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1 # Glucocorticoid-induced attenuation of the inflammatory
2 # 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.

45 # Introduction

46

47 Glucocorticoids (GCs) regulate a wide range of biological processes, such as our immune
48 response, metabolism, growth, reproduction, vascular tone, bone formation, and brain function
49 (1-6). Because of their anti-inflammatory effects, they are widely used clinically for the
50 treatment of many immune-related diseases, like asthma, rheumatoid arthritis and leukemia (7,8).
51 These effects are mediated by the glucocorticoid receptor (GR), which acts as a ligand-activated
52 transcription factor. In its inactive state, the GR resides within the cytoplasm, and upon GC
53 binding it translocates to the nucleus, where it acts as a transcription factor and orchestrates gene
54 expression (9). GRs may occupy glucocorticoid response elements (GREs) and recruit
55 transcriptional coregulators, which results in a positive or negative regulation of the transcription
56 rate of nearby target genes. GRs may also interact with other transcription factors, e.g. NF-κB or
57 AP-1, and repress their activity (1,2,4,10-12). This mode of action has long been considered the
58 main mechanism by which GCs exert their anti-inflammatory effects, since it results in a
59 downregulation of the expression of a large number of inflammatory mediators (1,2,9-13).
60 However, recent evidence shows that the picture appears to be more complex (14,15). For
61 example, repression of genes is commonly a result of GRE occupancy as well, and GR
62 interaction with transcription factors like NF-κB or AP-1 appears to enhance gene transcription
63 in about half of all cases where this interaction was observed (14).

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

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

75 The aim of the present study is to establish and exploit a robust *in vivo* model to
76 investigate in detail the molecular mechanism of the anti-inflammatory action of GCs. A better
77 understanding of the complex interplay of GR with the different components of the immune
78 response would be of great importance to improve GC therapies, since the clinical use of GCs is
79 currently limited by the deleterious side effects and the occurrence of resistance to GC treatment
80 (23,24).

81 Over the last decade, the zebrafish has emerged in biomedical research as an important
82 model system for a variety of human diseases (25-27). The zebrafish immune system remarkably
83 resembles that of mammals (28), thus providing an excellent system for modeling various
84 molecular and cellular elements of inflammation such as host-pathogen interactions during
85 infectious diseases and immune cell migration to wound sites (29,30). In the present study,
86 zebrafish larvae are used at three days post fertilization (dpf). At this stage, two types of
87 leukocytes are present which constitute the innate immune system, macrophages and neutrophils
88 (31-35). Cells representing the adaptive immune system, like lymphocytes, do not mature before
89 the second week of zebrafish development (36-38). Furthermore, the zebrafish is used as a model
90 organism for GC research (39-44). Zebrafish have a single GR gene which encodes a GR protein

91 that upon activation mediates gene transcription in a similar way as its human equivalent
92 (39,42,45-48). Local inflammation can be modeled in zebrafish by amputation of the tail fin of
93 zebrafish larvae (49). Amputation induces the expression of many pro-inflammatory mediators at
94 the wound site and migration of neutrophils and macrophages, towards the site of amputation
95 (46,49-53). Interestingly, it has been demonstrated that this migration is inhibited by
96 glucocorticoid treatment and therefore this model system enables studying of the anti-
97 inflammatory action of glucocorticoids in an *in vivo* situation (46,51).

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

105 # Materials & Methods

106

107 ## Zebrafish, husbandry & egg collection

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

115

116 ## Tail amputation &chemical treatments

117 Three-day-old embryos were anesthetized in egg water containing 0.02% buffered aminobenzoic
118 acid ethyl ester (tricaine, Sigma) and aligned in Petri dishes coated with 2% agarose for
119 subsequent partial amputation of the tail fin as shown in Fig.1A. Amputation was performed
120 using a 1mm sapphire blade (World Precision Instruments) using a Leica M165C stereo-
121 microscope and a micromanipulator. Amputated and non-amputated embryos were pretreated for
122 2h with either 25µM beclomethasone (Sigma) or vehicle (0.05% DMSO) prior to amputation and
123 again for a specified period of time after amputation. The relatively high dose of beclomethasone
124 was chosen based on studies by Mathew et al. (51), who showed this dose to be maximally
125 effective in zebrafish. Cycloheximide treatment (50 µg/ml, Sigma) was performed similarly. For
126 gene expression analysis samples were collected in TRIzol® reagent (Invitrogen), for ELISA

127 samples were snap frozen in liquid nitrogen, and for migration studies samples were fixed in 4%
128 paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and stored at 4°C.
129
130 **RNA isolation & cDNA synthesis**
131 Total RNA was extracted using TRIzol® reagent (Invitrogen) according to the manufacturer's
132 instructions (Invitrogen). RNA was dissolved in water and denatured for 5min at 60°C. Samples
133 were treated with DNAse using the DNA-*free*™ kit (Ambion). For microarray analysis, RNA
134 was further purified using the RNeasy MinElute™ Cleanup kit from Qiagen and its integrity was
135 checked with a lab-on-chip analysis using the 2100 Bioanalyzer (Agilent Technologies). For
136 subsequent cDNA synthesis, 1μg of total RNA was added as a template for reverse transcription
137 using the iSCRIPT™ cDNA Synthesis Kit (Biorad).
138
139 **Microarray design**
140 A 4x180k microarray chip platform (customized by Agilent Technologies, (Design ID:028233))
141 was used in this study. This array consists of all probes already present in an earlier 45.219
142 custom-made array (55), and another 126.632 newly designed zebrafish probes had been added
143 as described in (56). A total of 16 samples (4 experimental groups from 4 replicate experiments)
144 were processed for transcriptome analysis and were hybridized against a common reference
145 sample, consisting of a mixture of all samples used in this study.
146
147 **Microarray amplification & labeling**
148 Amplification and labeling of RNA was performed at the MicroArray Department (MAD) of the
149 University of Amsterdam (Amsterdam, The Netherlands). Per sample, 0.5μg total RNA was

150 amplified and combined with Spike A according to the Agilent Two-Color Microarray-Based
151 Gene Expression Analysis kit (Agilent technologies). As a common reference sample an
152 equimolar pool of all test samples was made and 0.5µg samples were amplified similarly as the
153 test samples with the exception that Spike B was used. Amino-allyl modified nucleotides were
154 incorporated during the aRNA synthesis (2.5mM of each GTP, ATP, UTP (GE Healthcare),
155 0.75mM CTP (GE Healthcare), 0.3mM AA-CTP (TriLink Biotechnologies)). Synthesized aRNA
156 was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit (Omega Bio-Tek). The quality
157 was inspected on the BioAnalyzer (Agilent Technologies) with the Agilent RNA 6000 kit
158 (Agilent Technologies). Test samples were labeled with Cy3 and the reference sample was
159 labeled with Cy5. Five µg of aRNA was dried out and dissolved in 50mM carbonate buffer pH
160 8.5. Individual vials of Cy3/Cy5 from the mono-reactive dye packs (GE Healthcare) were
161 dissolved in 200µl DMSO. To each sample, 10µl of the appropriate CyDye dissolved in DMSO
162 was added and the mixture was incubated for 1h. Reactions were quenched with the addition of
163 5µl 4M hydroxylamine (Sigma-Aldrich). The labeled aRNA was purified with the E.Z.N.A.
164 MicroElute RNA Clean Up Kit. Yields of aRNA and CyDye incorporation were measured on the
165 NanoDrop ND-1000.

166

167 **Microarray hybridization, scanning & data processing**

168 Each hybridization mixture was made up from 825ng Test (Cy3-labeled) and 825ng Reference
169 (Cy5-labeled) material. Hybridization mixtures were using the Agilent Two-Color Microarray-
170 Based Gene Expression Analysis kit according to the manufacturer's instructions (Agilent
171 technologies). The samples were loaded onto the microarray chips and hybridized for 17h at
172 65°C. Afterwards the slides were washed and scanned (20 bit, 3µm resolution) in an ozone-free

173 room with the Agilent G2505C scanner. Data was extracted with Feature Extraction (v10.7.3.1,
174 Agilent Technologies) with the GE2_107_Sep09 protocol for two-color Agilent microarrays.
175 The Agilent output from the 16 hybridizations was then imported into the Rosetta Resolver 7.2
176 software (Rosetta Biosoftware, Seattle, Washington) and subjected to a factorial design with a
177 re-ratio with common reference application. Data analysis was performed setting cutoff for the p-
178 value of $<10^{-10}$ and for fold change of either >2 or <-2. The raw data were submitted to the Gene
179 Expression Omnibus (GEO) database under accession number GSE69444.
180

181 **Gene Ontology analysis**

182 Gene ontology analysis of the microarray results was performed as described previously (44). As
183 a starting point, clusters of genes were analyzed using the online functional classification tool
184 DAVID (http://david.abcc.ncifcrf.gov/summary.jsp). In addition, for genes not classified by
185 DAVID, information was gathered on their function (using the websites GeneCards
186 (http://www.genecards.org/), NCBI (http://www.ncbi.nlm.nih.gov/gene), Genetics Home
187 Reference (http://www.ncbi.nlm.nih.gov/gene) and Wikipedia (http://en.wikipedia.org/wiki/).
188 Using this information, all genes were classified in one of the categories assigned by DAVID, or
189 in a new category.
190

191 **Quantitative Polymerase Chain Reaction (qPCR)**

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

196 at 60°C and 30sec at 72°C. Ct values (cycle number at which a threshold value of the
197 fluorescence intensity was reached) were determined for each sample. A dissociation protocol
198 was added, determining dissociation of the PCR products from 65°C to 95°C, allowing
199 discrimination of specific products. In all qPCR experiments, a water-control was included. Data
200 shown are means (± s.e.m.) of four individual experiments. In each experiment, cDNA samples
201 were assayed in duplicate. Sequences of all primers used for qPCR analysis are presented in
202 Suppl. Table 1, and a phylogenetic tree showing all zebrafish *arachidonate lipoxygenase (alox)*
203 genes is shown in Suppl.Fig.1.
204
205 **LTB4 and LXA4 ELISA**
206 For each data point, six samples (20 larvae each) were collected. All liquid was removed and
207 samples were snap frozen in liquid nitrogen. For ELISA, 250µl 1x PBS and 0.2 SSB02 stainless
208 steel beads (Next advance) were added to each sample. Larvae were homogenized using the
209 Bullet blender® (Next advance) for 3min on speed 8. The samples were then centrifuged at 3500
210 rpm for 5min. The supernatant was collected and centrifuged again at 5000 rpm for 5 min after
211 which the supernatant was collected again. An LTB4 ELISA kit (Enzo Life Sciences), and LXA4
212 ELISA kit (Cloud-Clone) were used according to the manufacturer's instructions. All samples
213 were measured in duplicate (100 µl used per measurement), and the data from the duplicates was
214 averaged. Data shown are the averages (± s.e.m.) from six replicates.
215
216
217

218 **Myeloperoxidase staining and whole mount immunohistochemistry for visualization of**
219 **macrophages and neutrophils**
220 Embryos were fixed in 4% PFA overnight at 4°C and following washes with PBS containing
221 0.1% Tween 20 (PBST), the Myeloperoxidase (mpx) activity was detected using the Leukocyte
222 Peroxidase kit (Sigma) according to the manufacturer’s instructions. Mpx staining was always
223 performed prior to L-plastin immunohistochemistry. For this purpose, embryos were washed in
224 PBST, gradually dehydrated with methanol in PBS and stored in 100% methanol overnight at
225 4°C. The next day embryos were rehydrated with graded series of methanol in PBS containing
226 0.8% Triton X-100 (PBS-TX) and incubated with 10µg/ml Proteinase K (Roche) for 10min at
227 37°C. Embryos were then incubated in PBS-TX blocking buffer (containing 1% BSA) for 2h at
228 RT and subsequently in blocking buffer containing a rabbit anti-L-plastin polyclonal antibody
229 (provided by Dr. A. Huttenlocher (57), 1:500 dilution) overnight at 4°C. Following washes with
230 PBS-TX, embryos were incubated again in blocking buffer for 1h at RT prior to incubation with
231 goat anti-rabbit Alexa Fluor® 568 dye–labeled secondary antibody (Invitrogen) for 2h at RT
232 (1:200 dilution in blocking buffer).
233 Imaging of the embryos was performed using a Leica MZ16FA fluorescence stereo-
234 microscope supported by the LAS version 3.7 software. Macrophages were detected based on the
235 red fluorescent labeling by the immunohistochemistry and neutrophils were detected based on
236 their dark brown appearance as a result of the Mpx assay (although they are stained by both
237 methods, the L-plastin immunolabeling is hard to detect in these cells due to the dark staining of
238 the Mpx assay). To determine the number of cells that had migrated to the wounded area, the
239 cells posterior to the caudal vein were counted (see also Suppl.Fig.6). Data shown are means (±

240 s.e.m.) of three individual experiments. In each experiment, treatment groups consisted of at least
241 20 larvae.
242
243 **Statistical analysis**
244 Statistical analyses (one- or two-way ANOVAs with Bonferroni post-hoc tests) were performed
245 using the GraphPad Prism version 4.00 (GraphPad Software, La Jolla, USA).

246 # Results

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

249 In order to study the anti-inflammatory action of GCs in zebrafish, we set up a tail fin amputation
250 assay using 3 day post fertilization (dpf) larvae that were exposed to either vehicle or the
251 synthetic GC beclomethasone (25µM) for 2h. Tail fins were amputated and vehicle or
252 beclomethasone treatment was continued. Total RNA samples were collected at 4 h post
253 amputation (hpa). This way, four experimental groups were generated: control treated with
254 vehicle (con/vehicle), amputated treated with vehicle (4hpa/vehicle), control treated with
255 beclomethasone (con/beclo), and amputated treated with beclomethasone (4hpa/beclo). The
256 samples were used in a microarray experiment to analyze the transcriptional response to
257 wounding as well as how this response was affected by beclomethasone treatment.
258

259 **The effects of amputation on gene transcription**

260 First, we identified 380 probes to be significantly regulated due to amputation (comparison
261 con/vehicle vs. 4hpa/vehicle). Gene annotation demonstrated that these probes corresponded to
262 279 genes, of which 201 were upregulated and 78 downregulated due to amputation. Gene
263 ontology analysis revealed that 31 genes in this cluster were involved in the immune system. Of
264 these 31 genes, 3 encoded anti-inflammatory proteins, 9 were involved in chemokine or cytokine
265 signaling, and 4 were involved in prostaglandin or leukotriene signaling. Furthermore, 29 genes
266 encoding transcription factors (or other proteins involved in transcriptional regulation) were
267 present in this amputation-regulated cluster. The two most strongly upregulated transcription
268 factor genes (*fos* and *atf3*) are both members of the AP-1 transcription factor family, and another

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

276

277 **The effects of beclomethasone on gene transcription**

278 Subsequently, we investigated which genes responded to beclomethasone treatment in non-
279 amputated larvae. A cluster of 927 probes was identified to be significantly regulated due to
280 beclomethasone treatment (comparison con/vehicle vs. con/beclo). Gene annotation
281 demonstrated that these probes corresponded to 506 genes (Fig.1B), of which 420 were
282 upregulated and 86 downregulated due to beclomethasone. Gene ontology analysis showed that
283 90 genes in this cluster were involved in metabolic processes, of which 19 in the metabolism of
284 carbohydrates, 28 in protein metabolism, and 13 in lipid metabolism. Other gene ontology
285 groups overrepresented in this cluster were those containing genes involved in membrane
286 transport (37 genes), cell cycle and apoptosis (30), and genes encoding transcription factors (30).
287 An overview of the gene ontology analysis of this cluster is presented in Suppl.Fig.2A and B,
288 and detailed information is presented in Suppl.Table3. A number of 32 genes were present in
289 both the amputation- and the beclomethasone-regulated cluster of genes (Fig.1C and
290 Suppl.Table3). This cluster may represent the genes that are regulated upon amputation due to
291 increased cortisol levels.

292

293 **The effects of amputation and beclomethasone on gene transcription**

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

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

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

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

332

333 **The effects of amputation and beclomethasone on leukotriene biosynthesis**

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

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

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

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

360 LXA4 data (Figs.5C-D). The qPCR results showed that amputation decreases the expression of
361 these genes and this decrease is only affected by beclomethasone for *alox12*.
362
363 **The tail fin amputation assay to study GC effects on leukocyte migration**
364 Previous studies in zebrafish larvae have shown that leukocytes migrate to wound sites,
365 representing an inflammatory response, and that this response is impaired upon treatment with
366 GCs (46,51). In order to study this in more detail, tail fins were amputated upon vehicle or
367 beclomethasone treatment as described above. Larvae were fixated at 0, 2, 4, 8, 16 and 24hpa
368 and neutrophils and macrophages were labeled and counted. To determine the number of cells
369 that had migrated to the wounded area, cells posterior to the caudal vein were counted (area
370 indicated by the red box in Fig.6A).
371 In order to label the populations of neutrophils and macrophages in 3dpf larvae we
372 employed Myeloperoxidase (Mpx) histochemistry, followed by immunofluorescent labeling of
373 L-plastin. At this stage of development two populations of leukocytes are present: neutrophils,
374 which are Mpx- and L-plastin-positive, and macrophages, which are Mpx-negative and L-
375 plastin-positive (31,33-35,58). Although neutrophils are stained by both methods, the L-plastin
376 immunofluorescence is hard to detect in these cells due to the dark staining of the Mpx assay
377 which hides the fluorescent signal. Using this approach, the number of macrophages and
378 neutrophils were determined in the tail fins at different time points upon amputation. The results
379 showed that macrophages migrated more to the posterior end of the tail where they appeared to
380 line up at the actual wound site, whereas neutrophils were more randomly located in the vicinity
381 of the wound (Fig.6B and 6C).
382

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

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

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

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

424 ## Discussion

425

426 In the present study, we have used zebrafish larvae in order to study the effects of GC signaling
427 on the inflammatory response to tail fin amputation, both at the molecular and the cellular level.
428 First, we looked for transcriptional changes at 4hpa and we identified 279 genes of which the
429 expression was significantly altered upon amputation. The largest gene ontology group in this
430 cluster of genes was formed by genes involved in the immune system, indicating that many of
431 the observed changes are related to the induction of an inflammatory response. In a similar study
432 by Yoshinari et al. (59), in which 2dpf embryos were tail fin amputated and samples were
433 collected at a much later time point (16hpa), transcriptome analysis revealed that the largest
434 fraction of regulated signaling routes were metabolic pathways (40%) and only a small fraction
435 (2%) of signaling cascades regulated were immune-related. Thus, it appears that at 4 hours after
436 injury, immune-related pathways are heavily activated at the transcriptional level, while 12 hours
437 later amputation-induced changes in gene expression no longer reflect an inflammatory response.
438 This is in line with the observed decline in neutrophil migration after 4hpa in our study. The
439 second largest group was formed by genes encoding transcription factors, encompassing
440 members of the AP-1 family and several other pro-inflammatory transcription factors.

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

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

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

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

470 of eicosanoids like LTB4 was observed (66-68). In line with these data, we found that the
471 amputation-induced increase in LTB4 concentration was inhibited by beclomethasone, although
472 the steroid did not clearly affect the transcriptional regulation of proteins involved in LTB4
473 biosynthesis. We also studied whether GCs stimulate conversion of LTA4 to lipoxinA4 (LXA4),
474 an anti-inflammatory lipid which could contribute to the resolution of the inflammatory response
475 (69,70). It was found that GCs did not affect LXA4levels, and did not have a clear effect on the
476 mRNA levels of genes involved in LXA4 biosynthesis. Apparently, the LXA4 pathway is not a
477 target for GCs, whereas LTB4 induction is inhibited by GCs.

478 Finally, we examined the effect of GC treatment on the migration of leukocytes towards
479 injured sites. Our analysis showed that beclomethasone treatment had a significant inhibitory
480 effect only on the migration of neutrophils. Hence, the zebrafish model recapitulates the
481 inhibitory effects of glucocorticoids on neutrophil migration towards inflamed tissues, that have
482 been well established in mammalian models (71). However, macrophage migration was not
483 inhibited by beclomethasone, in line with previously observed GC effects on leukocytes in 3dpf
484 zebrafish larvae that were shown to be specifically suppressive regarding the recruitment of
485 neutrophils but not of macrophages (51). It must be noted that macrophages are not a
486 homogeneous cell population, but rather encompass distinct phenotypes. Macrophages with pro-
487 inflammatory activities are generally called M1 and those displaying anti-inflammatory action,
488 thereby encouraging tissue repair, are called M2 (72). Interestingly, it has been shown that GC
489 exposure induced a gene expression profile in human monocytes in which not only expression of
490 pro-inflammatory genes was inhibited, but moreover expression of anti-inflammatory genes was
491 induced (73). GC treatment has been shown to induce a highly phagocytic monocyte-derived
492 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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704

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

751 beclomethasone treatment). **D.** Expression levels of *alox5a* determined by qPCR. A significant

752 effect of amputation was observed. **E.** Expression levels of *lta4h* determined by qPCR. A

753 significant effect of both amputation and beclomethasone treatment was observed. * Statistically

754 significant difference compared to control treatment (Bonferroni post hoc comparison, p<0.01).

755

756 **Figure 5. A.** Lipoxin A4 (LXA4) biosynthesis pathway. LXA4 can be synthesized from LTA4.

757 In zebrafish, three genes encode enzymes that may be involved in this conversion: *arachidonate*

758 *12-lipoxygenase* (*alox12*), *alox12b*, and *arachidonate 15-lipoxygenase b* (*alox15b*). These

759 enzymes may also convert AA to 15-Hydroxyicosatetraenoic acid (15S-HETE), which can

760 subsequently be converted into LXA4 by Alox5 (70). **B.** Whole body LXA4 concentrations

761 measured in 3dpf larvae by ELISA. Statistical analysis (ANOVA) showed an effect of

762 amputation (p=0.01). **C.** Expression levels of *alox12* determined by qPCR. A significant

763 interaction between amputation and beclomethasone treatment was observed. **D.** Expression

764 levels of *alox12b* determined by qPCR. A significant effect of amputation was observed. **D.**

765 Expression levels of *alox15b* determined by qPCR. A significant effect of amputation was

766 observed. * Statistically significant difference compared to control treatment (Bonferroni post

767 hoc comparison, p<0.05).

768

769 **Figure 6. A.** Schematic drawing of a zebrafish larvae at 3dpf, indicating the area selected for

770 counting the number of neutrophils and macrophages that had migrated to the wounded area (red

771 box, CV=caudal vein). **B**. Leukocyte staining upon tail fin amputation in a 3dpf embryo by

772 immunohistochemistry against the pan-leukocyte marker L-plastin (shown in red). **C.** Staining of

773 neutrophils specifically by Mpx staining (shown in black). Neutrophils are stained by both

methods, but the L-plastin immunolabeling is hard to detect in these cells due to the dark staining of the Mpx assay. Therefore, the number of neutrophils was determined by counting in the cells stained by the Mpx assay (shown black in B and C) and the number of macrophages was determined by counting the number of cells stained by the L-plastin immunohistochemistry (shown red in B). Further details on the analysis of this labeling can be found in Suppl.Fig.6.

Figure 7. Leukocyte migration upon tail fin amputation in 3dpf zebrafish larvae, and the effect of beclomethasone treatment on this migration. **A.** The number of macrophages in the wounded area as a function of time after amputation. Statistical analysis by two-way ANOVA revealed a migratory response of macrophages over time ($p<0.001$), but no effect of beclomethasone on this response. **B.** The number of neutrophils in the wounded area as a function of time after amputation. Statistical analysis by two-way ANOVA revealed that both beclomethasone treatment and time had a significant effect on the number of neutrophils (both $p<0.001$), and that the neutrophil number was significantly increased at 4hpa compared to the 0hpa time point ($p<0.001$). **C.** Neutrophil migration in GR mutant (gr^{s357}) larvae. The number of neutrophils in the wounded area is shown at 4 hours post amputation in wild type and gr^{s357} larvae. No effect of beclomethasone was observed in the mutant larvae, whereas beclomethasone significantly decreased the number of neutrophils in the wild types. **D.** The effect of cycloheximide treatment on macrophage and neutrophil migration. Macrophage and neutrophil numbers in the wounded area are shown at 4hpa, after vehicle (black bars) or cycloheximide (grey bars) treatment. Statistical analysis by ANOVA revealed a significant effect of cycloheximide treatment on both the macrophage and the neutrophil migration ($p<0.05$). * Statistically significant difference compared to vehicle treatment ($p<0.05$).