Fish and Shellfish Immunology 130 (2022) 591–601

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Role of the melanocortin system in zebrafish skin physiology

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ARTICLE INFO

Keywords:
Agouti-signalling protein
Melanocyte-stimulating hormone (MSH)
LPS
Cytokine
Mucus
Immune response
Stress

ABSTRACT

The agouti-signaling protein (ASIP) acts as both a competitive antagonist and inverse agonist of melanocortin receptors which regulate dorsal-ventral pigmentation patterns in fish. However, the potential role of ASIP in the regulation of additional physiological pathways in the skin is unknown. The skin plays a crucial role in the immune function, acting as a physical limitation against infestation and also as a chemical barrier due to its ability to synthesize and secrete mucus and many immune effector proteins. In this study, the putative role of ASIP in regulating the immune system of skin has been explored using a transgenic zebrafish model over-expressing the asip1 gene (ASIPzf). Initially, the structural changes in skin induced by *asip1* overexpression were studied, revealing that the ventral skin of ASIPzf was thinner than that of wild type (WT) animals. A moderate hypertrophy of mucous cells was also found in ASIPzf. Histochemical studies showed that transgenic animals appear to compensate for the lower number of cell layers by modifying the mucus composition and increasing lectin affinity and mucin content in order to maintain or improve protection against microorganism adhesion. ASIPzf also exhibit higher protein concentration under crowding conditions suggesting an increased mucus production under stressful conditions. Exposure to bacterial lipopolysaccharide (LPS) showed that ASIPzf exhibit a faster pro-inflammatory response and increased mucin expression yet severe skin injures and a slight increase in mortality was observed. Electrophysiological measurements show that the ASIP1 genotype exhibits reduced epithelial resistance, an indicator of reduced tissue integrity and barrier function. Overall, not only are ASIP1 animals more prone to infiltration and subsequent infections due to reduced skin epithelial integrity, but also display an increased inflammatory response that can lead to increased skin sensitivity to external infections.

1. Introduction

The melanocortin system is one of the most complex hormonal systems in vertebrates. The principal melanocortin peptides are the melanocyte-stimulating hormones (MSHs) and adrenocorticotropic hormone (ACTH), all encoded by a common precursor named proopiomelanocortin (POMC), mostly expressed in the pituitary gland [1]. Melanocortin signaling is mediated by a family of G protein-coupled receptors. In tetrapod species, five different subtypes (MC1R-MC5R) have been characterized, yet numbers diverge in fish species [2]. The genome of perciform fish has at most four receptors lacking MC3R, whereas zebrafish has six receptors with two copies of MC5R [3]. MCRs differ in their spatial distribution and pharmacological properties and regulate a wide plethora of physiological functions ranging from adrenal steroidogenesis to pigment synthesis [4]. These functions are exerted through a complex interplay of signaling ligands involving endogenous antagonists which compete with melanocortin agonists for MCR binding [3].

Agouti-signaling protein (ASIP1) is mostly expressed in the skin, where it competes by binding to MC1R, whereas agouti-related protein

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https://doi.org/10.1016/j.fsi.2022.09.036
Received 11 July 2022; Received in revised form 9 September 2022; Accepted 15 September 2022
Available online 20 September 2022

(AGRP) is expressed in the hypothalamus to regulate energy balance by binding to the central MC3R and MC4R [5]. In mammals, MC1R fills the extension (*E*) locus which is critical for the regulation of melanin synthesis [6]. ASIP1 binding to MC1R promotes the switch from eumelanin (dark/brown pigment) to pheomelanin (yellow/red pigment) synthesis [7,8]. The usage of alternative gene promoters discriminates the spatio-temporal expression of *asip,* thus directing constitutive mRNA production to the ventral region yet episodic synthesis during the hair formation cycle to the dorsal region of agouti coat phenotype [9]. Our studies have shown that the ASIP1/MC1R tandem also regulates fish pigmentation, notably the dorsoventral pigmentation pattern, yet using a different cellular mechanism involving proliferation of iridophore and a reduction in the number of melanophores [10,11].

Skin plays a crucial role in the immune function, acting as a physical deterrent against infestation and also as a chemical barrier due to its ability to synthesize and secrete mucus and a wide range of immune effector proteins [12]. Fish mucus is a matrix composed of lectins, lysozymes, complement proteins and antimicrobial peptides which contributes to the neutralization of pathogens [13] and is therefore part of the innate immunity that constitutes the first line of defense [12]. The skin also contains multiple cell types including mucus-secreting cells (e.g., goblet cells), lymphocytes (B and T cells), granulocytes, macrophages, melanocytes, and Langerhans-like cells [14,15]. This combination of innate and adaptive cell subsets coordinately responds to the continuous exposure to a wide range of non-pathogenic and pathogenic microorganisms present in the aquatic environment [16,17].

The melanocortin system has been shown to regulate inflammatory process, which constitutes a key point in the defense response against damaged factors. It is an important component of the innate immune system [18]. The regulatory role of α-MSH on inflammatory response functions by activating several molecular pathways, with cAMP signaling being a common underlying mechanism mediating the anti-inflammatory effects of melanocortins [18]. The nuclear factor kappa B (NF-κB) appears to be one of the major cellular pathways regulating the anti-inflammatory effects of melanocortins, as *in vitro* α-MSH treatment of human monocytes stimulated with different inflammatory agents significantly decreases NF-κB activation, in both a cAMP-dependent and independent manner [19]. In mammals, the anti-inflammatory effects of α-MSH also appear to act by modulating the cytokine metabolism. Thus, melanocortin receptors inhibit the proinflammatory effects of interleukin 1, 6, 8 (IL-1, IL-6, IL-8) as well as the tumor necrosis factor α (TNF-α) [20], yet stimulates the production and release of IL-10 [21]. Fish genomes possess every one of the (canonical) pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and downstream effectors such as IL-8 and IL-12, in addition to anti-inflammatory cytokines such as IL-10 and TGF-β1, thus a balanced inflammatory response to initially clear an infection is feasible [22].

Evidence that the melanocortin system plays a role in the immune response of fish is scarce. *In vitro* assays have shown that α-MSH is able to stimulate phagocytic and respiratory burst activity of rainbow trout and carp head kidney phagocytes [23–28]. Our recent studies using a zebrafish strain overexpressing *asip1* [10] have demonstrated the involvement of the melanocortin system in the regulation of skin physiology. This transgenic model provides a key opportunity to evaluate the potential role of the melanocortins in the skin immune response and this is what we have undertaken in the current study.

2. Material and methods

2.1. Animals

Wild type (WT), Tubingen (TU), and *asip1* overexpressing [Tg(Xla.Eef1a1:Cau.ASIP1)iim04,] [10] zebrafish (*Danio rerio*) strains were bred at 28 °C, with 14 h light/10 h dark cycle. Prior to any manipulation, fish were netted and anaesthetized with tricaine methane-sulfonate (MS-222-, Sigma-Aldrich), and on demand, euthanized by an overdose of the same anesthetic. Experiments were carried out in accordance with the principles published in the European animal directive (86/609/EEC) for the protection of experimental animals, and approved by the ethics committee of the Spanish National Research Council (CSIC) (project number PID2019-103969RB-C33).

2.2. Experiment 1. Difference in skin histochemical properties between ASIP1 and WT genotypes

For histological purposes, adult zebrafish (n = 4) (BW = 0.51 ± 0.07 g) were euthanized by an overdose of tricaine-methane-sulfonate (MS-222-, Sigma-Aldrich. The specimens were placed in 4% paraformaldehyde (PFA) buffered with phosphate buffer 0.1 M pH = 7.4 overnight at 4 °C. Following dehydration, specimens were embedded in paraffin and sectioned (3–5 μm). Four histological sections were stained with haematoxylin–eosin and haematoxylin-VOF [29] from each specimen; the remaining sections were used to evaluate the histochemical properties of the skin. The following techniques were performed: Schiff, Periodic Acid Schiff (PAS) and Alcian Blue (AB) pH = 0.5 and pH = 2.5 according to Underwood [30]. In order to characterize the glucidic residues bound to glycoconjugates, the following horseradish peroxidase (HRP) conjugated lectins (Sigma-Aldrich, Spain) were used: *Canavalia ensiformes*/ConA (mannose and/or glucose), *Triticum vulgaris*/WGA (N-acetyl-D-glucosamine and/or N-acetylneuraminic acid, NeuNAc/sialic acid/NANA), *Ulexeuropeus*/UEA-I (L-Fucose), *Sambucusnigra*/SNA (NeuNAc/sialic acid/NANA) and *Glycine max*/SBA (α-N-acetyl-D-galactosamine). The sections were treated with 0.3% H_2O_2 for 10 min in Tris-buffered saline solution (TBS; pH 7.2) and incubated for 30 min at RT in HPR-lectin conjugated (20 μg mL^{-1}) dissolved in TBS. After three washes in TBS, peroxidase activity was visualized with TBS containing 0.05% 3,3-diaminobenzidine tetra-hydrochloride and 0.015% H_2O_2. The sections were washed under running tap water (10 min), dehydrated, cleared and mounted. Histochemical results were visualized with a light microscope (Leitzdiaplan), manually recorded and expressed as the semi-quantitative evaluation of colour intensity scores [0, negative; 1, weak; 2, moderate; 3, intense] of four independent observers.

2.3. Experiment 2. Effects of the genotype on stress-induced protein production in skin mucus secretion

In order to study the total protein concentration in skin mucus, thirty-six fish (18 WT and 18 ASIP1) were anaesthetized in MS222 (0.01%) then individually placed in sterile 5 ml tubes containing 4 ml of phosphate buffered saline solution (PBS). Tubes were slightly agitated for 1 min, subsequently, the fish were removed from the tube and placed back into their home tanks. The PBS containing fish mucus was then centrifuged for 10 min at 1500×*g* at 4 °C. Ultimately, the supernatants were transferred to fresh tubes and stored at −80 °C until further analysis. The total protein concentration was quantified by using a Pierce™ BCA Protein Assay (Thermo Scientific) in 96-well microtiter plates (Sarstedt), as indicated in the manufacturer's instructions. Optical density at 562 nm was recorded using an Infinite M Plex TECAN microplate reader. The effect of genotype on stress-induced protein concentration in the skin mucus secretion was then studied. Thirty-six animals (18 of each genotype), body weight (BW) = 0.27 ± 0.01g, were confined in 4 half-litre tanks (9 animals per tank) for 4 days (pH = 7.2, total dissolved solids (TSD) = 45 ppm). The animals were weighed at the beginning and on finalizing the experiment. Weight loss was calculated as a percentage of the initial body weight. Likewise, mucus samples were obtained as previously described at the beginning of the experiment and once again following the 4 experimental days.

2.4. Experiment 3. Effects of lipopolysaccharides (LPS) on mucus production and gene expression

The influence of the genotype (WT vs ASIP1) on skin response to bacterial LPS was evaluated. *Escherichia coli* O111:B4 (Sigma) LPS was diluted in osmotized water and administered by baths. In a first study, 27 animals of each genotype (BW = 0.53 ± 0.06 g) were distributed into 6 half-litre tanks (9 fish per tank) and treated with two doses of LPS (50 and 100 μg/ml) for four days. One group remained untreated as a control group. Following the experimental period, the fish were weighed and mucus samples collected from the skin of 7 animals from each treatment in PBS for protein quantification as described above. Five animals were euthanized for whole skin dissection. Skin samples were cleaned with sterile PBS, removing all the muscle tissue, then placed in Tri- reagent (Invitrogen), homogenized and stored a −80 °C for RNA extraction as indicated in the manufacturer's instructions. Total RNA was dissolved in RNase-free water, quantified using a Nanodrop 2000 sp ectrophotometer (Thermo Scientific) and stored at −80 °C Potential genomic DNA was removed with Turbo DNase-RNase-free (Thermo Scientific), according to the manufacturer's instructions. Subsequently, 1 μg of the total RNA was reverse transcribed using the SuperScript III reverse transcriptase (Thermo Scientific) and oligo $(dT)_{12\text{-}18}$ (0.5 μg/ml). The cDNA was diluted in nuclease-free water and stored at −20 °C until further use. Real-time PCR was performed on a CBX Connect™ Real-Time System instrument (Biorad) using Absolute SYBR Green fluorescein (Thermo Scientific) and specific primers, previously optimized (Table 1), in order to evaluate the expression of interleukin 1β, 6 and 10 (IL-1β, IL-6, IL-10), tumor necrosis factor alpha (TNF-α) and 5.1 and 5.2 mucins (Muc5.1 and Muc5.2). Each sample was amplified under the following conditions: 15 min at 95 °C followed by 40 amplification cycles (15 s at 95 °C, 30 s at 55 °C, and 30s at 72 °C). The expression of target specific genes was normalized to the relative expression of zebrafish β-actin and fold change gene expression was performed using the mean normalized expression (MNE) method of the Q-Gene application [31,32]. Negative controls without a template were included in each experiment. A melting curve was determined for each PCR by fluorescence reading at each degree between 55 °C and 95 °C to ensure the specific amplification.

A second trial was conducted in which fish were exposed only to the highest dose of LPS for a shorter period. Once again, 20 animals of each genotype were distributed in 4 half-litre tanks as previously described and treated for 5 h with LPS (100 μg/ml). Subsequently, the fish were transferred to clean 10-L tanks equipped with fresh water recirculation and heating systems. Skin samples were collected at both 24 h and 4 days following the immunological challenge. Skin samples were treated as described above and used for gene expression analyses.

2.5. Tissue resistance in ussing chambers

Skin caudal to the pectoral fin, anterior to the anal area, was isolated and transferred to a well-aerated zebrafish saline solution (NaCl 75 mM, KCl 3 mM, NaH_2PO4 1 mM, $MgSO_4{\cdot}7H_20$ 2 mM $CaCl_2$ 1.7 mM, Glucose 5 mM, Na-Gluconate 10 mM, $NaHCO_3$ 5 mM, HEPES 4 mM, Na-HEPES 10 mM; pH = 7.80). Excess muscle was removed by blunt dissection, and the cleansed skin was mounted as previously described [33,34] with apical (water side) and basal (muscle side) surfaces identified on a tissue holder of 0.09 cm2 and positioned between two half-chambers containing 2 ml of physiological saline solution. The mounted skin was gassed bilaterally with humidified air and kept at 27–28 °C. For epithelial resistance (Rt, Ω.cm2) calculation, the tissue was short-circuited to 0 mV. The experiments were performed for short periods (~30 min) until stable Rt measurements were reached. Using Ohm's law, Rt was manually calculated from the current deflections induced by a bilateral +2 mV pulse of 4 s per minute. Voltage clamping and current injections were performed using VCC MC6 voltage-clamp amplifiers (Physiologic Instruments, San Diego, USA). The bioelectrical parameters for each tissue were recorded continuously during the *in vitro* period onto Labscribe3 run on a Lenovo computer using IWorx188 and Lab-Trax-4 data acquisition systems.

2.6. Statistics

Statistical analyses of skin mucosal protein concentration under basal conditions of WT and ASIP1 fish, as well as weight loss percentage due to crowding stress (experiment 1), were carried out using a two-tailed Student's *t*-test with Welch's correction once the F test indicated that the variances of both groups differed significantly.

Changes in mucus protein concentration after exposure to stress (experiment 2) were compared by a one-way ANOVA analysis. The results obtained from protein concentration in skin mucus and transcriptional levels of cytokines and mucins after LPS challenge were analyzed using a two-way ANOVA to test differences in the mean effects of LPS doses and fish genotype. Statistical analyses and graphic representation were carried out using GraphPad Prism8. The differences were considered significant at $P < 0.05$.

3. Results

3.1. Experiment 1

This experiment was designed to recognize whether there were histological differences in the skin structure of transgenic and WT zebrafish by using routine staining and semi-quantitative techniques (Fig. 1). No gross morphological differences between the dorsal regions of both genotypes (Fig. 1. A, E) were found, however the ventral epidermis of ASIP1 fish was thinner than that of WT animals, exhibiting at best 2–3 layers (Fig. 1. G), compared to the 5–7 layers observed in the ventral epidermis of WT animals (Fig. 1. C). In addition, a moderate hypertrophy of mucosal cells was detected in the ASIP1 genotype (Fig. 1 F, H). Classical histochemical studies showed a weak presence of neutral and carboxylate-acid mucins yet a moderate presence in the mucus cells of the dorsal skin of WT fish (Table 2). No positive reaction for ionized sulfate groups was detected in the dorsal epithelium (data not shown) yet once more the presence was moderate in the mucosal cells. A slight increase of neutral, sulfated and carboxylate mucins in the dorsal mucosal cells of ASIP1 animals (Table 2) was exhibited. In the ventral

Table 1
List of primers used in this study for gene transcription evaluation.

Gene	Forward primer	Reverse primer
β-actin	5′- GATGAGGAATCGCTGCCCT-3′	5′- GTCCTTCTGTCCCATGCCAA-3
IL-1β	5′-TGGACTTCGCAGCACAAAATG-3′	5′-GTTCACTTCACGCTCTTGGATG-3′
IL-6	5′-AGACCGCTGCCTGTCTAAAA-3′	5′-TTTGATGTCGTTCACCAGGA-3′
IL-10	5′- AGCAAATCAAGCTCCCCCATA-3′	5′-CTTTAAAGCACTCCACAACCCCAA -3′
TNF-α	5′-GCTGGATCTTCAAAGTCGGGTGTA-3′	5′-TGTGAGTCTCAGCACACTTCCATC-3′
Muc5.1	5′-TGGCAACTTGGCTGATGATA-3′	5′-TCGTCACACGGACCAGTAGA-3′
Muc5.2	5′- GGTGTCTGTTCCGATCAATC-3′	5′-TCATCCTTGTCGCCATTGTA-3′

β-actin (AY222742).1IL-1β Interleukina 1β (NM_212844), IL-6 Interleukina 6 (JN698962), IL-10 Interleukina 10 (NM_001020785), TNF-α, Tumor necrosis factor α (NM_212859), Muc5.1 Mucin 5.1 (XM_021470624), Muc5.2 Mucin 5.2 (NC_007136).

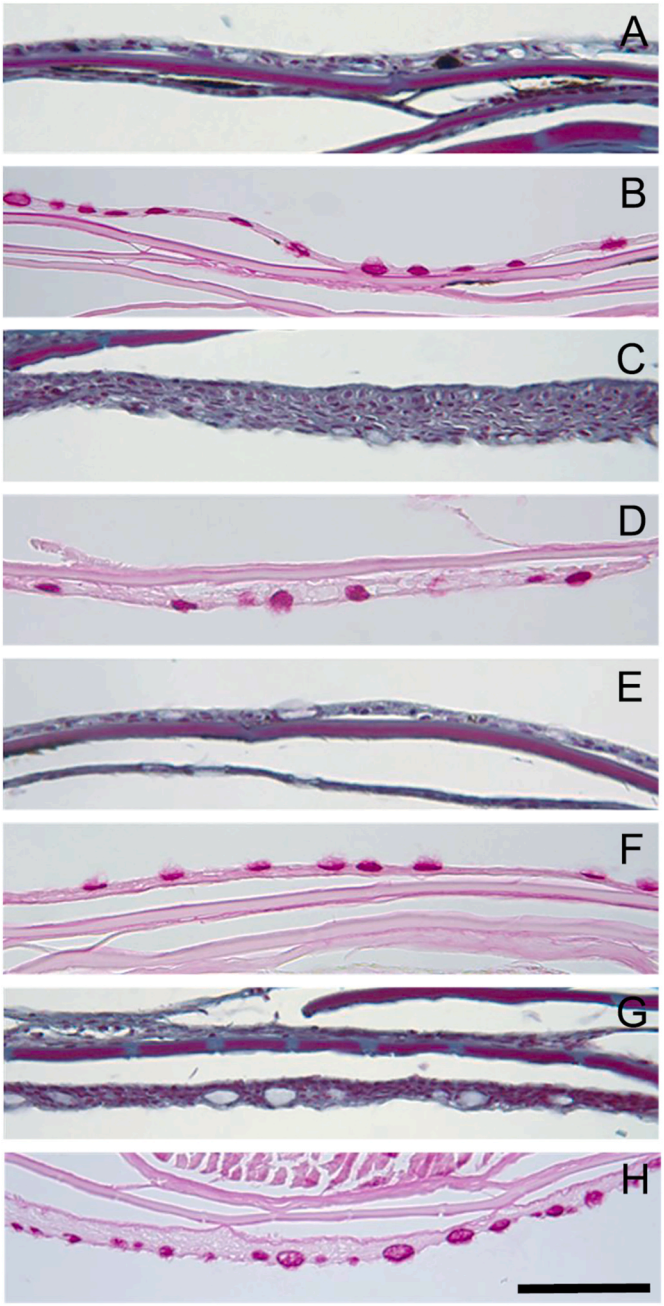


Fig. 1. Skin morphology. Histological sections stained with haematoxylin-eosin (B,D,F,H) and haematoxylin-VOF (A,C,E,G) showing dorsal and ventral skin morphology in WT and ASIP1 genotypes WT dorsal skin (A, B), WT ventral skin (C, D), ASIP1 dorsal skin (E, F) and ASIP1 ventral skin (G, H). Scale bar is 100 μm.

skin of WT fish, a weak reaction to acid carboxylate mucins containing sulfated groups was observed, in addition to a low number of neutral substances in the mucus. On the contrary, mucosal cells were moderate to intensely stained with neutral mucins as well as to carboxylate/sulfated mucins (Table 2). Regarding dorsal skin, a moderate increase in both neutral mucins/glycoproteins and carboxylate/sulfated mucins was observed (Table 2).

Lectin histochemistry revealed a low (epithelium, Fig. 2A1) or moderate (mucosal cells, Fig. 2A2) presence of glycidic residues rich in α/β N-acetyl-galactosamine (SBA) and weak affinity of lectins ConA and UEA-I (data not shown) in the dorsal skin of WT animals (Table 3). In addition, no reaction to lectins WGA (Fig. 2B1) and SNA (Fig. 2C1) was

Table 2
Histochemical characteristics of the skin of wild type and ASIP1 zebrafish, (*Danio rerio*) genotypes.

Dorsal/Ventral Skin	WT Epithelium	ASIP Epithelium	WT Mucous cells	ASIP Mucous cells
Neutral glycoproteins	1/1	1/1	2/2-3	2-3/3
Carboxylated glycoproteins	1/1	1/1	2/2-3	2-3/3
Strongly ionized sulfated glycoconjugates	0/1	0/1	2/2-3	2-3/3

Semi-quantitative assessment scoring based on color intensity scores: 0, negative (non-detected); 1, weak; 2, moderate; 3, intense.

detected in epithelial cells of the dorsal region, thus demonstrating the absence of N-acetyl-β-D-glucosamine/N-acetyl-neuraminic acid, respectively. Mucus cells also exhibited moderate WGA (Fig. 2B1) and SNA (Fig. 2C1) content even in the absence of ConA and UEA-I (data not shown) (Table 3).

In the ventral region, mucosal cells of ASIP genotype showed a higher affinity for SBA (Fig. 2D2) and SNA (Fig. 2F2) lectins, indicating a higher content in α/β N-acetyl-galactosamine and N-acetyl-neuraminic acid (Table 3). The affinity of WGA in mucosal cells was similar in both genotypes (Fig. 2B2, 2E2, Table 3). No ConA and UEA-I lectins were detected in the mucosal cells of either genotype.

3.2. Experiment 2

The afore-mentioned results suggest that transgenic zebrafish can produce more mucins in the skin mucosa under basal conditions or after stress/challenge conditions. To validate this hypothesis, the total protein in the skin mucosa in both conditions was measured. As shown in Fig. 3, total protein concentration in the skin mucosa was similar in both genotypes under basal conditions. However, WT fish showed a significant reduction in protein levels following 4-days of crowded conditions. This reduction in protein mucus levels was not observed in ASIP1 fish (Fig. 4). Weight reduction was also recorded during the experimental period. Remarkably, ASIP1 animals lost more weight than WT animals. Specifically, mean weight loss was 5.8% and 10.2% in WT and ASIP1 fish respectively (Fig. 5).

3.3. Experiment 3

The response to LPS challenges was studied in both genotypes. In the first trial, WT animals only lost weight after treatment with higher doses (100 μg/ml) yet ASIP1 fish always lost condition after challenging baths (0, 50 and 100 μg/ml) suggesting that ASIP1 are more prone to stress than WT, which in turn are more susceptible to a bacterial infection than transgenic animals (Fig. 6). The concentration of proteins present in the mucus of WT fish remained unchanged after LPS treatments, however, it increased dose-dependently in treated transgenic fish (Fig. 7). Additionally, LPS treatments induced lesions such as skin hemorrhages in the abdominal cavity of ASIP1 fish, which were unnoticeable in WTs (Fig. 8). Similar lesions were also observed in the opercular region and at the base of paired fins. One WT fish died with the highest doses of LPS following the experimental period, while three ASIP1 fish died after LPS treatments (1 fish at dose 50 μg/ml and 2 fish at 100 μg/ml LPS baths). Due to the differences obtained in both genotypes, it was subsequently decided to study the expression profiles of some immune and mucosal related genes in the skin (IL-1β, IL-6, IL-10, TNF-α, Muc5.1 and Muc5.2). Following a two-way ANOVA analysis($p < 0.05$), results showed similar values for almost every gene in both genotypes, with the only exception of IL-6 and TNF-α levels, which were significantly higher in transgenic fish (Fig. 9). The absence of a consistent response in both WT and ASIP1 genotypes, probably due to the prolonged exposure to bacterial LPS.

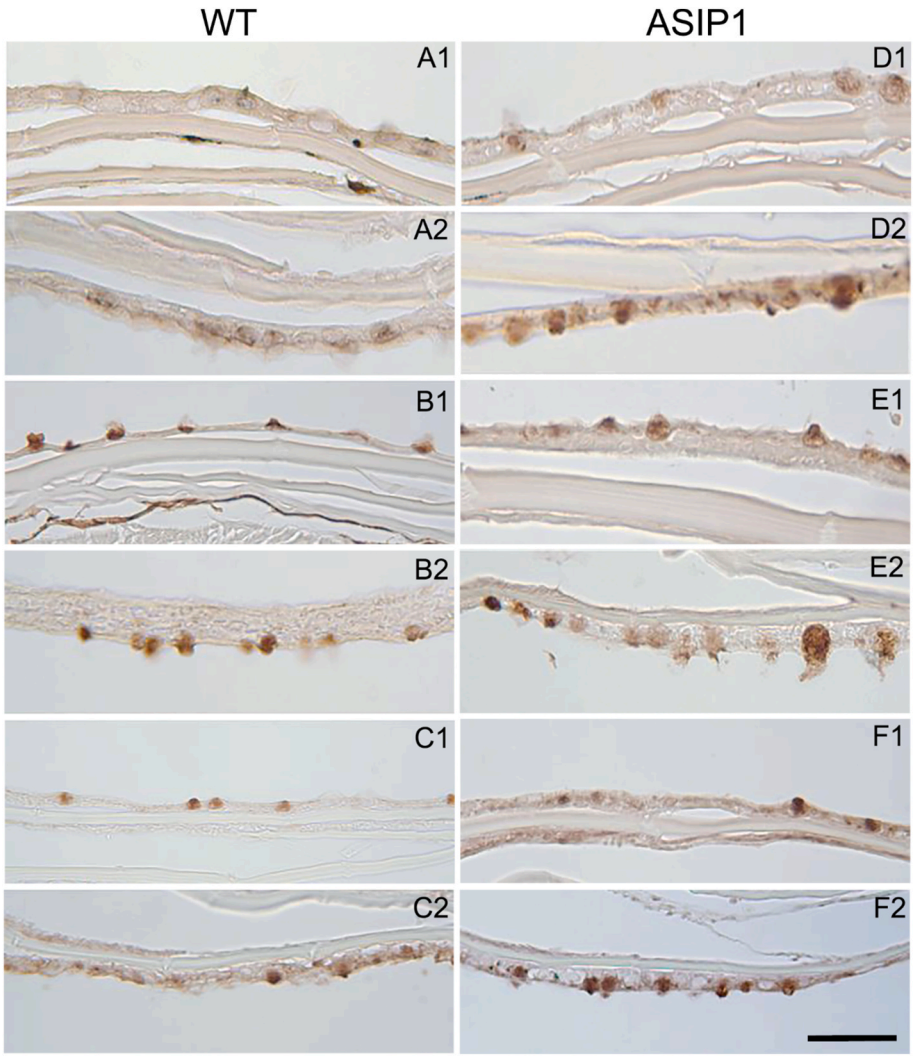


Fig. 2. Presence of lectins in skin. Histological sections showing glucidic residues bound to glycoconjugates in wild type (WT) and ASIP1-overexpressing transgenic zebrafish. The following horseradish peroxidase (HRP) conjugated lectins were used: *Canavalia ensiformes*/ConA (mannose and/or glucose), *Triticum vulgaris*/WGA (N-acetyl-D-glucosamine, *Ulexeuropeus*/UEA-I (L-Fucose), *Sambucusnigra*/SNA (NeuNAc/sialic acid/NANA) and *Glycine max*/SBA (α-N-acetyl-D-galactosamine). A) WT/SBA, B) WT/WGA, C) WT/SNA, D) ASIP1/SBA, E) ASIP1/WGA and F) ASIP1/SNA. (1) and (2) indicate dorsal and ventral respectively. Scale bar is 100 μm.

Table 3
Lectin affinity of the skin of wild type and ASIP1 zebrafish, (*Danio rerio*) genotypes.

Dorsal/Ventral Skin	WT Epithelium	ASIP Epithelium	WT Mucous cells	ASIP Mucous cells
ConA	0-1/0-1	0-1/0-1	0/0	0/0
WGA	0/0	0/0	2/2-3	2/2-3
UEA-I	0-1/0-1	0-1/0-1	0/0	0/0
SBA	1/1	1/1	2/2-3	2-3/3
SNA	0/0	0/0	2/2-3	2-3/3

Semi-quantitative assessment scoring based on color intensity scores: 0, negative (non detected); 1, weak; 2, moderate; 3, intense.

This led us to modify the experimental conditions by shortening the drug exposure to the first 5 h (5h vs 4 days), and using only the highest LPS dose (100 μg/ml). Samples were collected at two time points (1- and 4-days post-treatment) in order to analyze the expression levels of the same genes. This experiment focused on exploring genotype differences in the immune response and, accordingly, genotype-induced differences in the expression of IL-1β, IL-10, TNF-α and Muc5.2 following a two-way ANOVA ($p < 0.05$) test were found. Time-induced differences were found in IL-1β, IL-10 and Muc5.1 expression (Fig. 10). On the whole, ASIP1 fish showed a more rapid immune response, as gene expression

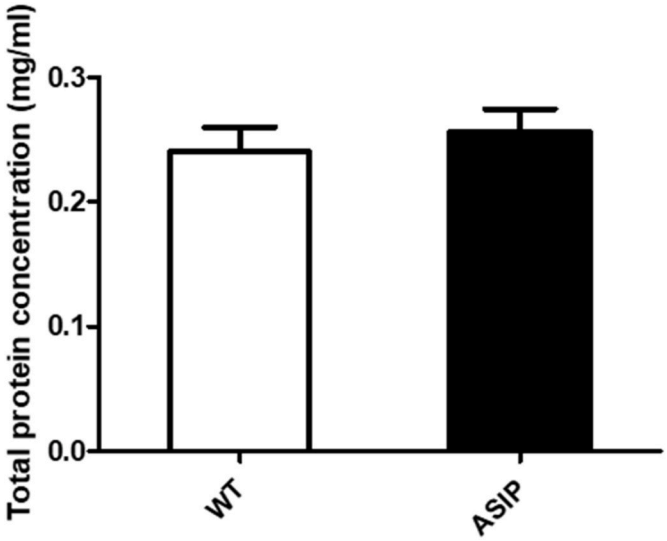


Fig. 3. Total protein concentration in skin mucus of wildtype and ASIP1 zebrafish kept under undisturbed basal conditions. The total protein concentration in mucus using a BCA protein assay. Each bar represents mean ± SEM ($n = 25$). Data were analyzed with two-tailed Student's *t*-test and differences considered significant when $p < 0.05$.

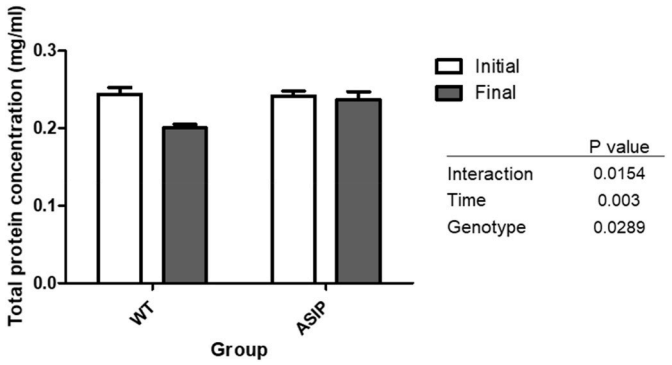


Fig. 4. Total protein concentration in skin mucus after stress exposure. Skin mucus was collected (n = 18 each genotype) and total protein concentration quantified as before. … Data were analyzed with a two-way ANOVA test and differences were considered significant when $p < 0.05$.

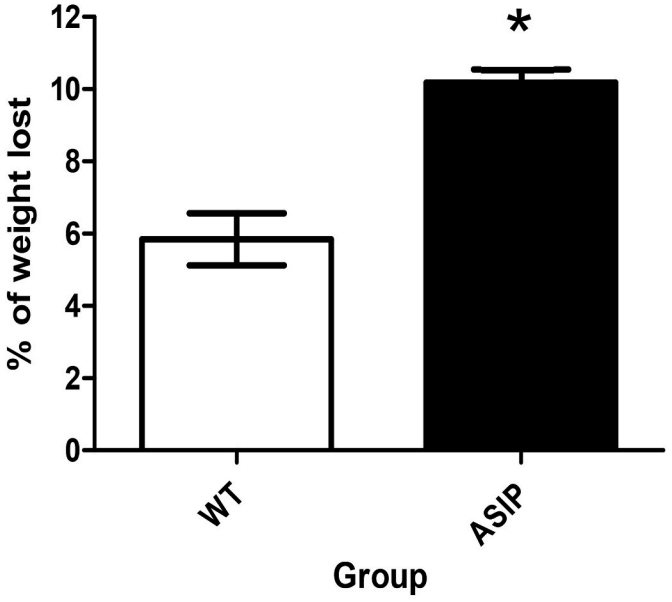


Fig. 5. Effects of stress on body weight of WT and ASIP1 zebrafish. Each bar represents the mean of weight loss ± SEM (n = 72). Statistical analysis was carried out using the two-tailed Student's-test and the difference was considered significant when P < 0.005.

levels were always higher than those observed in WT fish at 24h, with the exception of Muc5.1. The response at 24 h was also greater in ASIP1 fish for IL-1β, IL-6 IL-10, TNF-α and Muc5.2 than found in WT animals. In all cases, expression levels in ASIP1 fish decreased following 4 experimental days which emphasizes the stimulated expression at 24 h. In WT fish, the expression response was more modest than observed in ASIP1 fish at 24 h, as indicated by significant interactions of both variables (time x genotype) particularly for IL-1β, IL-6, IL-10, TNF-α and Muc5.2. Interaction between two variables indicates that at a given combination, a different response is induced in the gene expression. Suggestively, gene expression for IL-6, TNF-α and Muc5.2 at 4 days post-treatment was higher than that observed after 24 h, somewhat highlighting the potential delay of the immune response in WT compared to ASIP1 fish.

3.4. Tissue resistance in the ussing chambers

Rt values for each genotype are shown in Fig. 11. The skin of wild-type fish shows Rt values averaging ~115 Ω cm^2, overexpression of

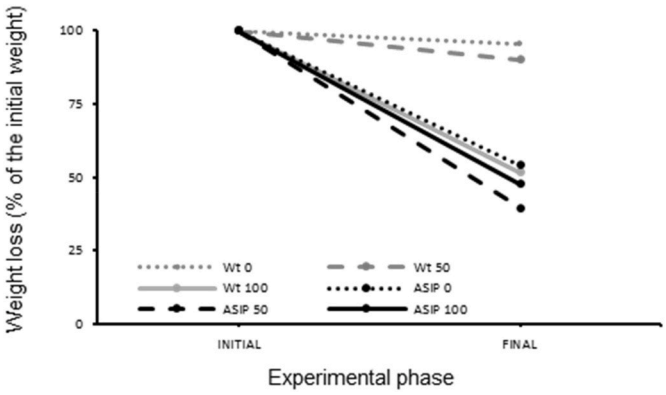


Fig. 6. Percentage of weight loss after the challenge with lipopolysaccharide (LPS) . Each line represents the percentage of loss of the initial body weight after LPS exposition (0, 50 and 100 μg/ml) during 4 days in wild type (Wt) and ASIP1 animals at the end (final) of the experiment (9 animals per group).

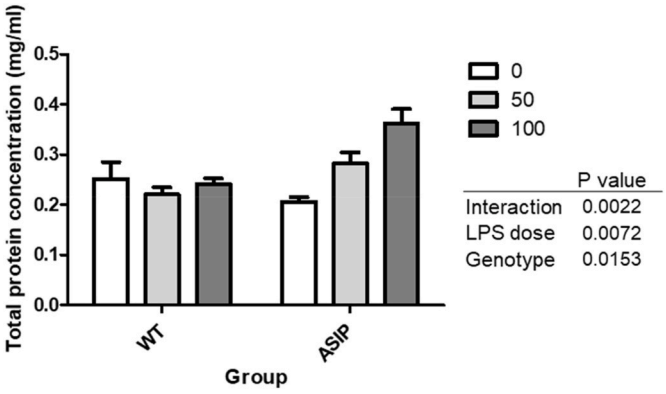


Fig. 7. Total protein concentration in skin mucus after LPS challenge. White and grey bars indicate protein mucus levels at the beginning (initial) and end (final) of the experiment. Each bar represents the mean ± SEM (n = 7). Data were analyzed with a two-way ANOVA test and differences were considered significant when $p < 0.05$..

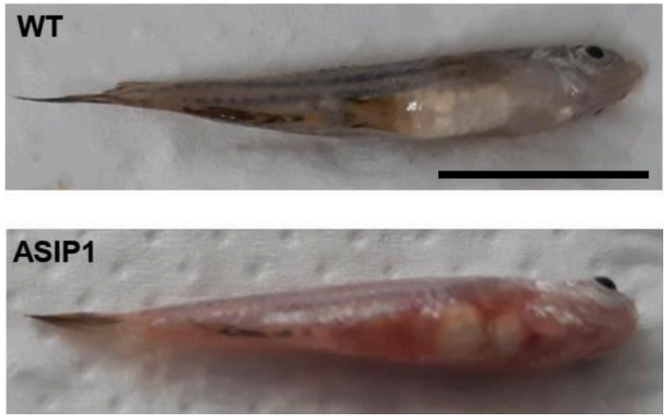
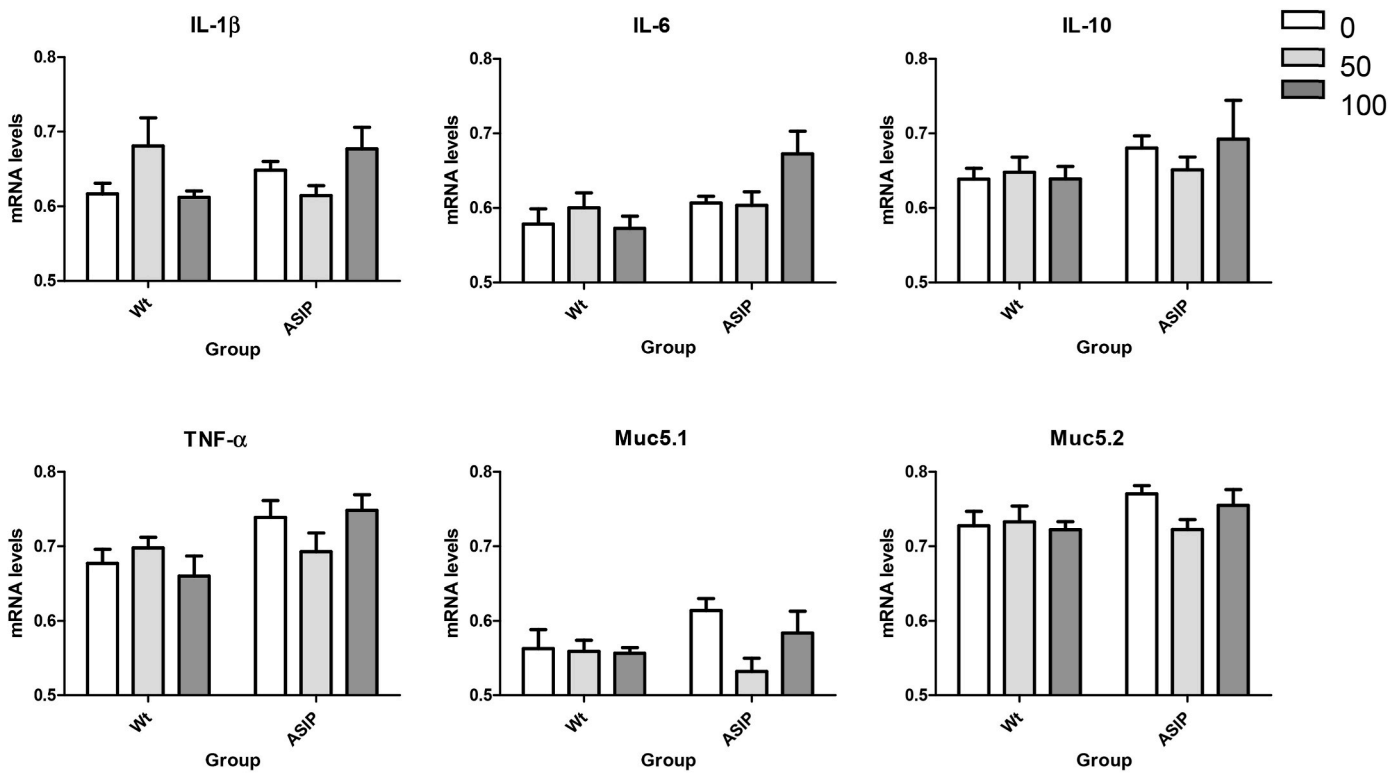


Fig. 8. Damage due to LPS challenge. Representative pictures showing skin damages after LPS exposition (100 μg/ml) for 5 h in wild type (WT) and ASIP1 fish. After LPS treatment fish were transferred to untreated water and 24 h later the pictures were taken focusing the ventral skin. . Scale bar is 1 cm.



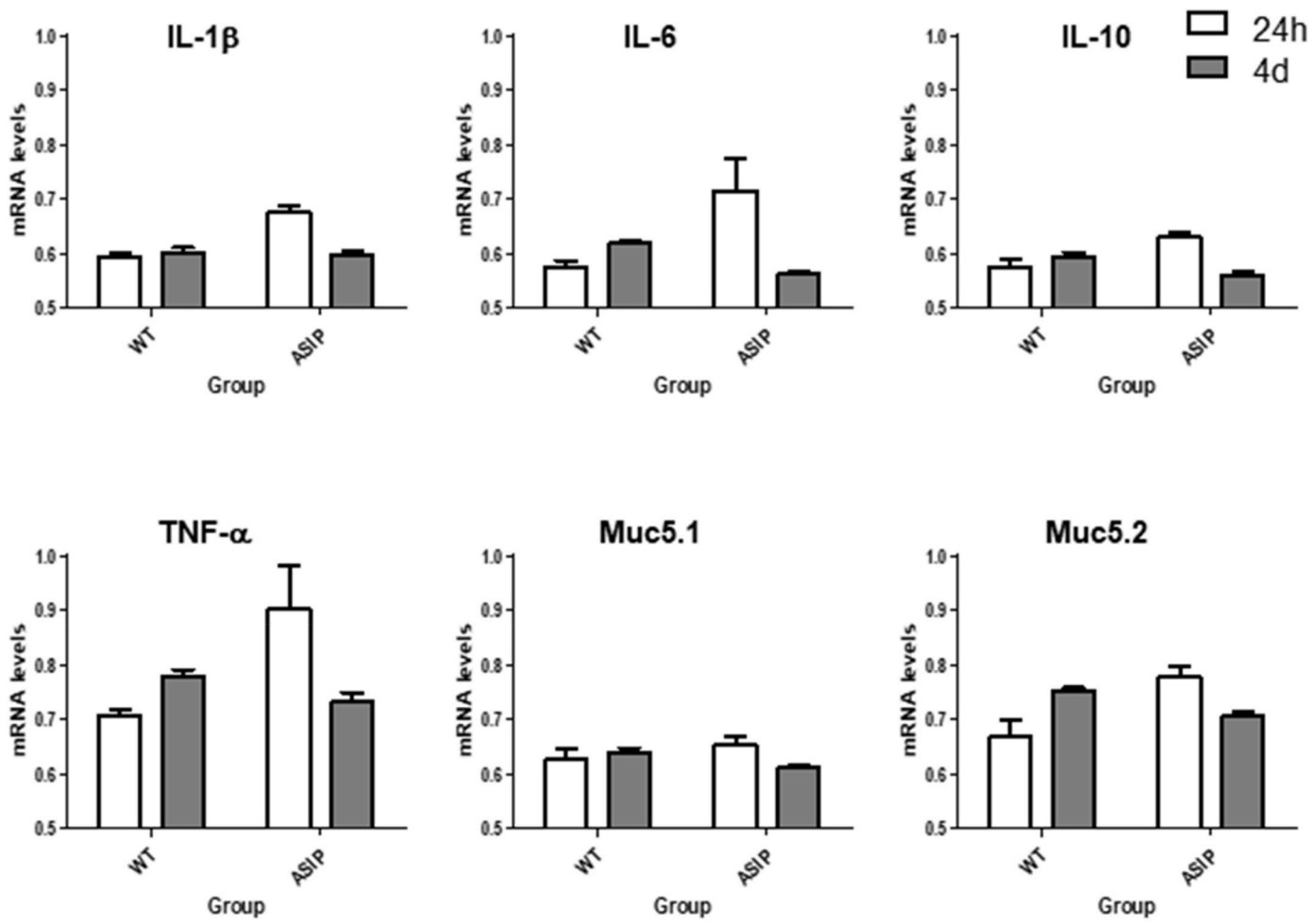
P value	IL-1β	IL-6	IL-10	TNF-α	Muc5.1	Muc5.2
Interaction	**0.0144**	0.0643	0.5786	0.1047	0.1606	0.2623
Dose	0.7662	0.3355	0.8116	0.8395	0.1205	0.4559
Genotype	0.5796	**0.0149**	0.1285	**0.011**	0.3171	0.1239

Fig. 9. Transcriptional levels of cytokines and mucins after exposure to different doses of LPS. Zebrafish (WT and ASIP1) were exposed to a challenge bath with different doses of LPS (0, 50 or 100 μg/ml). After four days, five zebrafish from each group were sacrificed and skin samples were taken for RNA extraction to determine the levels of expression of some pro-inflammatory cytokines and two mucins using real-time PCR. Data are shown as mean gene expression relative to endogenous control β-actin expression mean ± SEM (n = 5). Data were analyzed with a two-way ANOVA test and the differences were considered significant when $p < 0.05$.

ASIP1 induced a reduction in epithelial resistance suggesting a decreased tissue integrity and selectivity in the transgenic genotype.

4. Discussion

The Melanocortin System plays a critical role in fish skin pigmentation by conferring a hormonal environment that drives chromatophore differentiation by inhibiting melanogenesis and promoting iridophore proliferation [10]. This role is covered by the inverse agonism of ASIP1 on MC1R [11], however, the potential function of ASIP1 in other aspects of skin physiology remained unexplored. In this work, we studied the plausible participation of the melanocortin system in the regulation of the immune function in fish skin using a transgenic model that overexpresses *asip1* and, by extension exhibits reduced melanocortinergic signaling. It has been demonstrated that *asip1* overexpression can induce changes in the anatomical structure of the skin by reducing the number and thickness of epidermis cell layers in the ventral region. Suggestively, *asip1* expression is fully polarized toward the ventral skin, where it blocks melanogenesis via MC1R to promote the dorsoventral pigmentation pattern visible in most vertebrates, including fish [10,35]. Semi-quantitative data also suggests that ASIP1 fish show moderately hypertrophied mucous cells in the ventral skin. Mucous cells include goblet, club and sacciform cells that produce the mucous matrix of the outermost layer of the skin [12]. The mucous layer is part of a permanent mechanical and chemical barrier against pathogens. It is an important component of the innate immune system that prevents adherence and colonization of pathogens [36], but it also accumulates factors such as lysozyme, immunoglobulins, complement proteins, lectins, C-reactive protein (CRP), proteolytic enzymes, transferring and alkaline phosphatase (ALP) among others [36–41]. Therefore, the presence of carbohydrate-binding proteins or lectins was explored, as they are highly specific for sugar groups of cognate molecules and yet mediate the adhesion and binding of bacteria, viruses and fungi to their targets. N-acetil-galactosamine is significant for the systemic function, as well as for different stages of disease, because it has been associated to cell-cell communication processes [42]. Some monosaccharides, such as fructose, mannose and galactose, play a decisive role in inhibiting bacterial adhesion in the gill mucus of yellow croacker (P*seudoscianea crocea)* [43], sialic acid is also involved in microorganism adhesion and bacterial protection [44]. Our study revealed an increased affinity for SNA and SBA lectins in the mucous cells of ASIP1 fish, compared to WT animals, suggesting that transgenic fish could be further protected against potential pathogens present in water. Ultimately, mucins in the dorsal and ventral skin of both genotypes were identified and quantified, yielding similar values to those observed for lectins. The mucins detected in this study correspond to high molecular weight molecules,



P value	IL-1β	IL-6	IL-10	TNF-α	Muc5.1	Muc5.2
Interaction	**0,0002**	**0,0029**	**0,0002**	**0,0075**	**0,038**	**0,0005**
Time	**0,0009**	0,0893	**< 0,0001**	0,0584	**0,0109**	0,371
Genotype	**< 0,0001**	0,1213	**0,0222**	**0,0285**	0,1797	**0,0305**

Fig. 10. Transcriptional levels of cytokines and mucins following exposure to LPS. In a subsequent experiment to that shown in Fig. 9, zebrafish (WT and ASIP1) were exposed to a challenge bath with 100 μg/ml of LPS during 4 h. Animals were then transferred to fresh water and skin samples (n = 10) were taken after 1 and four days, t for RNA extraction to determine the levels of expression of some pro-inflammatory cytokines and two mucins by real-time PCR. Data are shown as mean gene expression relative to endogenous control β-actin expression mean ± SEM (n = 10). Data were analyzed with a two-way ANOVA test. The differences were considered significant when $p < 0.05$.

which are highly glycosylated and form approximately 50% of the mucus content. Such molecules exhibit adhesive and viscoelastic properties that play a crucial role as a mechanical barrier against pathogens, as they also contain a wide range of antimicrobial molecules [45–47]. A higher number of neutral mucins and carboxylate and sulfate mucoid substances in the dorsal and ventral skin of ASIP1 fish than in non-transgenic animals was observed. All such components of the fish mucus are essential for protecting the mucosa from a bacterial attack [48]. These results are significant as skin mucosal glycoconjugates are involved in cell agglutination and binding of pathogenic structures, thus, opsonizing and enhancing phagocytic capacity through complement activation [49–51]. After long-term exposure to LPS, our expression studies showed no genotypic effects on mucin expression, however, Muc5.2 was overexpressed in ASIP1 animals after short exposure (5h). The significant interaction between both factors (genotype x time) suggests that ASIP1 animals may respond earlier to LPS exposure than WT animals. Therefore, transgenic animals seem to compensate for the lower number of cell layers in the ventral skin by modifying the mucus composition to maintain or improve protection against microorganism adhesion.

The level of total proteins in the skin mucus was also studied as a coarse measure of mucus composition under both basal and stressful conditions. The total protein concentration was similar in both genotypes under basal conditions, yet once fish were stressed by crowding, only WT animals significantly reduced protein concentration in mucus. Consequently, our histological studies showed that ASIP1 fish contain more mucus cells showing a moderate hypertrophy than WT animals, suggesting that ASIP1 fish can potentially secrete a greater amount of mucus under demanding events such as stress. Previous studies revealed that acute stress induces modifications in the amount of mucus secretion in European sea bass (*Dicentrarchus labrax*) [52] while chronic stress in sea bream (*Sparus aurata*) alters the levels and composition of proteins in

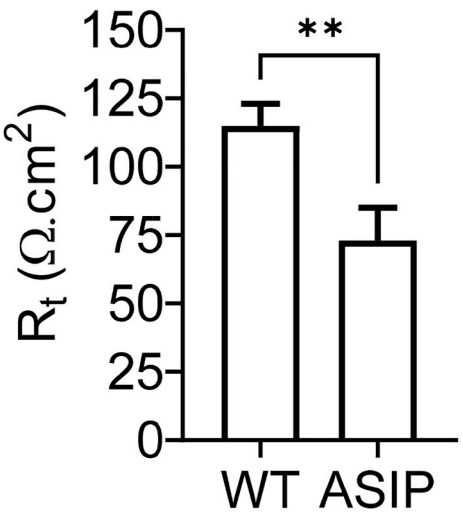


Fig. 11. *Trans*-epithelial electrical resistance (Rt, Ω cm2) in zebrafish skin recorded *in vitro*. Each column represents the mean ± SEM of 11 determinations in the wild type (WT) and 9 ASIP zebrafish. Asterisks represent significant effects ($p < 0.001$) after Student's *t*-test.

skin mucus [53,54]. In addition, some enzymes like cathepsin b and phosphatase alkaline modify their activity in mucus subject to the duration of the stress, showing low and high levels under short- and long-term stress respectively, in Atlantic salmon (*Salmo salar)* [55]. Remarkably, we were unable to document any significant change in total mucus protein levels in WT animals after 4 days of LPS treatment, but mucus protein levels increased in a dose-dependent manner in ASIP1 animals. In mammals, LPS challenges induce mucus hypersecretions in human bronchial epithelial cells [56] as well as in the lung of mice [57]. Consequently, increased intestinal mucins have been reported as a consequence of bacterial infections in several fish species, including Atlantic cod (*Gadus morhua*) [58], European sea bass [59], seabream [60], lump sucker (*Cyclopterus lumpus*) [61]and brown-marbled grouper (*Epinephelus fuscoguttatus*) [62]. A sea lice infestation also produces a similar increase in protein responses in Atlantic salmon [63]. However, experiments with seabream revealed decreased intestinal expression of a number of mucins following parasitic challenges [53]. This data again suggests that ASIP1 animals may respond more readily to emulsified bacterial infections by treatment with LPS, modifying mucus composition and/or quantity and thus conferring more protection against waterborne pathogens [62,64].

The skin immune response was then studied by determining the transcript levels of some genes involved in immunity. This showed that the time of exposure to LPS was essential. Only IL-6 and TNF-α showed significant differences between genotypes after 4 days of continuous exposure to LPS. However, IL-1β, IL-10 and Muc5.2, along with TNF-α, also showed genotypic differences after a 5 h treatment. It is plausible that the prolonged exposure (trial 1, 4 days) could dissipate time-dependent differences in the expression of immune genes. Suggestively, in our short-term experiment (5h exposure), increased expression of all pro-inflammatory cytokine and mucins after LPS challenge in transgenic fish compared to WT animals at 24 h post-treatment was observed, as indicated by significant interactions between both factors (genotype x time). However, expression levels in ASIP1 animals decrease at 4 days post-treatment reaching levels statistically similar to those exhibited by WT fish. The results obtained are in agreement with those reported in different fish and mammalian species, in which LPS treatment stimulates both IL1-β and TNF-α transcription and other inflammatory cytokines [65]. *In vitro* and *in vivo* studies in trout have shown that recombinant IL-1β induces the expression of a number of immune genes, including cyclooxygenase-2 (COX-2) and major histocompatibility complex class II (MHC II), in addition to stimulating phagocytosis, leucocyte bactericidal activity and leucocyte migration [22]. However, IL-1β-induced IL-6 and COX-2 expression can be inhibited by cortisol, the main glucocorticoid in fish stress response [66].

The IL-6 subfamily of cytokines is known to be a major participant in hematopoiesis, and contains pro- and anti-inflammatory properties, causing effects on macrophages and B cells in rainbow trout (*Oncorhynchus mykiss)* among other species [67], including the development and differentiation of lymphocytes, cell proliferation, cell survival, and apoptosis signaling [68]. Previous work has reported that the expression levels of this cytokine increase in early stages of the infection, subsequently decreasing over time, and its production could be reduced as a consequence of the cortisol secretion [66,67].TNF-α plays a key role in the regulation of inflammation and also shows overlapping functions with IL-1β. It stimulates chemokine expression in zebrafish and gilthead seabream endothelial cells [69] and is strongly overexpressed in intestinal granulomas surrounding bacteria following infection with *M. marinum* in zebrafish [70]. In summary, the results suggest that ASIP1 fish respond more rapidly to bacterial infections than WT animals by overexpressing pro-inflammatory cytokine and mucins [71]. The interaction between immune and melanocortin systems is lacking studies which means providing a mechanistic explanation of ASIP1-induced immune effects is complex. It is known that α-MSH has immunomodulatory effects in several cell types [18,72]. MSH inhibits the production of different pro-inflammatory cytokines such as IL-1β and chemokines such as TNF-α, it also increases the levels of IL-10 [18]. In humans, α-MSH is involved in the inhibition of NF-kB pathway activation and has been suggested to exhibit antimicrobial effects [18,72,73]. ASIP1 functions as a competitive antagonist and inverse agonist of MC1R and MC4R [74]. Consequently, overexpression *of asip1* is intended to induce opposite effects to those of MSH treatments, thus stimulating pro-inflammatory cytokine synthesis, as previously reported.

Remarkably, external morphological analysis of ASIP animals revealed more severe skin injures along with a slight increase in mortality following LPS exposure. In light of the afore-mentioned results, it is difficult to provide a potential explanation. It is plausible that the inflammatory response of LPS-challenged ASIP1 genotype is excessive as evidenced by the synthesis of pro-inflammatory cytokine. This disproportionate response, together with the reduced thickness and number of epidermal cell layers induced by ASIP1 overexpression, could be responsible for the observed increased number of injuries. Furthermore, the electrophysiological measurements show that the ASIP1 genotype exhibits reduced epithelial resistance (likely an indicator of reduced tissue integrity and barrier function), pointing in the same direction. Overall, ASIP1 animals are more prone to infiltration and subsequent infections due to reduced skin epithelial integrity and display an increased inflammatory response that can lead to increased skin sensitivity to external infections. The effect of *asip1* overexpression on epithelial permeability and electrogenic transport is not specific to skin epithelia since similar impacts in the intestinal epithelium were observed (unpublished results, E Leal, AR Angotzi, J Fuentes and JM Cerdá-Reverter).

ASIP1 animals show enhanced growth parameters [75,76], however, they do not appear to exhibit a differential feeding response following stressful conditions [75]. Remarkably, ASIP1 animals lost more weight than WT animals once subjected to confinement or treated with doses of LPS. Our unpublished results (Godino-Gimeno A, Rocha A and Cerdá-Reverter JM) have shown through different behavioral tests that ASIP1 fish exhibit higher levels of anxiety than WT animals, in all likelihood mediated by a deficiency in central serotonin levels. This behavioral difference could result in a higher metabolic rate which during could explain the greater weight loss in ASIP1 fish during the experimental starvation.

In conclusion, our results demonstrate that *asip1* overexpression not only induces alterations in skin pigmentation, as previously demonstrated [10], but also in structural morphology and immune responses

by regulating the expression of both mucin and pro-inflammatory cytokines. ASIP1 fish show a faster pro-inflammatory response and increased mucin expression that could help to overcome bacterial infections more easily than WT fish. However, overexpression of ASIP1 also decreases the integrity of the epithelia, making animals more susceptible to infections by reduction of the barrier function. The increased inflammatory and secretory response may be the result of the reduced epithelium integrity once animals are exposed to LPS. Undoubtedly, further research is required to explore the interface between the melanocortin and immune systems, yet fish overexpressing *asip1* would be an excellent model for exploration of such interactions.

CRediT authorship contribution statement

E. Leal: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **A.R. Angotzi:** Methodology, Investigation, Writing – review & editing. **S.F. Gregório:** Methodology, Investigation, Data curation, Writing – review & editing. **J.B. Ortiz-Delgado:** Methodology, Data curation, Writing – review & editing. **J. Rotllant:** Methodology, Writing – review & editing. **J. Fuentes:** Methodology, Data curation, Writing – review & editing. **C. Tafalla:** Writing – review & editing. **J.M. Cerdá-Reverter:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

This study was financed by funds from MCIN/AEI/10.13039/501100011033 (PID2019-103969RB-C33 to J.M.C.-R. RTI2018-093909-B-100 to J.B. O.-D. and AGL2017–89648P to J.R.) and, as appropriate, by "ERDF A way of making Europe", by the "European Union" or by the "European Union Next Generation EU/PRTR". CCMar receives Portuguese national funds from FCT - Foundation for Science and Technology through projects UIDB/04326/2020, UIDP/04326/2020 and LA/P/0101/2020.

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