study how beclomethasone changes the amputation-induced changes in gene expression, we plotted the level of regulation by amputation and beclomethasone (comparison con/veh vs. amp/beclo) against the regulation by amputation (comparison con/veh vs. amp/veh) for all probes significantly regulated upon amputation (Fig.2). The resulting scatter plot shows that of all probes regulated by amputation, 86% shows an attenuation of this regulation upon amputation in the presence of beclomethasone. This indicates that beclomethasone has a dramatic inhibitory effect on the amputation-induced changes in gene expression, affecting almost the entire transcriptional response to amputation. For comparison, a similar plot was made in which the level of regulation by amputation and beclomethasone (comparison con/veh vs. amp/beclo) was plotted against the regulation by beclomethasone (comparison con/veh vs. con/beclo). This plot (Suppl.Fig.3) shows that the regulation by beclomethasone was attenuated

upon amputation and beclomethasone treatment in only 62% of probes. Thus, the effect of beclomethasone on amputation-induced changes is much stronger than the effect of amputation on the total group of beclomethasone-regulated genes.

The regulation of immune system-related genes by amputation and beclomethasone was subsequently studied in more detail. Of the 31 immune-related genes that were regulated by amputation, we plotted the regulation by amputation (con/veh vs. amp/veh), beclomethasone (con/veh vs. con/beclo), and the combination of amputation and beclomethasone (con/veh vs. amp/beclo). As expected, the results show that most amputation-induced changes in immune gene expression are attenuated upon amputation in the presence of beclomethasone (Fig.3). By means of qPCR, the regulation of 4 immune-related genes was verified (Suppl.Fig.4). Additionally, we plotted the regulation of the 29 transcription factor genes that were observed to be induced by amputation (Suppl.Fig.5). The induction of only 6 transcription factor genes was resistant to beclomethasone treatment. Of the 23 other transcription factor genes (among which many known to have pro-inflammatory action) the induction was attenuated by beclomethasone. For 4 immune-related genes the induction upon amputation was not attenuated by beclomethasone treatment. Of these 4 genes, 2 encoded anti-inflammatory proteins (*cd22* and *anxa1a*), and 2 encoded pro-inflammatory proteins (*alox5ap* and *tlr4bb*).

The effects of amputation and beclomethasone on leukotriene biosynthesis

The observed regulation of the *alox5ap* (*arachidonate 5-lipoxygenase-activating protein*) gene is particularly interesting since Alox5ap activates the Alox5 protein. Alox5 is known to be involved (together with Leukotriene A4 hydrolase (Lta4h)) in the biosynthesis of Leukotriene B4 (LTB4), which plays an important role as a chemoattractant for leukocyte migration

(biosynthesis pathway shown in Fig.4A). Therefore, it was studied whether the observed *alox5ap* gene regulation was translated into altered LTB4 levels. An LTB4 ELISA was performed on homogenates taken from control and amputated larvae in the absence and presence of beclomethasone at 4hpa. The results show an almost three-fold increase in LTB4 concentration upon amputation, and interestingly this increase is abolished in the presence of beclomethasone (Fig.4B).

Subsequently, we studied whether transcriptional regulation of the expression of enzymes involved in the LTB4 biosynthesis pathway could explain the alterations in LTB4 levels. For this purpose, we determined mRNA levels for *alox5ap*, *alox5a*, and *lta4h* using qPCR (*alox5b.1-3* mRNA levels were too low to be detected by qPCR). The regulation of the *alox5ap* gene as observed in the microarray was verified (Fig.4C). Furthermore, *alox5a* and *lta4h* mRNA levels were decreased by amputation, and beclomethasone increased the expression of *lta4h* (Fig.4D and E). Thus, although the amputation-induced increase in *alox5ap* mRNA expression (observed in the microarray and confirmed by qPCR) was not inhibited by beclomethasone, the increase in LTB4 levels upon amputation was blocked by beclomethasone treatment. This discrepancy could not be explained by the regulation of other genes involved in the LTB4 biosynthesis.

Alternatively, beclomethasone may regulate eicosanoid biosynthesis downstream of LTA4 as well, and could for example stimulate conversion of LTA4 to lipoxinA4 (LXA4) (pathway shown in Fig.5A). An LXA4 ELISA was performed to test this hypothesis. The results showed that amputation decreased the LXA4 concentrations and that beclomethasone did not affect this decrease (Fig.5B), thereby falsifying the hypothesis. Expression of three genes involved in this pathway, *alox12*, *alox12b* and *alox15b*, determined by qPCR could explain the

LXA4 data (Figs.5C-D). The qPCR results showed that amputation decreases the expression of these genes and this decrease is only affected by beclomethasone for *alox12*.

The tail fin amputation assay to study GC effects on leukocyte migration

Previous studies in zebrafish larvae have shown that leukocytes migrate to wound sites, representing an inflammatory response, and that this response is impaired upon treatment with GCs (46,51). In order to study this in more detail, tail fins were amputated upon vehicle or beclomethasone treatment as described above. Larvae were fixated at 0, 2, 4, 8, 16 and 24hpa and neutrophils and macrophages were labeled and counted. To determine the number of cells that had migrated to the wounded area, cells posterior to the caudal vein were counted (area indicated by the red box in Fig.6A).

In order to label the populations of neutrophils and macrophages in 3dpf larvae we employed Myeloperoxidase (Mpx) histochemistry, followed by immunofluorescent labeling of L-plastin. At this stage of development two populations of leukocytes are present: neutrophils, which are Mpx- and L-plastin-positive, and macrophages, which are Mpx-negative and L-plastin-positive (31,33-35,58). Although neutrophils are stained by both methods, the L-plastin immunofluorescence is hard to detect in these cells due to the dark staining of the Mpx assay which hides the fluorescent signal. Using this approach, the number of macrophages and neutrophils were determined in the tail fins at different time points upon amputation. The results showed that macrophages migrated more to the posterior end of the tail where they appeared to line up at the actual wound site, whereas neutrophils were more randomly located in the vicinity of the wound (Fig.6B and 6C).

The effect of GC treatment on amputation-induced leukocyte migration

The results of the experiment described above revealed that both neutrophils and macrophages migrate towards the wounded area, but that their migratory behavior and response to beclomethasone are remarkably different. Analysis of our data revealed a migratory response of macrophages over time (as shown by a significant effect of time in an ANOVA ($p<0.001$)), but no effect of beclomethasone treatment was observed (Fig.7A). Macrophage migration increased rapidly after amputation, especially in the first 2 hours (9.7 ± 0.2 at 2hpa versus 4.0 ± 0.1 0hpa), and no decline was observed until 24hpa. For neutrophils, a migratory response was observed as well, which was inhibited by beclomethasone treatment (as shown by significant effects of time and beclomethasone treatment (both $p<0.001$)). Neutrophil migration reached a peak at 4hpa (7.4 ± 2.0 cells compared to 0.6 ± 0.1 at 0hpa) and rapidly decreased after this time point to 3.4 ± 0.6 at 8hpa after which it remained stable at this level until 24hpa (Fig.7B). Beclomethasone treatment had a significant inhibitory effect on the neutrophil migration at 4hpa (4.3 ± 0.4 cells in the presence of beclomethasone). Based on these results, we concluded that both neutrophils and macrophages migrate towards wound sites, but that beclomethasone exhibits an inhibitory effect only on neutrophil migration. To establish whether beclomethasone specifically affects the migration of neutrophils rather than their total number, cells in the entire tail fin area (posterior to the yolk extension) were counted. The results of these countings did not show any significant difference in the number of neutrophils between vehicle- and beclomethasone-treated larvae upon amputation (Suppl.Fig.7), indicating a specific effect of beclomethasone on the neutrophil migration towards the wound site.

In order to study whether the inhibition of neutrophil migration by beclomethasone was mediated by the GR, a mutant line gr^{s357} was used which has a point mutation in the gene

encoding the GR. This mutant receptor has been shown in *in vitro* studies to be unable to regulate gene transcription (54). Using this mutant line, neutrophil migration at 4hpa was determined in the absence and presence of beclomethasone. The results showed that beclomethasone had no effect on neutrophil migration in the mutant larvae (Fig.7C), indicating that the beclomethasone effect on the migration of neutrophils is mediated by the GR.

Looking for differences between neutrophil and macrophage migration which may help to explain the difference in glucocorticoid responsiveness, we studied whether this migration was dependent on *de novo* protein synthesis. For this purpose, we administered the protein synthesis inhibitor cycloheximide and studied the effect of this treatment on macrophage and neutrophil migration at 4hpa (Fig.7D). Cycloheximide appeared to significantly inhibit both the macrophage and the neutrophil migration (as shown by a significant effect of treatment in an ANOVA ($p=0.007$ and $p=0.013$ respectively)). Apparently, the migration of both macrophages and neutrophils upon amputation depends on *de novo* protein synthesis.

In summary, macrophage migration appears to be dependent on *de novo* protein synthesis and is not inhibited by beclomethasone treatment. Therefore, macrophage migration must be dependent on the upregulation of genes of which this upregulation is not inhibited by beclomethasone. The most likely candidates are the four immune-related genes *cd44*, *alox5ap*, *anxa1* and *tlr4bb*.

Discussion

In the present study, we have used zebrafish larvae in order to study the effects of GC signaling on the inflammatory response to tail fin amputation, both at the molecular and the cellular level. First, we looked for transcriptional changes at 4hpa and we identified 279 genes of which the expression was significantly altered upon amputation. The largest gene ontology group in this cluster of genes was formed by genes involved in the immune system, indicating that many of the observed changes are related to the induction of an inflammatory response. In a similar study by Yoshinari et al. (59), in which 2dpf embryos were tail fin amputated and samples were collected at a much later time point (16hpa), transcriptome analysis revealed that the largest fraction of regulated signaling routes were metabolic pathways (40%) and only a small fraction (2%) of signaling cascades regulated were immune-related. Thus, it appears that at 4 hours after injury, immune-related pathways are heavily activated at the transcriptional level, while 12 hours later amputation-induced changes in gene expression no longer reflect an inflammatory response. This is in line with the observed decline in neutrophil migration after 4hpa in our study. The second largest group was formed by genes encoding transcription factors, encompassing members of the AP-1 family and several other pro-inflammatory transcription factors.

In contrast, in the presence of beclomethasone the transcriptional response to amputation is dramatically inhibited. From the 279 genes regulated by amputation, only 61 were still significantly regulated in the presence of beclomethasone, and for 86% of all amputation-regulated probes an attenuated response to amputation was observed in the presence of beclomethasone. It must be noted that our data show that in general the transcriptional responses to tail fin injury are not completely blocked by beclomethasone, but that they are dampened.

When we focused on the regulation of immune-related genes, it was found that the amputation-induced regulation of only 4 genes was not attenuated by beclomethasone. Two of those genes, *cd22* and *anxa1a*, are known to encode anti-inflammatory genes, but the other two, *tlr4bb* and *alox5ap*, encode proteins considered to be pro-inflammatory.

In human cells, GCs have been shown to alter TLR signaling at different levels (60). The expression of the human *tlr4* gene (like the *trl2* gene) has been shown to be positively regulated by GCs in multiple human cell types *in vitro* (21,61). However, since GCs suppress the downstream signaling of these receptors, e.g. by inducing MKP-1 and GILZ/TCS22D1 or inhibiting transcription factors like AP-1, NF-κB and IRF (60), it has been argued that GCs ready the innate immune system by increasing the expression of TLRs, but repress inflammation by inhibiting the downstream signaling of these receptors (16). TLR ligands have been shown to stimulate cortisol secretion in mouse and human adrenal cells, which is abolished in TLR4-deficient mice. It has therefore been suggested that the induction of *tlr2* and *tlr4* in the adrenal glands by GCs serves as a positive feedback loop, resulting in an increased cortisol release upon exposure to TLR ligands, which will eventually elicit mainly anti-inflammatory effects (60).

Alox5ap is the activating protein for the enzyme alox5 which catalyzes the conversion of arachidonic acid (AA) into 5(S)-hydroperoxyeicosatetraenoic acid (5-HPETE) and LTA4 that can further be converted into LTB4, which plays an important role in the inflammatory response by acting as a chemoattractant for leukocytes. In several human and rat cell types, the expression of Alox5 and/or Alox5ap has been shown to be increased at the mRNA and protein level by dexamethasone treatment (62-65). However, the effect of GC treatment on the synthesis of pro-inflammatory eicosanoids like LTB4 is less clear. In several *in vivo* and *ex vivo* studies on cells from human asthma patients, either no effect of GC treatment or a decrease in the concentration

of eicosanoids like LTB4 was observed (66-68). In line with these data, we found that the amputation-induced increase in LTB4 concentration was inhibited by beclomethasone, although the steroid did not clearly affect the transcriptional regulation of proteins involved in LTB4 biosynthesis. We also studied whether GCs stimulate conversion of LTA4 to lipoxinA4 (LXA4), an anti-inflammatory lipid which could contribute to the resolution of the inflammatory response (69,70). It was found that GCs did not affect LXA4levels, and did not have a clear effect on the mRNA levels of genes involved in LXA4 biosynthesis. Apparently, the LXA4 pathway is not a target for GCs, whereas LTB4 induction is inhibited by GCs.

Finally, we examined the effect of GC treatment on the migration of leukocytes towards injured sites. Our analysis showed that beclomethasone treatment had a significant inhibitory effect only on the migration of neutrophils. Hence, the zebrafish model recapitulates the inhibitory effects of glucocorticoids on neutrophil migration towards inflamed tissues, that have been well established in mammalian models (71). However, macrophage migration was not inhibited by beclomethasone, in line with previously observed GC effects on leukocytes in 3dpf zebrafish larvae that were shown to be specifically suppressive regarding the recruitment of neutrophils but not of macrophages (51). It must be noted that macrophages are not a homogeneous cell population, but rather encompass distinct phenotypes. Macrophages with pro-inflammatory activities are generally called M1 and those displaying anti-inflammatory action, thereby encouraging tissue repair, are called M2 (72). Interestingly, it has been shown that GC exposure induced a gene expression profile in human monocytes in which not only expression of pro-inflammatory genes was inhibited, but moreover expression of anti-inflammatory genes was induced (73). GC treatment has been shown to induce a highly phagocytic monocyte-derived macrophage phenotype, characterized by an increased expression of the scavenger receptor

493 CD163 (73,74). We therefore suggest that the lack of effect of beclomethasone on macrophage
494 migration should not be interpreted as a pro-inflammatory pathway that is resistant to GC
495 treatment. However, GCs may induce differentiation of these macrophages towards an anti-
496 inflammatory phenotype, which may contribute to the resolution of the inflammation (75).
497 Interestingly, in a recent study it has been shown that Anxa1 is able to recruit monocytes, by
498 signaling through ALX/FPR2, which is the receptor for LXA4 (76). This suggests that the
499 amputation-induced upregulation of *anxa1* in our study which is not inhibited by beclomethasone
500 may play an important role in the chemoattraction of macrophages.

501 In summary, the zebrafish embryonic model of tail fin amputation and GC treatment
502 constitutes a suitable system for studying GR signaling with respect to the innate immune
503 response. In our model GCs appear to have a suppressive effect on the large majority of changes
504 in gene transcription at 4hpa, which are mainly pro-inflammatory in nature, and this suppressive
505 effect is reflected in a decreased neutrophil migration after 4hpa. Macrophage migration is not
506 inhibited by GC treatment, and this migration may be a result of Anxa1 upregulation and
507 increased production of anti-inflammatory eicosanoids. As a result, these macrophages may
508 rather act anti-inflammatory, thereby resolving inflammation.

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Figure legends

Figure 1. A. The tail fin amputation assay. Schematic drawing of a zebrafish larvae at 3dpf, indicating the site of the tail fin amputation (red line). **B.** Analysis of microarray experiment. Gene ontology groups represented in the clusters of genes regulated upon amputation. The results show that amputation mainly regulated genes involved in the immune system, genes encoding transcription factors, and genes involved in metabolism. Details on individual genes are presented in Suppl.Table2. **C.** Venn diagram showing overlaps between clusters of genes significantly regulated by amputation (amp), beclomethasone (beclo) and the combined amputation/beclomethasone treatment (amp+beclo). The diagram shows that there is a large overlap between the cluster of beclo-regulated genes and amp+beclo-regulated genes, but very little overlap between the amp-regulated cluster and the amp+beclo-regulated cluster. Data analysis was performed setting cutoffs for the p-value of $<10^{-10}$ and for fold change of either >2 or <-2

Figure 2. Scatter plot showing the effect of beclomethasone treatment on amputation-induced alterations in gene expression. For all 2539 probes showing significant regulation upon amputation (comparison con/vehicle vs. 4hpa/vehicle, cutoff for the p-value of $<10^{-10}$ and no cutoff for fold change), the fold change due to beclomethasone and amputation treatment (con/vehicle vs. 4hpa/beclo) was plotted as a function of the fold change due to amputation (con/veh vs. 4hpa/veh). The grey dashed line indicates the point at which beclomethasone treatment does not affect amputation-induced changes. Of the 2539 probes showing regulation by amputation (upregulation at right side of y-axis, downregulation at left side of y-axis), 86% shows an attenuation of this regulation in the presence of beclomethasone (indicated by red

728 markers, probes of which the regulation is not attenuated by beclomethasone are indicated by
729 green markers). These results show that in the vast majority of cases beclomethasone dampens
730 the effects of amputation on gene expression.
731
732 **Figure 3.** Regulation of genes involved in the immune system, determined using microarray
733 analysis. For all 31 genes of which at least one probe was regulated significantly upon
734 amputation, the average fold change due to amputation (amp, black bars), beclomethasone
735 (beclo, black bars) and the combined amputation/beclomethasone treatment (amp+beclo, grey
736 bars) was determined by averaging the fold change for all probes representing this gene present
737 on the microarray. The results show that beclomethasone dampens the amputation-induced
738 expression of 27 genes, but for 4 genes (indicated by grey boxes) amp+beclo treatment results in
739 higher fold change compared to amp treatment.
740
741 **Figure 4. A.** Leukotriene B4 (LTB4) biosynthesis pathway. Arachidonic acid (AA) is converted
742 into 5(S)-hydroperoxyeicosatetraenoic acid (5-HPETE) by Arachidonate 5-lipoxygenase
743 (Alox5). In zebrafish, four genes (*alox5a*, *alox5b.1-3*) encode four different Alox5 isoforms. 5-
744 HPETE is converted into LTA4, which can be converted into LTB4 by Leukotriene A4
745 hydrolase (LTA4H). **B.** Whole body LTB4 concentrations measured in 3dpf larvae by ELISA.
746 Statistical analysis (ANOVA) showed a significant increase upon amputation only in the vehicle-
747 treated groups. An interaction between amputation and beclomethasone treatment was observed
748 (p=0.01). **C.** Validation of *alox5ap* gene regulation by qPCR. Statistical analysis showed that
749 *alox5ap* mRNA expression was significantly altered by amputation (p=0.04), and that there was
750 no effect of beclomethasone treatment (and no interaction between amputation and

beclomethasone treatment). **D.** Expression levels of *alox5a* determined by qPCR. A significant effect of amputation was observed. **E.** Expression levels of *lta4h* determined by qPCR. A significant effect of both amputation and beclomethasone treatment was observed. * Statistically significant difference compared to control treatment (Bonferroni post hoc comparison, $p<0.01$).

Figure 5. A. Lipoxin A4 (LXA4) biosynthesis pathway. LXA4 can be synthesized from LTA4. In zebrafish, three genes encode enzymes that may be involved in this conversion: *arachidonate 12-lipoxygenase* (*alox12*), *alox12b*, and *arachidonate 15-lipoxygenase b* (*alox15b*). These enzymes may also convert AA to 15-Hydroxyicosatetraenoic acid (15S-HETE), which can subsequently be converted into LXA4 by Alox5 (70). **B.** Whole body LXA4 concentrations measured in 3dpf larvae by ELISA. Statistical analysis (ANOVA) showed an effect of amputation ($p=0.01$). **C.** Expression levels of *alox12* determined by qPCR. A significant interaction between amputation and beclomethasone treatment was observed. **D.** Expression levels of *alox12b* determined by qPCR. A significant effect of amputation was observed. **D.** Expression levels of *alox15b* determined by qPCR. A significant effect of amputation was observed. * Statistically significant difference compared to control treatment (Bonferroni post hoc comparison, $p<0.05$).

Figure 6. A. Schematic drawing of a zebrafish larvae at 3dpf, indicating the area selected for counting the number of neutrophils and macrophages that had migrated to the wounded area (red box, CV=caudal vein). **B**. Leukocyte staining upon tail fin amputation in a 3dpf embryo by immunohistochemistry against the pan-leukocyte marker L-plastin (shown in red). **C.** Staining of neutrophils specifically by Mpx staining (shown in black). Neutrophils are stained by both

774 methods, but the L-plastin immunolabeling is hard to detect in these cells due to the dark staining
775 of the Mpx assay. Therefore, the number of neutrophils was determined by counting in the cells
776 stained by the Mpx assay (shown black in B and C) and the number of macrophages was
777 determined by counting the number of cells stained by the L-plastin immunohistochemistry
778 (shown red in B). Further details on the analysis of this labeling can be found in Suppl.Fig.6.
779
780 **Figure 7.** Leukocyte migration upon tail fin amputation in 3dpf zebrafish larvae, and the effect
781 of beclomethasone treatment on this migration. **A.** The number of macrophages in the wounded
782 area as a function of time after amputation. Statistical analysis by two-way ANOVA revealed a
783 migratory response of macrophages over time ($p<0.001$), but no effect of beclomethasone on this
784 response. **B.** The number of neutrophils in the wounded area as a function of time after
785 amputation. Statistical analysis by two-way ANOVA revealed that both beclomethasone
786 treatment and time had a significant effect on the number of neutrophils (both $p<0.001$), and that
787 the neutrophil number was significantly increased at 4hpa compared to the 0hpa time point
788 ($p<0.001$). **C.** Neutrophil migration in GR mutant (gr^{s357}) larvae. The number of neutrophils in
789 the wounded area is shown at 4 hours post amputation in wild type and gr^{s357} larvae. No effect of
790 beclomethasone was observed in the mutant larvae, whereas beclomethasone significantly
791 decreased the number of neutrophils in the wild types. **D.** The effect of cycloheximide treatment
792 on macrophage and neutrophil migration. Macrophage and neutrophil numbers in the wounded
793 area are shown at 4hpa, after vehicle (black bars) or cycloheximide (grey bars) treatment.
794 Statistical analysis by ANOVA revealed a significant effect of cycloheximide treatment on both
795 the macrophage and the neutrophil migration ($p<0.05$). * Statistically significant difference
796 compared to vehicle treatment ($p<0.05$).