Glucocorticoid modulation of the immune response: Studies in zebrafish

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Chapter 3

Glucocorticoids inhibit macrophage differentiation towards a pro-inflammatory phenotype upon wounding without affecting their migration

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Abstract

Glucocorticoid drugs are widely used to treat immune-related diseases, but their use is limited by side effects and by resistance, which especially occurs in macrophage-dominated diseases. In order to improve glucocorticoid therapies, more research is required into the mechanisms of glucocorticoid action. In the present study, we have used a zebrafish model for inflammation to study glucocorticoid effects on the innate immune response. In zebrafish larvae, the migration of neutrophils towards a site of injury is inhibited upon glucocorticoid treatment, while migration of macrophages is glucocorticoid resistant. We show that wounding-induced increases in expression of genes encoding neutrophil-specific chemoattractants (Il8 and Cxcl18b) are attenuated by the synthetic glucocorticoid beclomethasone, but that beclomethasone does not attenuate the induction of the genes encoding Ccl2 and Cxcl11aa, which are required for macrophage recruitment. RNA sequencing on Fluorescence-Activated Cell Sorting (FACS)-sorted macrophages shows that the vast majority of the wounding-induced transcriptional changes in these cells are inhibited by beclomethasone, whereas only a small subset is glucocorticoid-insensitive. As a result, beclomethasone decreases the number of macrophages that differentiate towards a pro-inflammatory (M1) phenotype, which we demonstrated using a *tnfa:eGFP-F* reporter line and analysis of macrophage morphology. We conclude that differentiation and migration of macrophages are regulated independently, and that glucocorticoids leave the chemotactic migration of macrophages unaffected, but exert their anti-inflammatory effect on these cells by inhibiting their differentiation to an M1 phenotype. The resistance of macrophage-dominated diseases to glucocorticoid therapy can therefore not be attributed to an intrinsic insensitivity of macrophages to glucocorticoids.

Introduction

Glucocorticoids (GCs) are a class of steroid hormones secreted by the adrenal gland, and the main endogenous glucocorticoid in our body is cortisol [1-3]. Glucocorticoids regulate a wide variety of systems in our body, including the immune, metabolic, reproductive, cardiovascular and central nervous system [4-7]. Due to their potent and well-established immunosuppressive effects, they are often prescribed to treat various immune-related diseases, including asthma, rheumatoid arthritis, dermatitis, leukemia, and several autoimmune diseases [8, 9]. However, their clinical use is limited by two issues. First, chronic glucocorticoid therapy can lead to severe side effects, like osteoporosis, muscle weakness, diabetes, infection, and hypertension [10]. Second, resistance to glucocorticoid drug treatment occurs in a large number (~10-30%) of patients [11, 12]. In order to develop novel glucocorticoid therapies that overcome these barriers and retain their therapeutic efficacy, more

insight into the molecular and cellular mechanisms of glucocorticoid modulation of the immune response is required.

Glucocorticoids exert their function through an intracellular receptor, the glucocorticoid receptor (GR) [13], which acts as a transcription factor, altering the transcription of a plethora of genes. The GR modulates the transcription of genes by several mechanisms [14]. It can bind directly to DNA, to glucocorticoid response elements (GRE) and enhance transcription upon recruitment of transcriptional cofactors. In contrast, binding to negative GREs (nGREs) has been shown to repress gene transcription [15]. Alternatively, the GR can bind indirectly to DNA through interaction with other transcription factors, like AP-1, NF-κB or STAT3. Through this 'tethering', it modulates the activity of these factors.

The tethering mechanism of the GR, resulting in the inhibition of transcription of immune-activating genes, is generally considered to be the main mechanism by which glucocorticoids exert their anti-inflammatory actions [16]. For example, TNF- or LPS-induced transcriptional responses in cultured cells can be repressed through tethering of the NF-κB subunit p65 [17-20]. Other mechanisms, like the activation of anti-inflammatory genes through GRE binding, and a reduction of NF-κB recruitment, contribute to the anti-inflammatory actions of GR as well, but the exact role of these mechanisms has not been fully established [21, 22]. Through these mechanisms, glucocorticoids exert strong suppressive effects on the inflammatory response [23]. At the initial stage of this response, they dampen signaling pathways downstream from Toll-like receptors (TLRs), inhibit the induction of genes encoding cytokines, upregulate the expression of anti-inflammatory proteins, and inhibit the generation of prostaglandins and leukotrienes [8, 24]. In addition, they reduce the blood flow to the inflamed tissue and inhibit vascular leakage. At subsequent stages, glucocorticoids attenuate the production of chemokines and adhesion molecules, thereby reducing leukocyte extravasation and migration towards the inflamed site [23, 24].

It has become clear that glucocorticoid action on the immune system is highly complex and requires further investigation. A complicating factor is that the effects of glucocorticoids have been shown to be highly cell type-specific [25]. Whereas they induce apoptosis of eosinophils and basophils, they promote the survival and proliferation of neutrophils [26, 27]. In monocytes, they induce an anti-inflammatory phenotype with increased mobility and phagocytic capacity [28]. Macrophages are often divided into two functional phenotypes: a classically activated, pro-inflammatory (M1) phenotype which contributes to the inflammatory response, and an alternatively activated (M2) phenotype which can be subdivided in several different phenotypes which have been shown to be involved in the resolution of inflammation and wound healing [29, 30] . In animal models for arthritis and acute lung injury, glucocorticoids have been shown to inhibit the differentiation of macrophages towards an M1

phenotype, whereas the effect on M2 differentiation is less clear [31, 32]. In addition to the cell type-specificity of glucocorticoid actions, it has become clear that the transcriptional regulation of immune-activating genes by the GR is not strictly suppressive [33]. Upregulation of various pro-inflammatory genes after glucocorticoid treatment has been observed in several cell types [34-38], and GR has been shown to activate pro-inflammatory genes in synergy with other signaling pathways [39-41]. In addition, some genes that are induced upon TNF or LPS treatment appear to be insensitive to the repressive action of GR [17-20].

In the present study, we have used the zebrafish as an *in vivo* model to study glucocorticoid effects on the inflammatory response. The immune system of the zebrafish is highly similar to that of humans. Like humans, the zebrafish has a thymus, innate immune cells (macrophages, neutrophils) and adaptive immune cells (T cells and B cells), and cells that bridge innate and adaptive immunity (dendritic cells) [42-44]. Besides, the innate immune system of zebrafish develops within a few days after fertilization, while the adaptive immune system only matures after two weeks, which means the innate immune system can be studied separately in larvae [43, 45]. Zebrafish larvae are widely used as a model system to study the inflammatory response [46-48]. Tail wounding-induced inflammation in zebrafish larvae is a well-established model in which amputation of the tail triggers the expression of many pro-inflammatory molecules and the recruitment of innate immune cells (neutrophils and macrophages) towards the wounded area [49, 50]. This model enables the investigation of cell-type specific inflammatory responses *in vivo* and has been widely used for research on leukocyte migration and infiltration and anti-inflammatory drug screening [51-53].

The zebrafish Gr is highly similar to its human equivalent in structure and function [54-56]. This makes the zebrafish a valuable model to study the molecular mechanisms of glucocorticoid action *in vivo* [56-58]. In previous work, we have studied the anti-inflammatory effects of glucocorticoids using the tail amputation model and found that glucocorticoid treatment attenuates the vast majority amputation-induced changes in gene expression, which were measured in lysates from whole larvae [59]. In addition, we observed that the recruitment of neutrophils to the wounded area is inhibited by glucocorticoids, but that the migration of macrophages is resistant to glucocorticoid treatment [59-61].

It has been shown that glucocorticoids are less effective in the treatment of inflammatory diseases dominated by macrophages, like chronic obstructive pulmonary disease (COPD), but the mechanisms underlying the limited responsiveness to glucocorticoid treatment remain poorly understood [62]. Therefore, in the present study, we sought to find a mechanistic explanation for our finding that glucocorticoids do not inhibit amputation-induced macrophage migration. We demonstrate that the

induction of genes encoding chemo-attractants involved in macrophage recruitment is insensitive to glucocorticoid treatment, providing an explanation for the resistance of macrophage migration to glucocorticoids. In addition, we show that macrophages should not be considered a generally glucocorticoid-insensitive cell type. In these cells, glucocorticoids attenuate almost all wounding-induced changes in gene expression. Through this modulation of the transcriptional response, glucocorticoids inhibit the differentiation of macrophages to a pro-inflammatory (M1) phenotype.

Results

Glucocorticoids inhibit migration of neutrophils, but leave macrophage migration unaffected

Using tail amputation in 3 dpf zebrafish larvae as a model for inflammation, we studied the effect of four glucocorticoids (beclomethasone, dexamethasone, hydrocortisone, prednisolone) on the migration of leukocytes towards a site of injury. To quantitate the migration of neutrophils and macrophages, we counted the number of these innate immune cells in a defined area of the tail at 4 hours post amputation (hpa, Figure 1A). All four glucocorticoids had a highly significant inhibitory effect on the migration of neutrophils, as previously observed [63] (Figure 1C). Three glucocorticoids (beclomethasone, dexamethasone and prednisolone) did not affect the migration of macrophages significantly, and one (hydrocortisone) induced a slight decrease (~12.5%, Figure 1B). These data are in line with a previous study from our group, in which we demonstrated that beclomethasone inhibited the migration of neutrophils and not of macrophages, that this effect was mediated through Gr, and that beclomethasone did not affect the total leukocyte numbers in the larvae [59].

To study the effects of beclomethasone on leukocyte migration in more detail, larvae were imaged using confocal microscopy between 1.5 and 12 hpa, and the leukocyte numbers in the wounded area were automatically determined using dedicated software. The results of this analysis showed that for the control group the average number of macrophages present in the wounded area increased from 16.7±2.6 to 32.3±3.2 cells between 1.5 hpa and 12 hpa (Figure 2A). No significant effect of beclomethasone on macrophage migration was observed (from 16.2±1.8 to 27.3±2.2 for the beclomethasone-treated group). For neutrophils, in the control group, a number of 10.8±1.8 was observed at 1.5 hpa, and their number reached a peak of 23.0±2.8 at around 5 hpa, then decreased and reached a level of 19.4±2.5 at 9 hpa which remained relatively constant until 12 hpa (Figure 2B). In the beclomethasone-treated group, a lower number of recruited neutrophils was observed in the wounded area at 5 hpa (15.9±1.8).

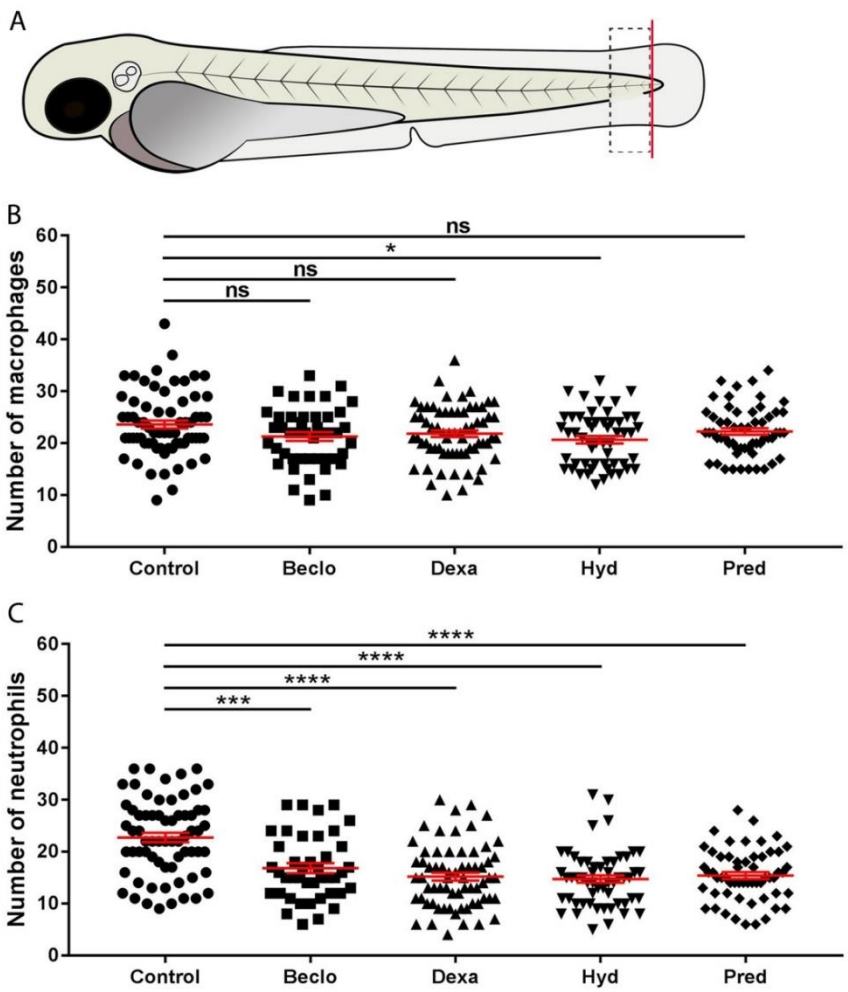

Figure 1. Effect of glucocorticoids on macrophage and neutrophil recruitment upon tail amputation in *Tg*(*mpx:GFP/mpeg1:mCherry-F*) larvae. A. Schematic drawing of a zebrafish larva at 3 dpf. The red line shows the site of amputation. The black dashed box shows the area in which cells were counted to quantitate the recruitment. B. The number of macrophages recruited to the wounded area at 4 hpa. In the Beclo (beclomethasone), Dexa (dexamethasone) and Pred (prednisolone) groups, no significant differences were observed compared the control group (vehicle-treated). In the Hyd (hydrocortisone) group, a significantly decreased number of macrophages was observed. C. The number of neutrophils recruited to the wounded area at 4 hpa. For all glucocorticoid-treated groups, a significantly reduced number of neutrophils was recruited compared to the control group. Statistical analyses were performed by ANOVA. Values shown are the means ± s.e.m. of data from three independent experiments. Statistical significance is indicated by: ns, non-significant; * P<0.05; *** P<0.001; **** P<0.0001.

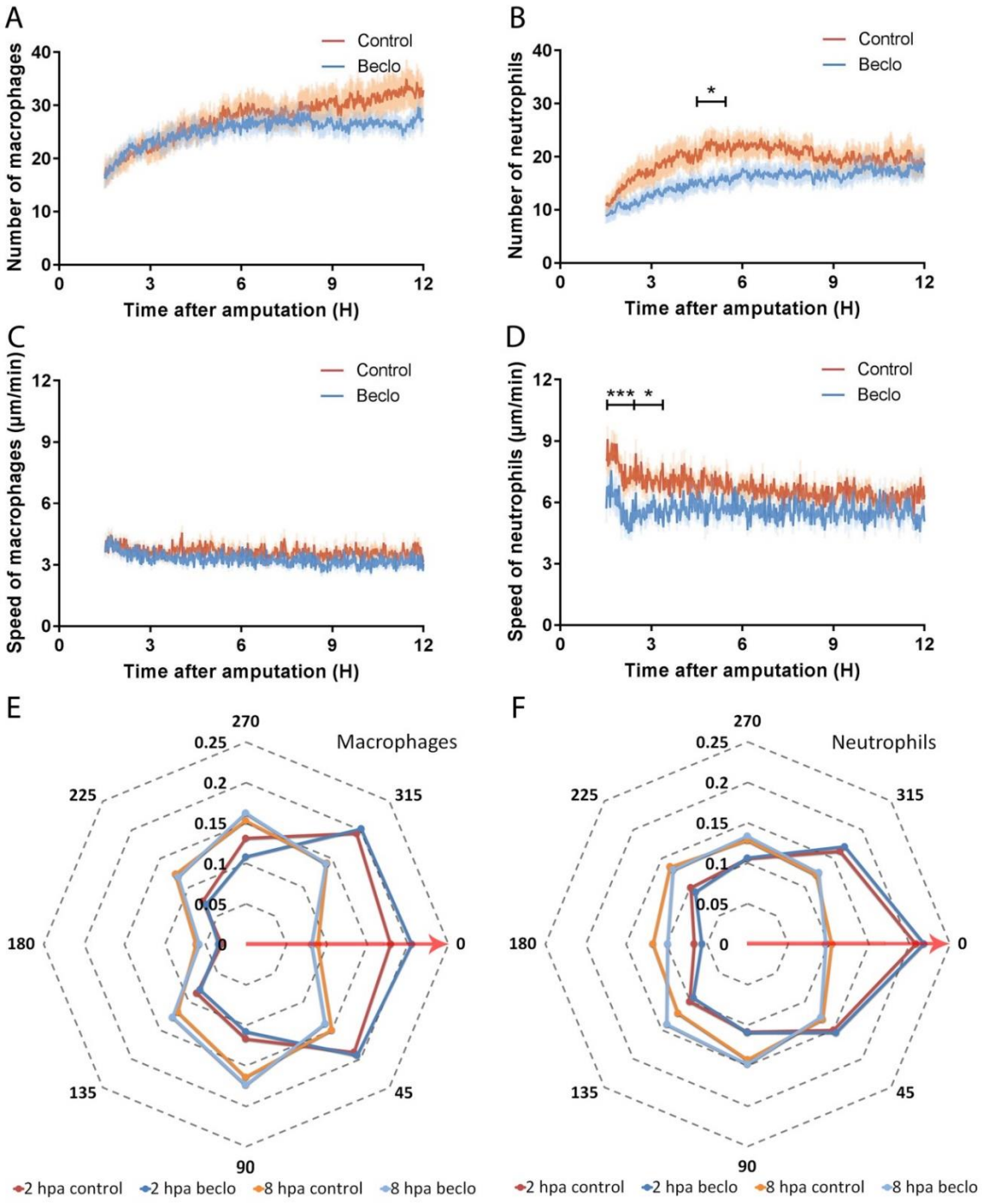

Figure 2. Live imaging and tracking of migrating macrophages and neutrophils upon tail amputation. A-B. The number of macrophages (A) and neutrophils (B) recruited to the wounded area from 1.5 hpa to 12 hpa in 3 dpf larvae in the vehicle-treated group (Control) and the beclomethasone-treated group (Beclo). Data were averaged for each hour to proceed statistical analysis by ANOVA with a Fisher's LSD post hoc test. No significant difference was observed for the number of recruited macrophages. A significantly reduced number of neutrophils were recruited in the beclomethasone treated group compared to the control group at 5 hpa. Values shown are the means ± s.e.m. from 10 larvae. Statistical significance is indicated by: * P<0.05. C-D. The velocity of macrophages (C) and neutrophils (D). Data were averaged for each hour to proceed statistical analysis by ANOVA with a Fisher's LSD post hoc test. No significant difference was observed for the velocity of macrophages. At 2 and 3 hpa, the velocity of neutrophils in the beclomethasone-treated group was significantly lower than

the velocity in the control group. Values shown are the means ± s.e.m. from 10 embryos. Statistical significance is indicated by: * P<0.05; *** P<0.001. E-F. The directionality of recruited macrophages (E) and neutrophils (F) at 2 hpa and 8 hpa. The circular x axis indicates the different angles made by cells, classified into 8 categories. Category 0 represents the direction towards the wound (including angles between 22.5 to -22.5 degrees, shown by the red arrows). The y axis indicates the size of the fraction of cells occurring within a category in that hour. Statistical analysis was performed by Kolmogorov-Smirnov test. No difference was observed between the control and beclomethasone treated groups. Values shown are the means from 10 embryos.

To further analyze the effects of beclomethasone, we used automated tracking of the leukocytes (see Movies 1-2 in Supplementary Information), and quantified the velocity and directionality of the migrating macrophages and neutrophils. The data showed that during the entire time frame, the velocity of the macrophages fluctuated around 3.5 μm/min for both the control and the beclomethasone-treated group (Figure 2C). For neutrophils, the velocity peaked at 1.5 hpa (8.12±0.56 μm/min for the control group and 5.70±0.72 μm/min for the beclomethasone-treated group) and decreased slowly afterwards (Figure 2D). At 2 hpa and 3 hpa, the velocity of neutrophils in the beclomethasone-treated group was significantly lower compared to the control group.

In addition, we measured the direction in which the macrophages and neutrophils moved and plotted the distribution of these directions measured at 2 and 8 hours after amputation (Figures 2E-F). The results showed that at none of these time points beclomethasone affected the directionality of either macrophages or neutrophils. At 2 hpa, most of the macrophages (~60%) moved towards the wounded area (angles 292.5°- 360°, and 0°- 67.5°) (Figure 2E). Only less than 20% of them moved in the opposite direction (angles 112.5°- 247.5°). At 8 hpa, the percentage of macrophages that moved towards the wounded area in the control and beclomethasone-treated group decreased to ~40%. For the neutrophils, the directionality showed a similar trend (Figure 2F). At 2 hpa, over 50% of the neutrophils moved towards the wounded area in both the control group and the beclomethasone-treated group, while at 8 hpa, this percentage decreased to ~35%. In conclusion, beclomethasone does not affect any of the migration parameters of macrophages but reduces the number of recruited neutrophils and their velocity.

Beclomethasone inhibits the induction of chemoattractants for macrophages

To unravel the molecular mechanisms underlying the difference between the effect of beclomethasone on macrophage and neutrophil migration, we first studied the expression of chemo-attractants that are known to be involved in the migration of these leukocytes. According to previous studies on leukocyte migration and infiltration, Ccl2 and Cxcl11aa are two of the key chemokines that stimulate the migration of macrophages, while Il8 and Cxcl18b are important for the stimulation of neutrophil migration [64-70]. Using qPCR on RNA samples from whole larvae, we determined the

expression levels of the genes encoding these four chemo-attractants (*ccl2*, *cxcl11aa, il8* and *cxcl18b)* at different time points after amputation, (Figure 3A-D). The results showed that at 4 hpa, the mRNA level of all four chemo-attractants was increased by amputation. At 2 hpa, the expression of *ccl2*, *cxcl11aa*, and *cxcl18b* was increased, and at 8 hpa the expression of *ccl2*, *il8* and *cxcl18b* showed an increase. In the presence of beclomethasone, amputation induced a smaller increase in *il8* and *cxcl18b* expression but the increase in the expression of *ccl2* and *cxcl11aa* was not inhibited. In addition, beclomethasone decreased the expression of *il8* independent of amputation. We previously observed a similar suppression under basal conditions by beclomethasone for *mmp3*, *mmp9* and *ilb* [59], indicating that for some immune-related genes, glucocorticoids downregulate the basal expression, in addition to attenuating their upregulation.

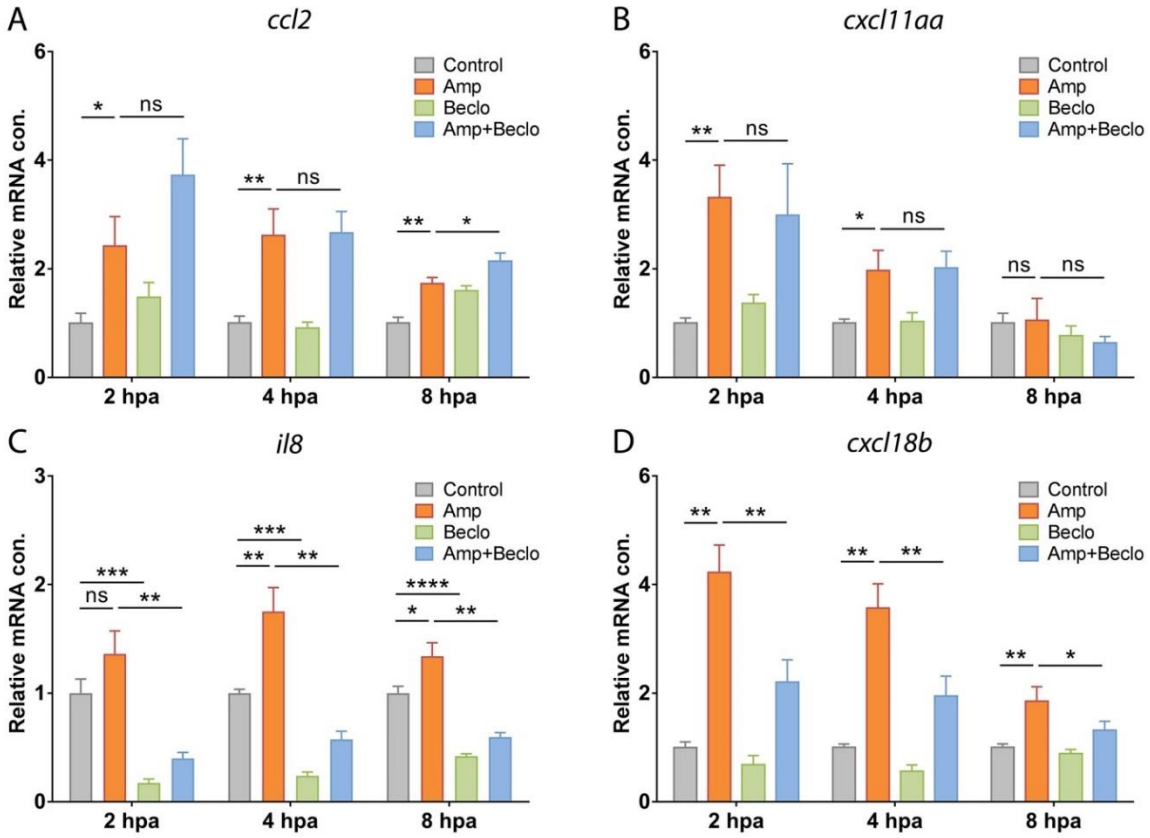

Figure 3. Expression levels of genes encoding chemo-attractants Ccl2 (A), Cxcl11aa (B), Il8 (C), and Cxcl18b (D) in whole larvae at 2hpa, 4hpa and 8hpa, determined by qPCR. Statistical analysis by ANOVA with a Fisher's LSD post hoc test showed that *ccl2* and *cxcl11aa* mRNA levels were significantly increased by amputation (Amp) and that the combined amputation/beclomethasone (Amp+Beclo) treatment resulted in a similar level of regulation, relative to the non-amputated, vehicle-treated group (Control). Expression levels of *il8*, and *cxcl18b* showed a significant increase upon amputation, and this effect was lower upon the combined treatment. Expression level of *il8* was significantly suppressed by beclomethasone (Beclo). Values shown are the means ± s.e.m. of three independent experiments. Statistical significance is indicated by: ns, non-significant; * P<0.05; ** P<0.01; *** P<0.001.

To demonstrate that the chemo-attractants Ccl2 and Cxcl11aa are required for macrophage recruitment in this tail amputation model, we analyzed their role in macrophage migration in our model. We used a previously described morpholino to create a knockdown of Ccr2, the receptor of Ccl2, in zebrafish larvae, which was shown not to affect the total number of leukocytes [65, 71]. In the *ccr2* morphants, a significantly decreased number of recruited macrophages was observed in the wounded area at 4 hpa (Figure 4A, C). However, the number of recruited neutrophils was identical to the number in the mock-injected controls (Figure 4B, D , the number of recruited neutrophils was unexpectedly high in these experiments (compared to data shown in Fig,1C, 2B and 4F), which we can only explain as an effect of the injections). For the receptor of Cxcl11aa, Cxcr3.2, we used a mutant fish line, and it was previously demonstrated that total numbers of leukocytes were not affected by the mutation [66]. The *cxcr3.*2 -/- larvae showed significantly decreased numbers of both macrophages (Figure 4E, G) and neutrophils (Figure 4F, H) recruited to the wounded area compared to the *cxcr3.2 +/+* controls (the number of recruited macrophages was slightly lower in these experiments (compared to data shown in Fig,1B, 2A and 4A), which may be due to the different genetic background of the used fish line).

These findings indicate that beclomethasone does not affect the amputation-induced increase in the expression of the genes encoding the chemo-attractants Ccl2 and Cxcl11aa, which are involved in macrophage recruitment upon tail amputation. This provides an explanation for the insensitivity of macrophage migration to glucocorticoid treatment.

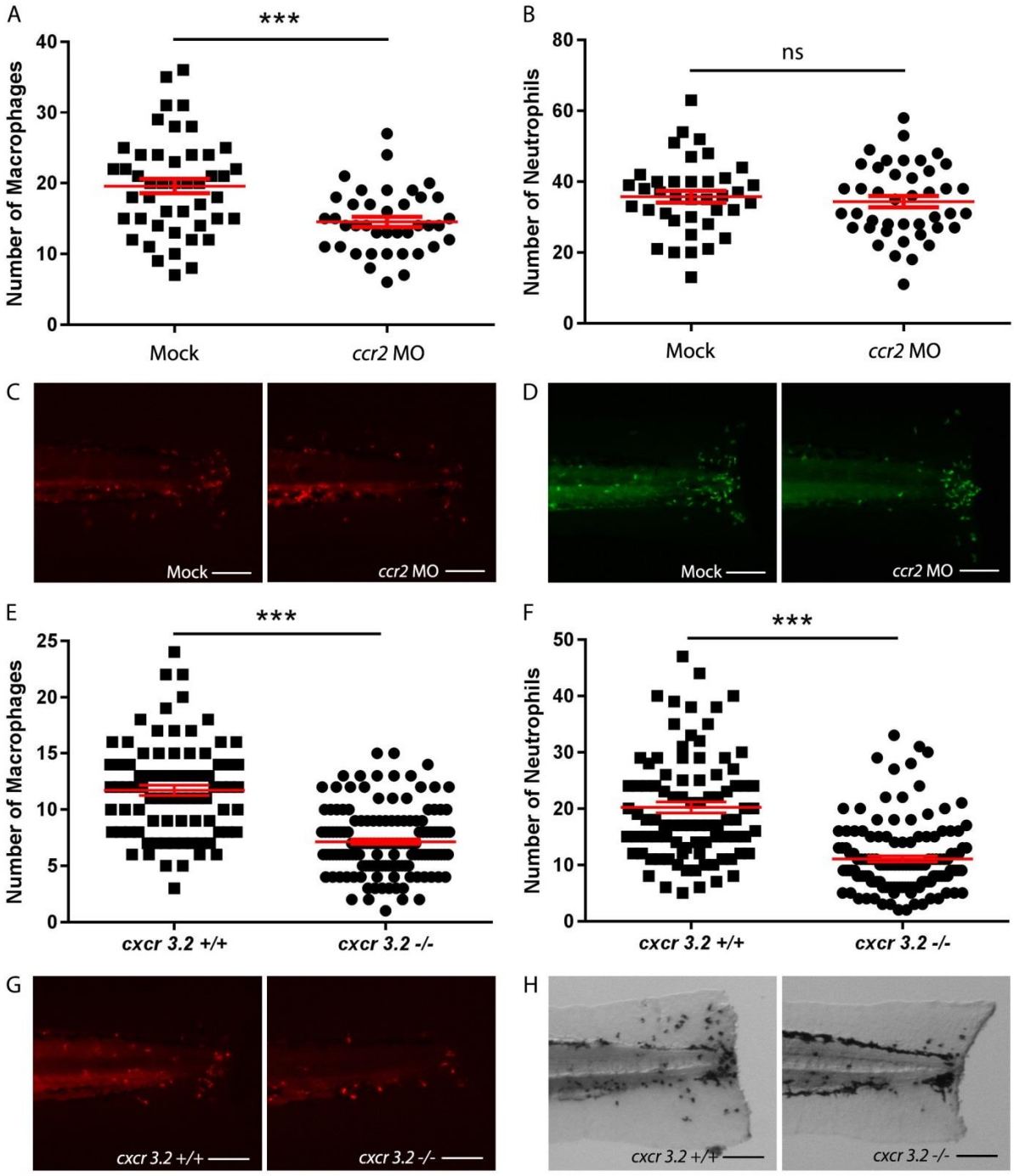

Figure 4. Effect of *ccr2* morpholino knockdown or *cxcr3.2* mutation on macrophage and neutrophil recruitment upon tail amputation in larvae. A-B. The number of macrophages (A) and neutrophils (B) recruited to the wounded area at 4 hpa in 3dpf *Tg*(*mpx:GFP/mpeg1:mCherry-F*) larvae. In *ccr2* morpholino injected larvae, a significantly reduced number of macrophages were recruited compared to the number in mock(vehicle)-injected larvae. Statistical analysis were performed by two-tailed t-test. No significant difference was observed for the number of recruited neutrophils. Data were pooled from 3 independent experiments. Means ± s.e.m. are indicated in red. Statistical significance is indicated by: ns, non-significant; *** P<0.001. C-D. Representative images of the macrophages (fluorescently labeled by mCherry) (C) and the neutrophils (fluorescently labeled by GFP) (E) of mock-injected and *ccr2* morpholino-injected larvae at 4 hpa. Scale bar = 100 µm. E-F. The number of macrophages (E) and neutrophils (F) that recruited to the wounded area at 4 hpa in 3 dpf *Tg*(*mpeg1:mCherry-F*)

larvae. Statistical analysis were performed by two-tailed t-test. A significantly reduced number of macrophages and neutrophils were recruited in *cxcr3.2* mutant larvae compared to the number in wild type controls. Data were pooled from 3 independent experiments. Means ± s.e.m. are indicated in red. Statistical significance is indicated by: *** P<0.001. G-H. Representative images of the macrophages (fluorescently labeled by mCherry) (G) and the neutrophils (stained using MPX assay) (H) of wild type and *cxcr3.2* mutant larvae at 4 hpa. Scale bar = 100 µm.

Beclomethasone attenuates almost all amputation-induced changes in gene expression in macrophages

To study whether glucocorticoid treatment changes the transcriptional response of macrophages to wounding, we performed a transcriptome analysis on FACS-sorted macrophages derived from larvae at 4 hpa. We found that 620 genes were significantly regulated by amputation, of which 411 genes were upregulated and 209 genes were downregulated (Figure 5A, D, E). When the larvae had been amputated and treated with beclomethasone, only 327 significantly regulated genes were identified, of which 260 genes were upregulated and 67 genes were downregulated (Figure 5B, D, E). Apparently, amputation-induced gene regulation in macrophages is attenuated by beclomethasone administration. To study the effect of beclomethasone on the amputation-induced changes in gene expression in macrophages in more detail, we plotted the level of regulation by the combined amputation and beclomethasone treatment against the regulation by amputation in the absence of beclomethasone, for all genes that were significantly regulated by at least one of these treatments (Figure 5F). The resulting scatter plot shows that 75.37% of the genes regulated by amputation showed attenuation of this regulation when amputation was performed in the presence of beclomethasone. These results indicate that beclomethasone has a very general and strong dampening effect on the amputation-induced changes in gene expression in macrophages, which is in contrast with the lack of inhibition of the migration of these cells towards the wounded area.

Interestingly, only a small overlap was observed between the cluster of 620 amputation-regulated genes and the cluster of 327 genes regulated by the combined amputation and beclomethasone treatment (Figure 5A, C, D, E). Only 60 and 11 genes were present in the overlap between these clusters for up-regulation and down-regulation respectively (Figure 5 D, E). A large overlap was observed between the gene cluster regulated by the combination treatment and the cluster regulated by beclomethasone (without amputation) (134 genes in total, Figure 5B, C, D, E, Supplementary Figure 1A). This indicates that the cluster of genes regulated by the combination treatment mainly contains genes that are regulated as a result of the beclomethasone treatment. Apparently, amputation hardly affects beclomethasone-induced changes in gene expression, whereas beclomethasone has a very strong effect on amputation-induced transcriptional changes. The smallest overlap was observed between the cluster of amputation-regulated genes and the cluster of beclomethasone-regulated

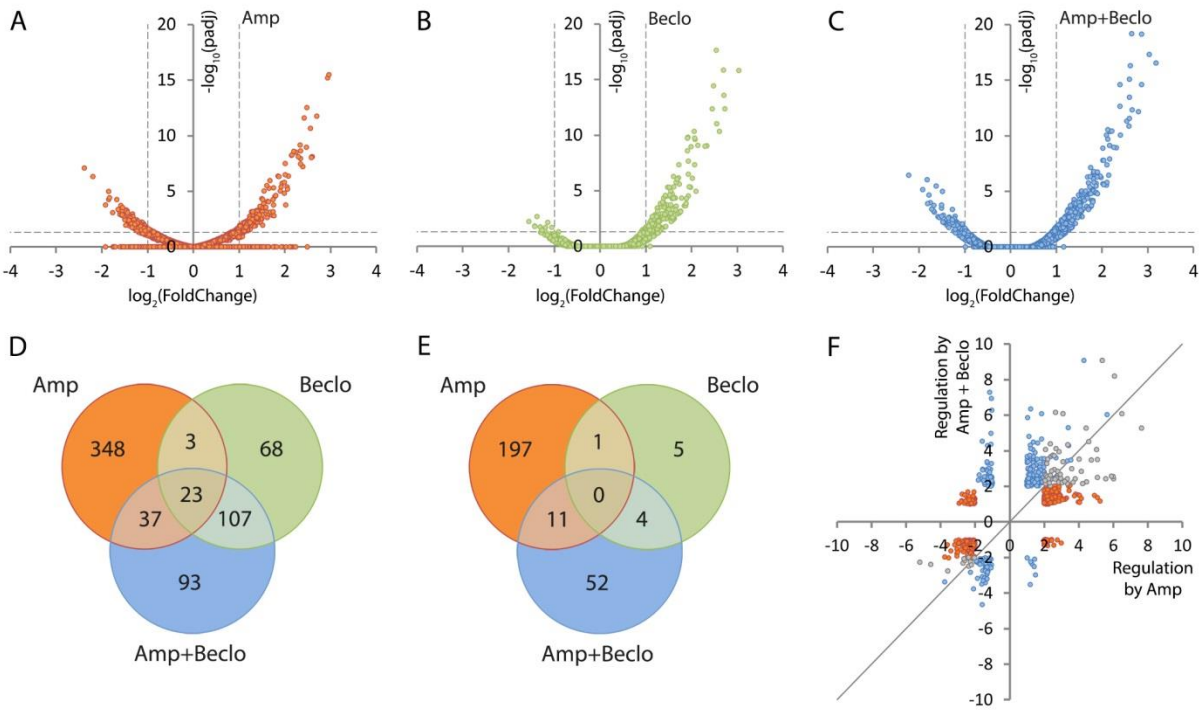

Figure 5. Macrophage-specific transcriptome analysis by RNA sequencing showing modulation of amputation-induced gene regulation by beclomethasone. A-C. Volcano plots indicating the fold change (x-axis) and p-value (y-axis) of the regulation for individual genes by amputation (A), beclomethasone (B) and the combined amputation/beclomethasone treatment (C), compared to the non-amputated, vehicle-treated control group. D-E. Venn diagrams showing overlaps between clusters of genes significantly upregulated (D) or downregulated (E) by amputation (Amp), beclomethasone (Beclo) and the combined amputation/beclomethasone treatment (Amp+Beclo). The diagrams show that there is a large number of genes regulated by amputation in macrophages. Beclomethasone affects the expression of a relatively small number of genes, but it decreases the number of genes significantly regulated upon amputation. F. Scatter plot showing the effect of beclomethasone treatment on amputation-regulated gene expression. For all genes showing significant regulation upon amputation (red and grey dots) or the combined beclomethasone and amputation treatment (blue and grey dots), the fold change due to beclomethasone and amputation treatment was plotted as a function of the fold change due to amputation. The grey dots represent the overlap between amputation and combination treatment. The grey line indicates the point at which beclomethasone treatment does not alter amputation-induced gene regulation. Of all the genes that were significantly regulated by amputation in macrophages, 75.37% showed attenuation in the presence of beclomethasone. Paired analysis were performed using DESeq (v1.26.0) R package by comparing each group to the control group (non-amputated/vehicle treated). Significantly regulated genes were selected by using a p.adj<0.05 and |FoldChange|>2 cutoff.

genes (Figure 5A, B, D, E, Supplementary Figure 1B), which suggests that upon amputation endogenous glucocorticoid signaling due to increased cortisol levels only regulates a small number of genes.

Using gene ontology analysis, we classified the regulated genes according to the KEGG-pathways they are involved in (Supplementary Figure 2, Supplementary Table 1). This analysis showed that the largest group of pathways regulated by amputation were involved in metabolism (16 pathways, 98 genes) and that 4 pathways (19 genes) involved in the immune system were altered. The combined amputation

and beclomethasone treatment affected a smaller number of pathways for both metabolism- and immune system-related pathways (12 pathways and 26 genes, and 1 pathway and 6 genes respectively). Only 5 of these pathways (Toll-like receptor signaling pathway, Insulin resistance, Biosynthesis of antibiotics, Galactose metabolism, Glycolysis/Gluconeogenesis) were both regulated by amputation and by the combination treatment. Beclomethasone treatment (without amputation) affected 7 pathways (5 metabolism-related), of which 6 were also regulated when the larvae were amputated in the presence of beclomethasone.

Among the significantly enriched metabolism-related KEGG pathways, we studied 3 specific pathways which are known to be associated with specific macrophage phenotypes: glycolytic metabolism which is increased in pro-inflammatory (M1) macrophages, and mitochondrial oxidative phosphorylation (OXPHOS) and tricarboxylic acid (TCA) cycle which are related to the anti-inflammatory (M2) phenotype [72]. We mapped the gene expression levels into these pathways (Supplementary Figure 3 A-C). The data showed that the vast majority of the mapped genes were up-regulated by amputation and this up-regulation was inhibited by beclomethasone treatment. We, therefore, conclude from the gene ontology analysis that amputation mainly up-regulates genes involved in metabolism and the immune system and that the vast majority of the amputation-induced changes in these gene ontology groups are attenuated by glucocorticoids.

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

Subsequently, we specifically analyzed the regulation of immune-related genes. For all immune-related genes that were significantly regulated by amputation, we plotted the regulation by amputation, by beclomethasone, and by the combination of amputation and beclomethasone (Figure 6). For the vast majority of these genes, the amputation-induced changes were attenuated by the administration of beclomethasone. Among those genes were 3 that are known to be associated with a pro-inflammatory (M1) phenotype of macrophages: *tnfa*, *il1b*, and *il6* [30, 73]. For 3 genes (*cd22*, *alox5ap*, and *tlr5b*), the amputation-induced regulation was enhanced by beclomethasone. These findings suggest that the differentiation of macrophages to a pro-inflammatory (M1) phenotype is sensitive to inhibition by glucocorticoids.

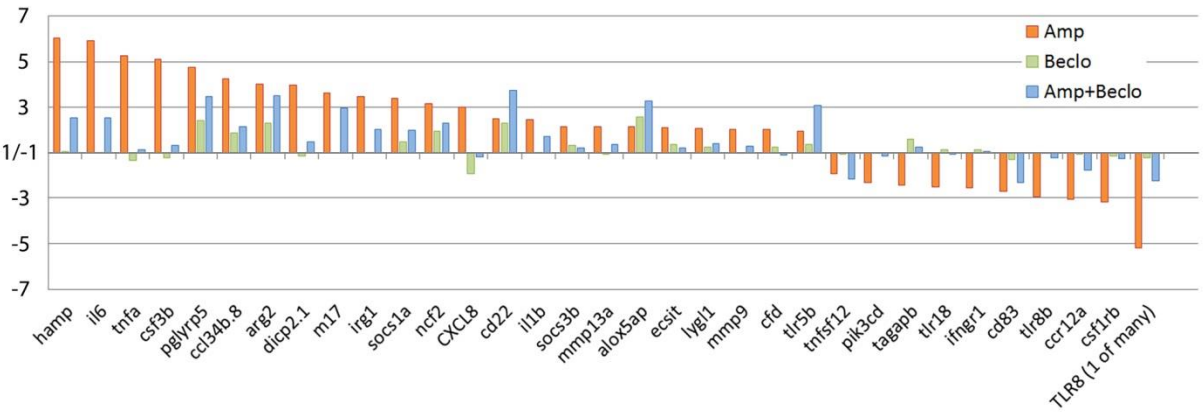

Figure 6. Regulation of immune-related genes in macrophages, determined by RNA sequencing analysis. For all genes significantly regulated upon amputation, the fold change due to amputation (Amp; red bars), beclomethasone (Beclo; green bars), and the combined amputation/beclomethasone treatment (Amp+Beclo; blue bars) is shown. The results show that beclomethasone dampens the amputation-induced expression of most genes, but for 4 genes (*cd22, alox5ap, tlr5b*) the combined treatment results in a higher fold change compared to the amputation treatment. Paired analysis were performed using DESeq (v1.26.0) R package by comparing each group to the control group (non-amputated/vehicle treated). Significantly regulated genes were selected by using a p.adj<0.05 and |FoldChange|>2 cutoff.

To study the glucocorticoid sensitivity of macrophage differentiation in more detail and validate some of the observed transcriptional changes, we performed qPCR on RNA samples isolated from FACS-sorted macrophages. At 4 hpa, the expression of 4 classic pro-inflammatory genes was measured: *il6, il1b, tnfa*, *mmp9*, of which the first 3 are markers for M1 macrophages and the 4th encodes a metalloproteinase that facilitates leukocyte migration by remodeling the extracellular matrix [30, 73, 74] (Figure 7A). The expression levels of *il6* and *il1b* showed an amputation-induced increase, and this increase was attenuated upon the combined beclomethasone and amputation treatment. The levels of *tnfa* and *mmp9* expression were not significantly increased by amputation, but the expression level of *tnfa* was significantly lower after the combination treatment compared to the amputation treatment.

In addition, we measured the expression levels of 4 markers for M2 macrophages, *arg2, cxcr4b, tgfb1* and *ccr2* [73, 75](Figure 7B). The expression level of *arg2* was induced by amputation at 4 hpa, and this induction was similar upon the combination treatment. The other genes were not upregulated by amputation at this time point, but upon beclomethasone treatment the expression of *cxcr4b* was increased. Since the M2 macrophage markers are expected to show increased expression levels during the resolution phase of the inflammatory response [73], we measured the expression of those genes in macrophages at 24 hpa as well (Supplementary Figure 4 A). However, no significant upregulation by amputation was observed for any of these 4 genes. Thus, in this experiment on M2 markers, we only

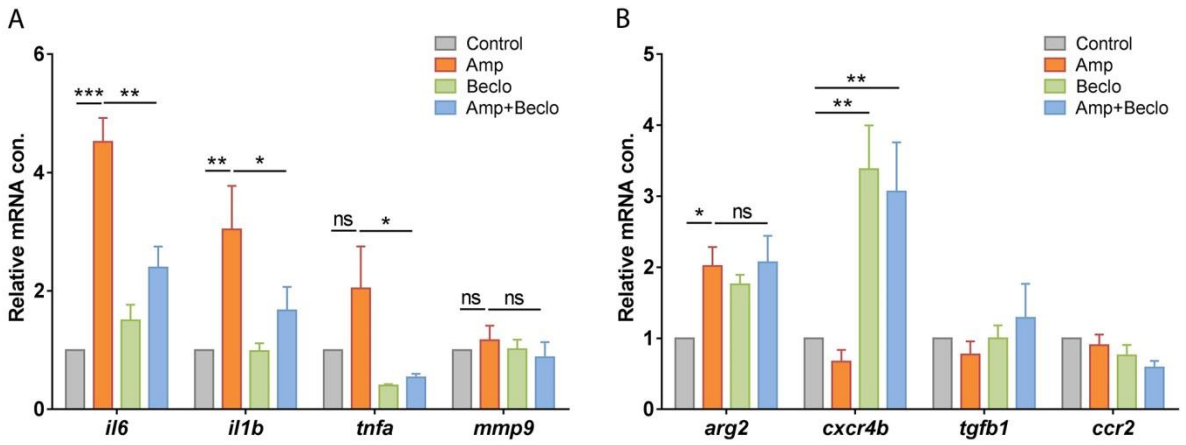

Figure 7. Expression levels of immune-related genes in FACS-sorted macrophages, determined by qPCR for *il6*, *il1b*, *tnfa, mmp9* (A) and for *arg2, cxcr4b, tgfb1, ccr2* (B) at 4hpa in 3 dpf larvae. Statistical analysis (ANOVA with a Fisher's LSD post hoc) showed the levels of *il6* and *il1b* expression were significantly increased by amputation, and this effect was inhibited by beclomethasone treatment. The expression level of *arg2* showed a significant increase upon amputation, and beclomethasone treatment did not affect this regulation. The expression level of *cxcr4b* was increased by beclomethasone treatment. Values shown are the means ± s.e.m. of three independent experiments. Statistical significance is indicated by: ns, non-significant; * P<0.05; ** P<0.01; *** P<0.001.

found an amputation-induced upregulation of the expression of *arg2* at 4 hpa, and this upregulation was insensitive to beclomethasone.

To further study the influence of beclomethasone on the differentiation of macrophages towards a pro-inflammatory (M1) phenotype, we used a reporter line for the expression of *tnfa*: the *Tg(mpeg1:mCherry-F/tnfa:eGFP-F)* fish line. Larvae from this line were amputated at 5 dpf, and at a more distal position than in the previous experiments to create a wound that recruits fewer macrophages which facilitates the visualization of individual *tnfa* expressing macrophages. We performed live confocal imaging at 2 and 4 hpa, and the GFP expression level in macrophages was used as a reporter for *tnfa* promoter activity *in vivo* (Figure 8 A-C). In the control group, an increase in the percentage of GFP-positive macrophages was observed between 2 and 4 hpa. During this time course, this percentage increased from 9.8±3.4% to 23.8±4.0%. The images show that GFP expression does not exclusively occur in macrophages that have reached the wounded area. In the beclomethasone-treated group, at both time points, a lower percentage of *tnfa* expressing macrophages was recruited to the wounded area compared to the control group (1.7±1.7% and 1.4±1.4% respectively).

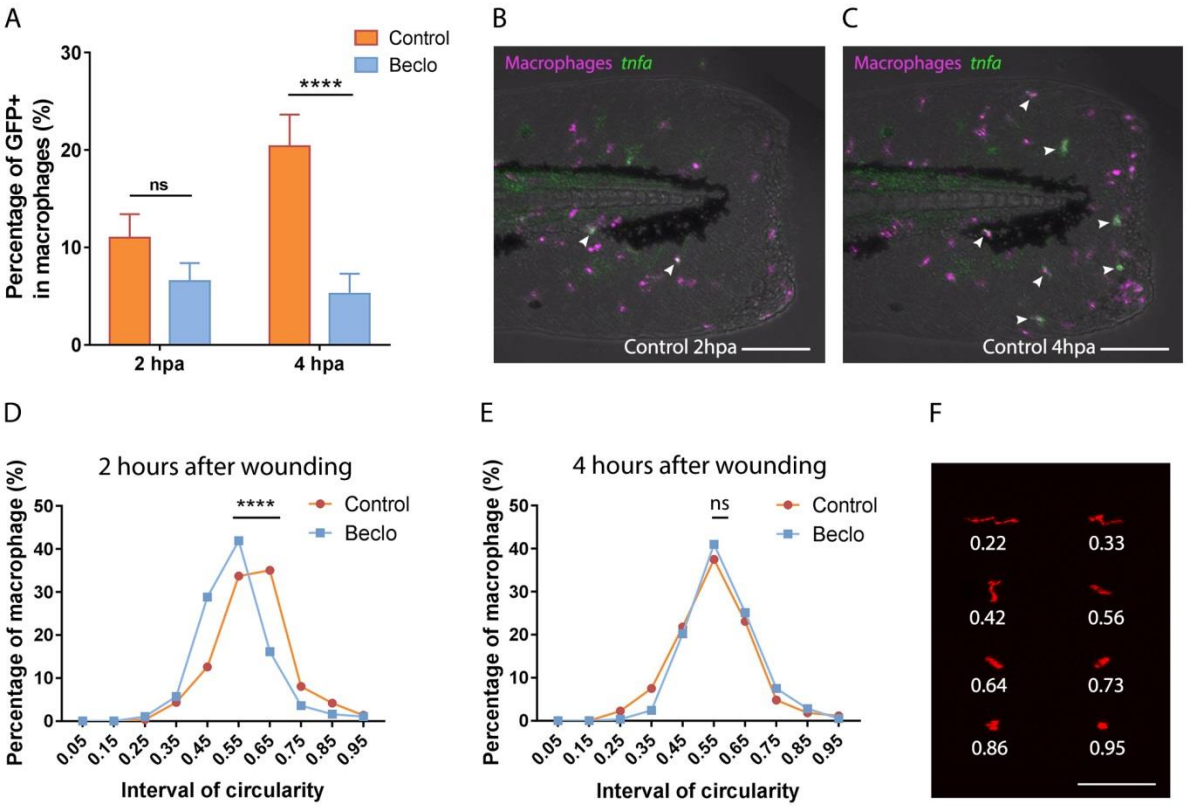

Figure 8. Effect of beclomethasone on the phenotype of macrophages. A. In *Tg(mpeg1:mCherry-F/tnfa:eGFP-F)* reporter larvae, the number of GFP-positive macrophages (as a percentage of the total number of macrophages) recruited to the wounded area were quantified at 2 and 4 hpa in 5 dpf larvae. Statistical analysis was performed by ANOVA with a Fisher's LSD post hoc. In the beclomethasone-treated group (Beclo), at 4 hpa, a significantly reduced percentage of the recruited macrophages was GFP-positive compared to the vehicle-treated group (Control). Values shown are means ± s.e.m. Statistical significance is indicated by: ns, non-significant; **** P<0.0001. B-C. Representative images of macrophages (fluorescently labeled by mCherry) and GFP-positive macrophages (fluorescently labeled by both mCherry and GFP) in the control group at 2 hpa (B) and 4 hpa (C). Scale bar = 100 µm. Arrow heads indicate macrophages displaying the GFP signal, which is a measure for activation of the *tnfa* promoter. D-E. The distribution of circularity of macrophages recruited to the wounded area at 2 h after wounding (a small hole was punched in the tail fin) (D) and at 4 h after wounding hpa (E) in 3 dpf *Tg(mpeg1:mCherry-F)* larvae. Statistical analysis were performed by Kolmogorov-Smirnov test. At 2 hpa, a significant difference of distribution pattern was observed between the two groups, with the beclomethasone-treated group (Beclo) shifted towards lower circularity. At 4 hpa, no significant difference was observed. Statistical significance is indicated by: ns, non-significant; **** P<0.0001. F. Representative images of macrophages analyzed in D and E and their corresponding circularity. Scale bar = 100 µm.

Finally, we analyzed the influence of beclomethasone on the morphology of macrophages, since macrophage morphology has been shown to be an indicator for their differentiation: M1 macrophages are generally less elongated and dendritic than M2 macrophages [73]. Instead of amputation, a small hole was punched in the tail fins of the larvae with a glass microcapillary needle to recruit a reduced number of leukocytes, which facilitated the visualization of individual cells. We performed live confocal imaging at 3 dpf with the *Tg(mpx:GFP/mpeg1:mCherry-F)* fish line and the circularity of mCherry-

positive macrophages was used to quantitate the morphology (Figure 8 D-E). In the control group, at 2 hpa, the percentage of macrophages with a high circularity (0.5-1.0) was relatively high (67.6±4.0%) and gradually decreased to 47.9±3.2% at 12 hpa (Supplementary Figure 5 A). In the beclomethasone-treated group, at 2 hpa the percentage of macrophages with a high circularity was lower (51.7±3.5%) and remained relatively stable until 12 hpa (Supplementary Figure 5 B). The most obvious difference between the control and beclomethasone-treated group was observed at 2 hpa. At this time point, the plot showing the distribution of circularity shows a clear shift towards a lower circularity in the beclomethasone-treated group (Figure 8 D). At 4 hpa, this difference of circularity distribution between the control group and beclomethasone-treated group had disappeared (Figure 8 E). The highly transient nature of the increased circularity is probably due to the small size of the wound in this experiment. These data from the analysis of the circularity may suggest an inhibitory effect of beclomethasone on the differentiation of macrophages towards a pro-inflammatory (M1) phenotype, in line with the data obtained using the *tnfa:eGFP-F* reporter line.

Discussion

Although glucocorticoids have been used as anti-inflammatory drugs for decades, their mechanism of action and the specificity of their effects have not been fully unraveled yet. Using the zebrafish tail amputation model, we have shown that the inflammatory response comprises glucocorticoid-sensitive and glucocorticoid-insensitive pathways. Glucocorticoids inhibit the migration of neutrophils towards a site of inflammation by inhibiting the induction of chemoattractants for this cell type. However, the migration of macrophages is not affected by glucocorticoids, since the induction of two chemoattractants that are critical for macrophage recruitment, *ccl2* and *cxcl11aa*, is insensitive to treatment with the glucocorticoid beclomethasone. Using RNAseq analysis we show that beclomethasone attenuates most transcriptional responses to amputation in macrophages and inhibit their differentiation towards a pro-inflammatory (M1) phenotype.

Chemoattractants are important trafficking signals that direct the movement of immune cells into and out of specific tissues [76]. In this study, we have demonstrated that glucocorticoids exert a specific inhibitory effect on the induction of the expression of two chemoattractants involved in neutrophil recruitment (Il8 and Cxcl18b). Using *in vitro* and *in vivo* models, it has been demonstrated that human and mouse neutrophil migration is dependent on the induction of Il8 expression [68, 77, 78] and that this induction is inhibited by glucocorticoids [79-81]. In mammals, Il8 has been demonstrated to signal through the chemokine receptors Cxcr1 and Cxcr2, whereas in zebrafish only Cxcr2 has been shown to mediate the effects of Il8 [66, 82]. Interestingly, our RNAseq data show that amputation increased the expression of *il8* in macrophages, and that this increase was strongly attenuated by beclomethasone.

These data suggest that the glucocorticoid inhibition of the neutrophil migration results at least partly from the suppression of chemoattractant expression in macrophages. Cxcl18b, a chemokine specific for fish and amphibian species, has also been shown to act as a ligand for Cxcr2 in zebrafish, thereby stimulating chemotaxis of neutrophils [67]. These findings suggest that Cxcr2 activation is crucial for the migration of neutrophils and that glucocorticoids inhibit this migration by attenuating the induction of the expression of Cxcr2 agonists like Il8 and Cxcl18b.

In contrast to the inhibitory effect on neutrophil migration, our study revealed that glucocorticoids leave the induction of chemoattractants involved in macrophage recruitment (Ccl2 and Cxcl11aa) unaffected. Ccl2, also known as monocyte chemoattractant protein-1 (MCP-1), and Cxcl11aa have been shown to be key chemokines implicated in macrophage migration and infiltration in humans and mice [64, 83-87]. In zebrafish, their role as chemoattractants for macrophages has been demonstrated during mycobacterial infection [65, 66, 71]. Our data show that these two chemoattractants also promote macrophage migration in the tail amputation model and that beclomethasone has no effect on the amputation-induced increase in their expression levels. The RNAseq analysis showed very low expression levels of *ccl2* and undetectable levels of *cxcl11aa* expression in macrophages, which suggests that the contribution of these cells to the increased expression of these chemokines is limited.

In line with our findings, it has been shown in bronchoalveolar lavage fluid of COPD patients that glucocorticoid treatment reduces neutrophil numbers, but that the number of macrophages was not decreased [88]. Contrary to our findings, in most of the studies carried out in humans and rats, the inflammation-induced Ccl2 level has been found to be inhibited by glucocorticoids [83, 89, 90], and this inhibition is related to a decreased p38 MAPK phosphorylation [90, 91]. Similarly, glucocorticoids have been shown to inhibit Cxcl11 upregulation in fluticasone propionate-stimulated peripheral blood monocytes, and in IFN-γ- or LPS-stimulated RAW 264.7 macrophages, as well as in multiple tissues of endotoxemia mice [28, 92]. Nevertheless, some studies do show an insensitivity of the mammalian Ccl2 or Cxcl11aa induction to glucocorticoid treatment. In a breast cancer cell line (T47D), glucocorticoid treatment has no effect on Il1-stimulated Ccl2 production [93], and in A549 epithelial cells, IFNγ-induced Cxcl11 is insensitive to glucocorticoid treatment [94]. These data suggest that the observed insensitivity of the *ccl2* and *cxcl11a* induction to glucocorticoids, which underlies the glucocorticoid insensitivity of macrophage migration, requires a specific context, which may involve factors like the activating signal, the glucocorticoid treatment regime, or the cell type and tissue involved.

Although glucocorticoids did not affect the migration of macrophages in our study, they did have a big impact on the transcriptional changes in these cells upon amputation. We showed by RNAseq analysis

in FACS-sorted macrophages that, similarly to our previous findings from a microarray analysis carried out on RNA isolated from whole larvae [59], most of the amputation-induced transcriptional changes are decreased by beclomethasone, whereas a small subset of transcriptional responses is insensitive to glucocorticoid treatment. Focusing on the regulation of immune-related genes, we found that, in line with our previous findings in whole larvae [59], beclomethasone suppressed the induction of almost all pro-inflammatory, M1 associated, genes, like *il6*, *tnfa*, *il1b*, *il8* and *mmp9*. In line with these data, many genes involved in glycolysis, a metabolic pathway often associated with an M1 phenotype [72, 95, 96], were upregulated upon amputation and this upregulation was mostly inhibited by beclomethasone. This inhibitory effect of glucocorticoids on the induction of pro-inflammatory genes in macrophages is in agreement with *in vitro* results obtained in LPS-stimulated primary mouse macrophages [17, 18, 97]. In addition, *in vivo* data obtained in mouse models for arthritis and acute lung injury demonstrated an inhibitory effect of glucocorticoids on the differentiation of macrophages towards a pro-inflammatory M1 phenotype [31, 32]. In the present study, we observed a reduction in the number of macrophages with activation of a *tnfa:eGFP-F* reporter gene upon beclomethasone administration, and a morphology characterized by a low circularity, which demonstrates that the macrophage differentiation to an M1 phenotype was inhibited by the glucocorticoid treatment. Taken together, these data strongly support the idea that glucocorticoids inhibit the differentiation of macrophages to an M1 phenotype by interfering at the level of transcription.

This glucocorticoid effect on macrophages may have great clinical relevance, since this cell type has been identified as the main target for glucocorticoid action in several animal models for inflammatory diseases [98-100]. In murine models for contact allergy and septic shock it has been shown that the anti-inflammatory effect of glucocorticoids depends on the presence of GR in macrophages, suppressing the induction of pro-inflammatory mediators like IL-1β [99, 101]. These glucocorticoid effects are absent in a mouse line with a deficiency in GR dimerization, suggesting that activation of anti-inflammatory gene transcription through GRE binding may be the main GR mechanism of action [99, 101]. Furthermore we conclude that the glucocorticoid resistance observed in macrophage-dominated inflammatory diseases like COPD cannot be attributed to a general insensitivity of macrophages to the immune-suppressive effects of glucocorticoids.

In addition to the effect of glucocorticoids on M1 differentiation, we investigated their effect on the differentiation of macrophages to an M2 phenotype. Previous studies in a mouse arthritis model showed that the induction of an M2 phenotype was not affected by glucocorticoids [31] and in an acute lung injury model [32] it was shown to be enhanced. In our RNAseq and qPCR analysis, the M2 marker *arg2* [30, 73, 75] was among the small subset of amputation-induced genes that were insensitive to beclomethasone, suggesting that the differentiation to an M2 phenotype is insensitive

to glucocorticoids. However, genes involved in the TCA cycle and OXPHOS, metabolic pathways associated with an M2 phenotype [72, 95, 96], were upregulated upon amputation and this upregulation was inhibited by beclomethasone, which would suggest that M2 differentiation is blocked by glucocorticoid treatment. In our qPCR analysis, we showed that the expression of various other M2 markers (*cxcr4b, tgfb1, ccr2*) was not increased upon amputation. The variation in responses of M2 markers to amputation and /or glucocorticoid treatment in our assay supports the idea that the M2 phenotype of macrophages may occur as various alternative differentiation states [29, 30]. Independent of the amputation, beclomethasone increased the expression of *cxcr4b* (and to a lesser extent *ccr2*), in line with previous observations that glucocorticoids induce the differentiation of human macrophages to an M2 phenotype *in vitro* [28, 102]. In summary, whereas the amputation-induced increases in the expression levels of M1 markers are consistently inhibited by beclomethasone, increased expression of M2 markers (when present in our assay) can be either insensitive or suppressed by glucocorticoid treatment.

In our tail amputation model for inflammation, the vast majority of macrophage transcriptional responses was suppressed by glucocorticoids and only a small subset of these responses was not affected. Studies in murine models for inflammatory diseases suggest that the anti-inflammatory GR action in macrophages depends on GRE-dependent transcriptional regulation, probably reducing the activation of a subset of pro-inflammatory transcription factors [98, 99, 101]. Alternatively, our data may indicate an important role for GR interaction ('tethering') with the transcription factor NF-κB, since in many studies it has been shown that the NF-κB-mediated transcriptional activation can be suppressed by GR or remains unaffected [17-20]. Recruitment of IRF3 to the transcription initiation complex has been shown to be associated with sensitivity to GR suppression [17, 97].

In conclusion, our *in vivo* study of the glucocorticoid modulation of the transcriptional responses to wounding using the zebrafish tail amputation model shows that the vast majority of these responses are sensitive to glucocorticoids, and only a small subset are insensitive. These insensitive responses play a role in the migration of macrophages and possibly their differentiation to an M2 phenotype, whereas the sensitive responses are involved in the migration of neutrophils and the differentiation of macrophages to an M1 phenotype. Our data demonstrate that these processes can be regulated independently, and that glucocorticoids exert their immunosuppressive effects on macrophages by modulating differentiation rather than migration.

Materials and methods

Zebrafish lines and maintenance

Zebrafish were maintained and handled according to the guidelines from the Zebrafish Model Organism Database (http://zfin.org) and in compliance with the directives of the local animal welfare committee of Leiden University. They were exposed to a 14 hours light and 10 hours dark cycle to maintain circadian rhythmicity. Fertilization was performed by natural spawning at the beginning of the light period. Eggs were collected and raised at 28°C in egg water (60 µg/ml Instant Ocean sea salts and 0.0025% methylene blue).

The following fish lines were used in this work: wild type (wt) strain AB/TL, the double transgenic lines *Tg(mpx:GFP/mpeg1:mCherry-F)* [49, 103] and *Tg(mpeg1:mCherry-F/tnfa:eGFP-F)* [73], and the combination of *Tg(mpeg1:mCherry-F)* and the homozygous mutants (*cxcr3.2*$^{-/-}$) or wt siblings (*cxcr3.2*$^{+/+}$) of the *cxcr3.2*^{hu6044} mutant strain [66].

Tail amputation and chemical treatments

After anesthesia with 0.02% aminobenzoic acid ethyl ester (tricaine, Sigma Aldrich), the tails of 3 days post fertilization (dpf) embryos were partially amputated (Figure 1A) with a 1mm sapphire blade (World Precision Instruments) on 2% agarose-coated Petri dishes under a Leica M165C stereomicroscope [49, 59]. In the experiment on larvae from the *Tg(mpeg1:mCherry-F/tnfa:eGFP-F)* line, the site of amputation was more distal, so the wound attracted a lower number of leukocytes, which facilitated the imaging of individual cells (Figure 8A-C). In the experiment in which we determined the morphology of the macrophages (Figure 8D-F), a hole was punched in the tail fin with a glass microcapillary needle (Harvard Apparatus, preparation of needles with 10-20 µm outer diameter described in [104], in order to make an even smaller wound and attract an even lower number of leukocytes. Wounded and non-wounded (control) embryos were pretreated for 2 hours with 25 µM beclomethasone (Sigma Aldrich) or vehicle (0.05% dimethyl sulfoxide (DMSO)) in egg water prior to amputation/wounding and received the same treatment after the amputation/wounding.

Imaging and image quantification

Images of fixed or live larvae were captured using a Leica M205FA fluorescence stereomicroscope, equipped with a Leica DFC 345FX camera. In all fish lines used, the macrophages were detected based on the fluorescence of their mCherry label. Neutrophils were detected based on either their fluorescent GFP label or their mpx staining. To quantify the number of macrophages and/or neutrophils recruited to the wounded area, the cells in a defined area of the tail (Figure 1A) were

counted manually. Data were pooled from two or three independent experiments, and the means ± s.e.m. of the pooled data are indicated.

Confocal microscopy and image analysis

For time lapse imaging and automated tracking of the leukocyte migration, the amputated larvae were mounted in 1.2% low melting agarose in egg water containing 0.02% tricaine and 25 µM beclomethasone or 0.05% DMSO on 40 mm glass bottom dishes (Willco-Dish) and covered with 1.5 ml egg water containing tricaine and the corresponding chemicals. Confocal microscopy was performed using a Nikon Eclipse Ti-E microscope with a Plan Apo 20X/0.75 NA objective. A 488 nm laser was used for excitation of GFP and a 561 nm laser was used for excitation of mCherry. Time-lapse microscopy was performed at 28 °C with an interval of approximately one minute. From the obtained z-stacks, aligned maximum projection images were generated using NIS-Elements, which were further analyzed using Image J with custom-made plugins, developed by Dr. Joost Willemse (Leiden University), for localizing and tracking of cells ('Local Maxima Stack' and 'Track Foci', algorithms described previously in [105] and determining their circularity (calculated as (area x 4π)/(circumference)^2).

Morpholino injection

A morpholino targeting the translational start site of the *ccr2* gene (5'AACTACTGTTTTGTGTCGCCGAC3', purchased from Gene Tools) [71] was prepared and stored according to the manufacturer's instructions. Injection of 1 nl (0.5 mM) of the morpholino solution was performed into the yolk of fertilized eggs at the 1-2 cell stage.

RNA isolation, cDNA synthesis and quantitative PCR (qPCR)

At different time points after amputation, larvae were collected (15-20 per sample) in QIAzol lysis reagent (QIAGEN) for RNA isolation, which was performed using the miRNeasy mini kit (Qiagen), according to the manufacturer's instructions. Extracted total RNA was reverse-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad). QPCR was performed on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) using iTaq™ Universal SYBR® Green Supermix (Bio-Rad). The sequences of the primers used are provided in Supplementary Table 2. Cycling conditions were pre-denaturation for 3 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and elongation for 30 s at 72°C. Fluorescent signals were detected at the end of each cycle. Cycle threshold values (Ct values, i.e. the cycle numbers at which a threshold value of the fluorescence intensity was reached) were determined for each sample. For each sample, the Ct value was subtracted from the Ct value of a control sample, and the fold change of gene expression was calculated and adjusted to the

expression levels of a reference gene (*peptidylprolyl isomerase Ab* (*ppiab*)). Data shown are means ± s.e.m. of three independent experiments.

Myeloperoxidase (Mpx) staining

Larvae were fixed in 4% paraformaldehyde (PFA, Sigma Aldrich) at 4°C overnight, and rinsed with PBS containing 0.05% Tween20. The myeloperoxidase (mpx) staining for the *cxcr3.2* mutant line was performed using the Peroxidase (Myeloperoxidase) Leukocyte kit (Sigma Aldrich), according to the manufacturer's instructions. To visualize both macrophages and neutrophils in the same larvae, the mpx staining was always performed after imaging of the fluorescent signal of the macrophages.

Fluorescence-Activated Cell Sorting (FACS) of macrophages

Macrophages were sorted from *Tg(mpeg1.4:mCherry-F)* embryos as previously described [106, 107]. Dissociation was performed with 100-150 embryos for each sample at 4 hours post amputation (hpa) using Liberase TL (Roche) and stopped by adding Fetal Calf Serum (FCS) to a final concentration of 10%. Isolated cells were resuspended in Dulbecco's PBS (DPBS), and filtered through a 40 µm cell strainer. Actinomycin D (Sigma Aldrich) was added (final concentration of 1 µg/ml) to each step to inhibit transcription. Macrophages were sorted based on their red fluorescent signal using a FACSAria III cell sorter (BD Biosciences). The sorted cells were collected in QIAzol lysis reagent (Qiagen) for RNA isolation. Extracted total RNA was either reverse-transcribed for qPCR or amplified using the SMART-seq V4 kit (Clontech) for sequencing.

Transcriptome analysis

A total of 12 samples (four experimental groups obtained from three replicate experiments) were processed for transcriptome analysis using cDNA sequencing. The RNA seq libraries generated with the SMART-seq V4 kit were sequenced using an Illumina HiSeq 2500 instrument according to the manufacturer's instructions with a read length of 50 nucleotides. Image analysis and base calling were done by the Illumina HCS version 2.2.68, and RTA version 1.18.66. cDNA sequencing data were analyzed by mapping the reads to the Danio rerio GRCz10 reference genome with annotation version 80 using Tophat (v2.1.0). Subsequently, the DESeq (v1.26.0) R package was used to test for differential expression. Before each analysis, the genes with low reads were removed (i.e. those genes for which the sum of reads from three replicates of the analyzed two groups was lower than 30). The output data were used for transcriptome analysis. Significant gene regulation was defined by using p.adj<0.05 and |FoldChange|>2 cutoffs. The raw data are available at Gene Expression Omnibus database under accession number GSE122643.

Gene ontology analysis was performed using the online functional classification tool Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/ summary.jsp). Further analysis of the macrophage transcriptomes was performed in R v3.4.3 using *Bioconductor* v3.6. Zebrafish Ensembl gene IDs were converted to Entrez Gene IDs using the R package *org.Dr.eg.db* v3.5.0. The enriched pathways in different groups were determined by comparing the statistically differentially expressed genes against the KEGG zebrafish database using the *kegga*() function from the *edgeR* package v3.20.7. Finally, gene expression data were mapped into significantly enriched KEGG pathways using *pathview* v1.18.0.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 by one-way or two-way ANOVA (Figure 1, Figure 2 , Figure 3, Figure 7, Figure 8A, Supplementary Figure 4), Kolmogorov-Smirnov test (Figure 8D, E) or two-tailed t-test (Figure 4). Significance was accepted at $p<0.05$ and different significance levels are indicated: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

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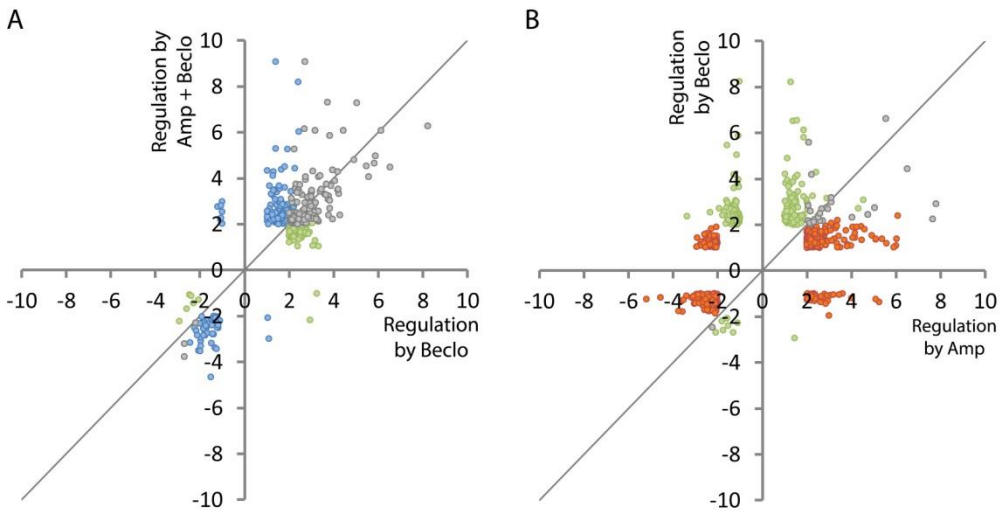
Supplementary Material

Online Movie 1 can be found at: http://movie.biologists.com/video/10.1242/dmm.037887/video-1

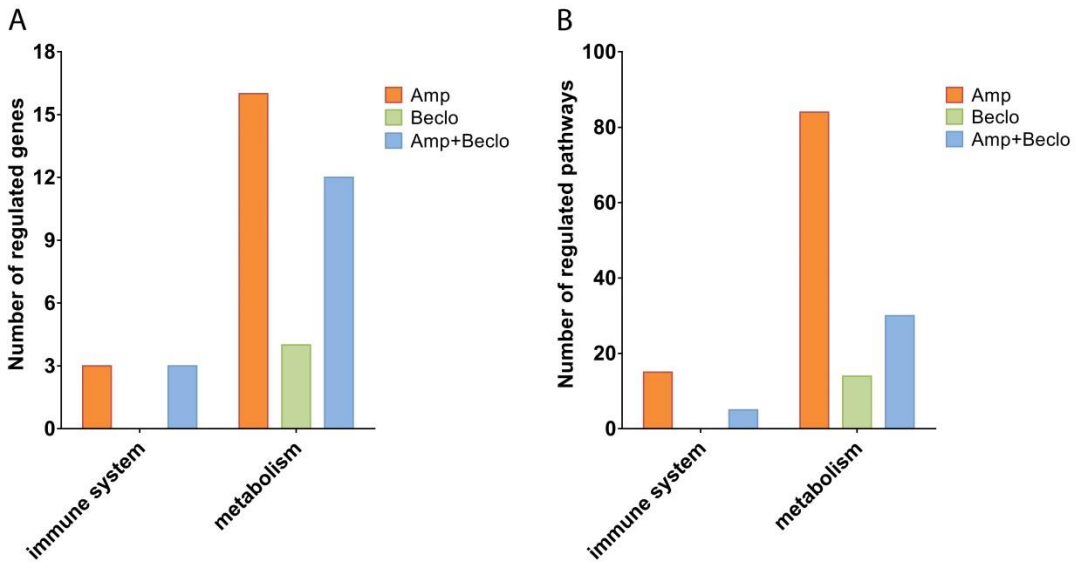
Online Movie 1. Macrophage migration upon tail amputation. Tracks of macrophages migrating between 1.5 and 5.5 hpa in 3 dpf larvae in the vehicle-treated group. Confocal microscopy images were analyzed using ImageJ with custom-made plugins, developed by Dr. Joost Willemse (Leiden University), for localization and tracking of cells.

Online Movie 2 can be found at: http://movie.biologists.com/video/10.1242/dmm.037887/video-2

Oneline Movie 2. Neutrophil migration upon tail amputation. Tracks of neutrophils migratimg between 1.5 and 5.5 hpa in 3 dpf larvae in the vehicle-treated group. Confocal microscopy images were analyzed using ImageJ with custom-made plugins, developed by Dr. Joost Willemse (Leiden University), for localization and tracking of cells.

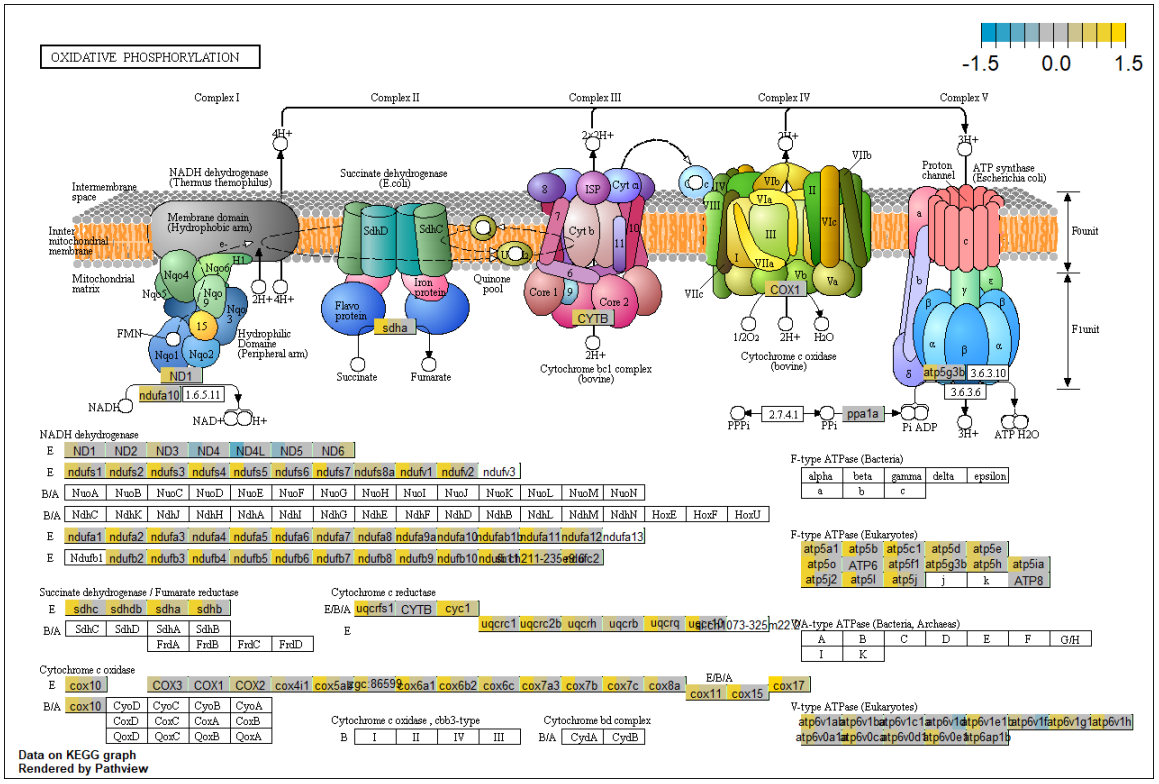


Supplementary Figure 1. Scatter plot showing the difference and overlaps in gene regulation between different treatments. A. Comparison between beclomethasone and combined beclomethasone and amputation treatment. For all genes showing significant regulation upon beclomethasone (green and grey dots) or the combined beclomethasone and amputation treatment (blue and grey dots), the fold change due to beclomethasone and amputation treatment was plotted as a function of the fold change due to beclomethasone. The plot shows a large group of genes (grey dots) regulated by both treatments. B. Comparison between amputation and beclomethasone treatment. For all genes showing significant regulation upon amputation (red and grey dots) or beclomethasone treatment (green and grey dots), the fold change due to beclomethasone treatment was plotted as a function of the fold change due to amputation. The plot shows a very small group of genes (grey dots) regulated by both treatments. The grey line indicates the point at which the two different treatment have the same effect. Significantly regulated genes were selected by using a p.adj<0.05 and |FoldChange|>2 cutoff.

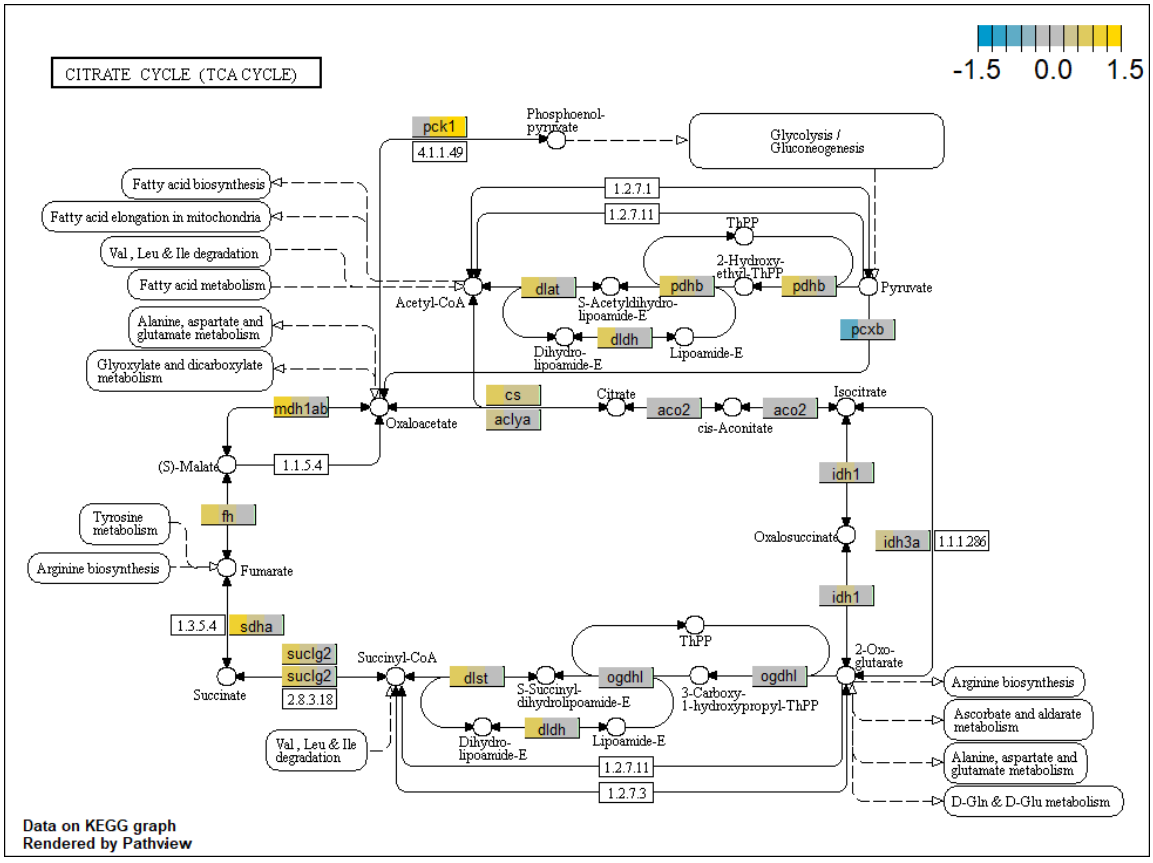


Supplementary Figure 2. Gene ontology analysis of RNA sequencing experiment. A. The number of KEGG pathways overrepresented in clusters of genes significantly regulated by amputation, beclomethasone, and amputation+beclomethasone. Amputation regulated pathways involved in the immune system and in metabolism. Amputation+beclomethasone treatment regulated a lower number of regulated pathways involved in both the immune system and metabolism. B. The number of regulated genes involved in the overrepresented pathways. Amputation regulated genes involved in immune- and metabolism-related pathways. Amputation+beclomethasone regulated a lower number of genes involved in these pathways.

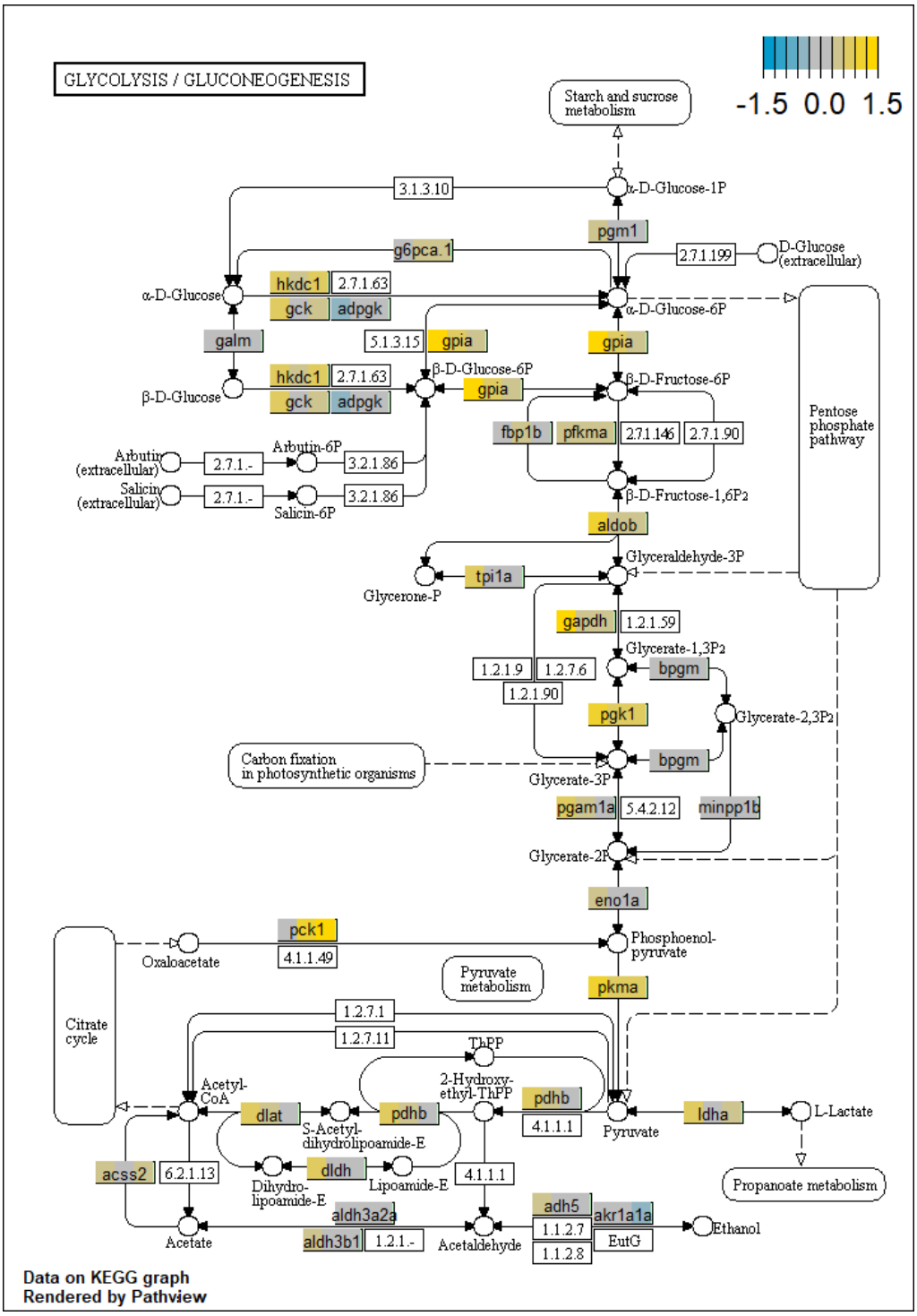
A


OXIDATIVE PHOSPHORYLATION
-1.5 0.0 1.5
Complex I
Complex II
Complex III
Complex IV
Complex V
NADH dehydrogenase (Thermus thermophilus)
Succinate dehydrogenase (E.coli)
Cytochrome bcl complex (bovine)
Cytochrome c oxidase (bovine)
ATP synthase (Escherichia coli)
Intermembrane space
Inner mitochondrial membrane
Mitochondrial matrix
Membrane domain (Hydrophobic arm)
Hydrophilic Domain (Peripheral arm)
Quinone pool
Proton channel
F1unit
Fo unit
Data on KEGG graph
Rendered by Pathview

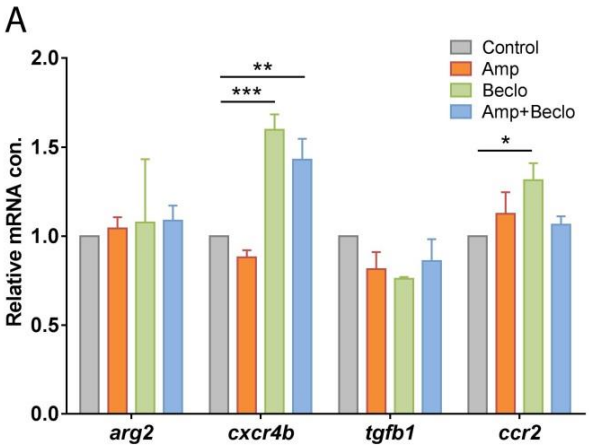
B


CITRATE CYCLE (TCA CYCLE)
-1.5 0.0 1.5
Phosphoenol-pyruvate
Glycolysis / Gluconeogenesis
Fatty acid biosynthesis
Fatty acid elongation in mitochondria
Val , Leu & Ile degradation
Fatty acid metabolism
Alanine, aspartate and glutamate metabolism
Glyoxylate and dicarboxylate metabolism
Acetyl-CoA
S-Acetyldihydro-lipoamide-E
Dihydro-lipoamide-E
Lipoamide-E
ThPP
2-Hydroxy-ethyl-ThPP
Pyruvate
Oxaloacetate
Citrate
cis-Aconitate
Isocitrate
(S)-Malate
Tyrosine metabolism
Arginine biosynthesis
Fumarate
Oxalosuccinate
Succinate
Succinyl-CoA
S-Succinyl-dihydrolipoamide-E
3-Carboxy-1-hydroxypropyl-ThPP
2-Oxo-glutarate
Arginine biosynthesis
Ascorbate and aldarate metabolism
Alanine, aspartate and glutamate metabolism
D-Gln & D-Glu metabolism
Data on KEGG graph
Rendered by Pathview

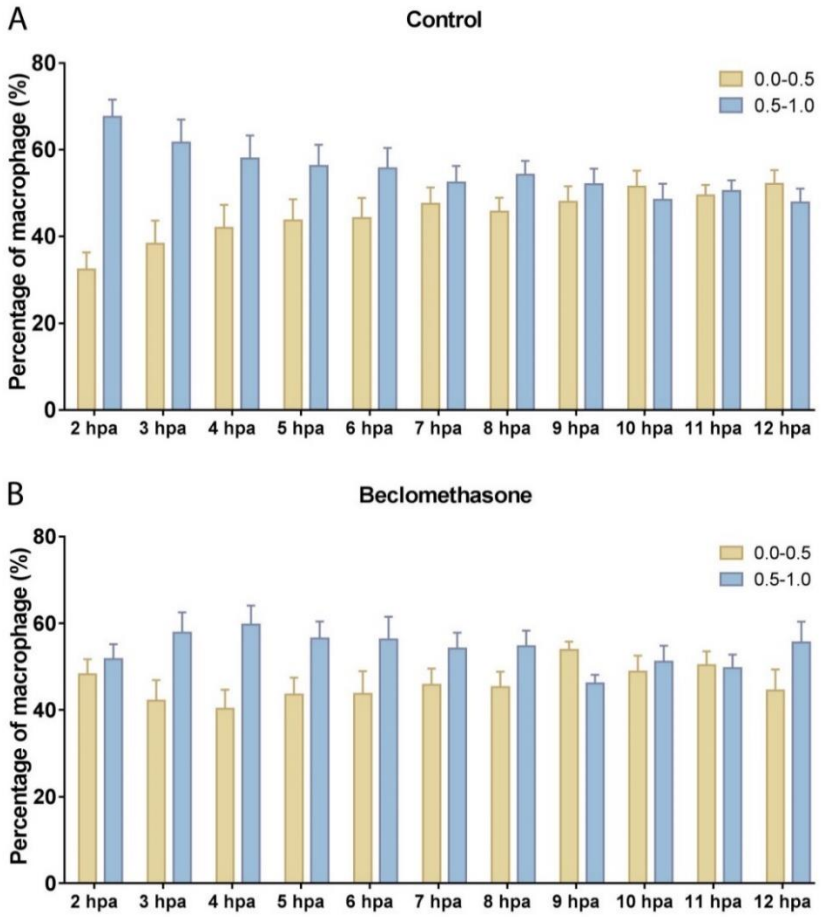
C



Supplementary Figure 3. Significantly enriched KEGG pathways mapped with gene expression level. In the oxidative phosphorylation (A), Citrate cycle (TCA cycle) (B) and Glycolysis/ Gluconeogenesis (C) pathways, the fold change of the genes is indicated by different colour and the intensity of the colour as shown in the scale. The three colour shown in one gene indicated the logarithm fold change of amputation, beclomethasone and amputation+beclomethasone treatment respectively. The maps show that the vast majority of the amputation-induced changes in gene expression are inhibited by beclomethasone treatment in these pathways.



Supplementary Figure 4. Expression levels of immune-related genes in FACS-sorted macrophages, determined by qPCR for *arg2, cxcr4b, tgfb1, ccr2* (A) at 24hpa in 3 dpf larvae. The expression level of *cxcr4b* was increased by beclomethasone treatment. Statistical analysis were performed by ANOVA with a Fisher's LSD post hoc. Values shown are the means ± s.e.m. of three independent experiments. Statistical significance is indicated by: * P<0.05; ** P<0.01; *** P<0.001.



Supplementary Figure 5. Effect of beclomethasone on the morphology of macrophages. The percentage of macrophages with different levels of circularity (0.0-0.5 and 0.5-1.0) were determined over time for the control group (A) and beclomethasone-treated group (B). In the control group, an increase in the percentage of macrophages with a circularity level between 0.0 and 0.5 and a decrease in the percentage of macrophages with a level between 0.5 and 1.0 was observed. In the beclomethasone-treated group, these changes were not observed. Values shown are means ± s.e.m..

Supplementary Table 1. KEGG-pathways, based on RNA sequencing analysis.

Amputation

Term	Count	%	P-value	Fold Enrichment
Immune system				
NOD-like receptor signaling pathway	6	1.03	2.89E-02	3.43
Toll-like receptor signaling pathway	8	1.37	6.13E-02	2.26
Cytokine-cytokine receptor interaction	11	1.89	6.58E-02	1.88
Jak-STAT signaling pathway	8	1.37	9.53E-02	2.04
Metabolism				
Oxidative phosphorylation	28	4.81	2.08E-13	5.60
Carbon metabolism	23	3.95	2.72E-10	5.15
Biosynthesis of antibiotics	31	5.33	4.11E-10	3.73
Metabolic pathways	83	14.26	1.20E-09	1.84
Glycolysis / Gluconeogenesis	17	2.92	3.07E-09	6.52
Biosynthesis of amino acids	13	2.23	3.87E-05	4.28
Arginine and proline metabolism	10	1.72	1.33E-04	5.00
Pentose phosphate pathway	7	1.20	6.51E-04	6.32
Citrate cycle (TCA cycle)	6	1.03	5.67E-03	5.09
Fructose and mannose metabolism	6	1.03	1.42E-02	4.10
Pyruvate metabolism	6	1.03	1.73E-02	3.91
Galactose metabolism	5	0.86	2.82E-02	4.24
Insulin resistance	10	1.72	4.62E-02	2.11
Glycine, serine and threonine metabolism	5	0.86	7.43E-02	3.11
Propanoate metabolism	4	0.69	7.54E-02	4.00
RNA degradation	7	1.20	8.06E-02	2.31
Others				
Cardiac muscle contraction	13	2.23	1.30E-04	3.79

Amputation & Beclomethasone

Term	Count	%	P-value	Fold Enrichment
Immune system				
Toll-like receptor signaling pathway	6	1.97	8.62E-03	4.64
Metabolism				
Insulin signaling pathway	10	3.28	3.19E-04	4.45
Starch and sucrose metabolism	5	1.64	6.92E-04	11.96
Insulin resistance	8	2.62	1.49E-03	4.60
Steroid hormone biosynthesis	4	1.31	1.16E-02	8.27
FoxO signaling pathway	7	2.30	2.16E-02	3.15
Biosynthesis of antibiotics	8	2.62	2.94E-02	2.63
Arginine biosynthesis	3	0.98	3.76E-02	9.57
Glyoxylate and dicarboxylate metabolism	3	0.98	6.69E-02	6.96
Galactose metabolism	3	0.98	6.69E-02	6.96
Glycolysis / Gluconeogenesis	4	1.31	6.72E-02	4.19
Retinol metabolism	3	0.98	8.94E-02	5.89
Alanine, aspartate and glutamate metabolism	3	0.98	9.33E-02	5.74
Others				
Adipocytokine signaling pathway	5	1.64	2.43E-02	4.45

Beclomethasone

Term	Count	%	P-value	Fold Enrichment
Metabolism				
FoxO signaling pathway	8	4.08	1.48E-03	4.57
Insulin signaling pathway	8	4.08	1.59E-03	4.51
Insulin resistance	6	3.06	1.06E-02	4.38
Glyoxylate and dicarboxylate metabolism	3	1.53	4.35E-02	8.82
Steroid hormone biosynthesis	3	1.53	5.35E-02	7.87
Others				
Focal adhesion	7	3.57	3.80E-02	2.74
Adipocytokine signaling pathway	4	2.04	5.57E-02	4.51

Supplementary Table 2. Sequences of Primers used in qPCR reactions.

Gene name	Gene accession	Sequence (5'-3')
ppail	ENSDARG00000103994	**Fw:** CATCCACAACCTTCCCGAACAC
		Rv: ACACTGAAACACGGAGGCAAAG
ccl2	ENSDARG00000041835	**Fw:** GTCTGGTGCTCTTCGCTTTC
		Rv: TGCAGAGAAGATGCGTCGTA
cxcl11aa	ENSDARG00000100662	**Fw:** ACTCAACATGGTGAAGCCAGTGCT
		Rv: CTTCAGCGTGGCTATGACTTCCAT
il8	ENSDARG00000104795	**Fw:** TGTGTTATTGTTTTCCTGGCATTTC
		Rv: GCGACAGCGTGGATCTACAG
cxcl18b	ENSDARG00000075045	**Fw:** TCTTCTGCTGCTGCTTGCGGT
		Rv: GGTGTCCCTGCGAGCACGAT
il6	ENSDARG00000102318	**Fw:** CGCTAAGGCAACTGGAAGAC
		Rv: CCAGACCACTGGGAAACACT
il1b	ENSDARG00000098700	**Fw:** TGTGTGTTTGGGAATCTCCA
		Rv: CTGATAAACCAACCGGGACA
tnfa	ENSDARG00000009511	**Fw:** ACCAGGCCTTTTCTTCAGGT
		Rv: TTTGCCTCCGTAGGATTCAG
mmp9	ENSDARG00000042816	**Fw:** CATTAAAGATGCCCTGATGTATCCC
		Rv: AGTGGTGGTCCGTGGTTGAG
arg2	ENSDARG00000039269	**Fw:** AAGGCCATTCTCAGCAGTGT
		Rv: AGGTTTCCCGAAGGTGAAGT
cxcr4b	ENSDART00000061499	**Fw:** GCGACCTCTCAGTCAGCAAT
		Rv: TCACAAGCACCACAAGTCCA
tgfb1	ENSDARG00000041502	**Fw:** CAACCGCTGGCTCTCATTTGA
		Rv: ACAGTCGCAGTATAACCTCAGCT
ccr2	ENSDARG00000079829	**Fw:** TGGCAACGCAAAGGCTTTCAGTGA
		Rv: AGGTTTCCCGAAGGTGAAGT