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Aeromonas salmonicida type III secretion system-effectors-mediated immune suppression in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Aeromonas salmonicida subsp. *salmonicida*, the etiologic agent of furunculosis, is a major pathogen in aquaculture. Together with other pathogens, it is characterized by the presence of a type 3 secretion system (T3SS). The T3SS is the main virulence mechanism of *A. salmonicida.* It is used by the bacterium to secrete and translocate several toxins and effector proteins into the host cell. Some of these factors have a detrimental impact on the integrity of the cell cytoskeleton, likely contributing to impair phagocytosis. Furthermore, it has been suggested that effectors of the T3SS are able to modulate the host's immune response. Here we present the first partial characterization of the immune response in rainbow trout (*Oncorhynchus mykiss*) infected with distinct strains of *A. salmonicida* either carrying (i) a fully functional T3SS or (ii) a functionally impaired T3SS or (iii) devoid of T3SS ("cured" strain). Infection with an *A. salmonicida* strain either carrying a fully functional or a secretion-impaired T3SS was associated with a strong and persistent immune suppression. However, the infection appeared to be fatal only in the presence of a fully functional T3SS. In contrast, the absence of T3SS was neither associated with immune suppression nor fish death. These findings suggest that the T3SS and T3SS-delivered effector molecules and toxins of *A. salmonicida* do not only impair the host cells' cytoskeleton thus damaging cell physiology and phagocytosis, but also heavily affect the transcription of critical immune mediators including the shut-down of important warning signals to recognize infection and induce immune defense.

1. Introduction

The type III secretion system (T3SS) is a complex nano-device used by several prokaryotes to deliver effector proteins and toxins to eukaryotic cells [1,2]. Of the best known bacteria carrying T3SS the best characterized are those of *Salmonella*, *Shigella* and *Yersinia* spp [2]. The T3SS has been proposed to have evolved from the flagellar apparatus [3], which through distinct evolutionary passages would have first lost its motility and then acquired the ability to secrete polypeptides [2]. The T3SS is composed of more than 20 proteins including the membrane secretion channel, a needle complex, which is the actual delivering device, several cytoplasmic proteins and the translocases, extracellular proteins that would be delivered by the T3SS into the host cell membrane where they are assembled into a protein channel [2]. Once the T3SS has been inserted into the host cell wall it can deliver a number of bacterially-produced effector proteins that reach the host cell cytosol thanks to a T3SS-associated ATPase, although alternative mechanisms have been proposed [2]. It appears that the delivery of the bacterial effector proteins requires a contact-activation between the tip of the needle and the host cell membrane. However, the dynamic of this process is largely unknown [2].

Besides the well-known T3SS systems present in *Salmonella*, *Shigella* and *Yersinia* spp, more recently, a T3SS has also been discovered in *A. salmonicida* subsp. *salmonicida* (*A. salmonicida*), the etiologic agent of furunculosis of fish, where it constitutes the main virulence mechanism [4,5]. Furunculosis is one of the most significant diseases of salmonids and is responsible for the death of large numbers of fish particularly when raised in aquaculture, accounting for significant losses in revenues [6]. The disease may have a sub-acute to chronic course or an acute one. The acute form is generally associated with mass mortality secondary to septicemia with multisystemic hemorrhages. Differently, the sub-acute to chronic

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form may be associated with low mortality and with the development of the typical "furuncles" consisting in collections of fluid admixed with necrotic tissue elevating the skin in multifocal nodular-like lesions. Clinical signs including anorexia, lethargy and abnormal swimming behavior have been reported in affected fish [7,8]. *A. salmonicida* is considered to enter the body of the host from multiple sites including the skin, the gills and the intestine to rapidly diffuse in the internal organs and leading to the death of the infected fish [8]. The T3SS of *A. salmonicida* is encoded by a large conjugative plasmid of 150 Kb [9–11], which can be lost or undergo insertion sequence dependent-rearrangements when grown in stressful conditions, including growth at temperatures above 20 °C [5,10–12]. The transcription of the T3SS in *A. salmonicida* is induced under Ca^{2+} limiting conditions and during the contact of *A. salmonicida* with the host [5]. A number of effector proteins have been shown to be encoded by *A. salmonicida* that can be delivered by the T3SS and include AexT, AopH, Ati2, AopP, AopO, AopN and ExsE [11]. One of these effectors, AexT, a bifunctional protein with actin ribosylating- and GTPase activity, has a detrimental effect on the cytoskeleton of the host cell, partially explaining the occurrence of cell rounding in target cells early after infection of fish cells *in vitro* [13]. Differently, another protein, AopP, inhibits the NF-kB signaling pathway, providing a major pro-apoptotic signal [14]. While the deletion of individual T3SS effector genes lead either to partial (*aopO*, *aexT* and *aopH*) or no attenuation at all (*aopO*) of *A. salmonicida*, the complete loss of the 150 Kb plasmid or the deletion of *ascV*, encoding a protein of the socket/cup of the T3SS in the inner membrane, results in complete avirulence [11,15,16]. Similarly, the deletion of the *ascC* gene encoding a protein for the outer membrane pore of the T3SS is associated with complete loss of pathogenicity [15,17]. These findings are highly suggestive of a major role of a functional T3SS in specifically targeting the virulence effectors to host cells.

Defense response against bacterial pathogens in teleost fish is funded on both innate and adaptive immunity [18,19]. A number of publications have highlighted the critical role of antimicrobial molecules including defensins [20], apolipoproteins [21], proteases, lectins, lysozyme, pentraxins and complement [22]. Further, innate cellular defenses have been documented to play a critical role against bacteria in fish. More specifically, macrophages and polymorphonucleated cells are a powerful first line of defenses against bacteria [22–24]. In particular the production of reactive oxygen (ROI) and nitrogen intermediates together with the direct and indirect iron deprivation mechanisms carried out by macrophages are important antibacterial mechanisms adopted by teleost fish [25]. Additionally to innate immunity, important antibacterial activity is provided also by the adaptive immunity. More specifically, direct antibacterial activity of $CD8^+$, $CD4^+$ T-cells and of sIgM + cells has been reported in fish [26].

The immune response toward *A. salmonicida* is not fully understood, however, it has been proposed that a protective immunity would be based on a balanced Th-1/Th-2 response [11,27,28].

A number of scientific articles support a relevant immunomodulatory activity of T3SS [11,16,27,29,30] as also exemplified by the induction of IL10 and IL12 expression in Atlantic salmon cell cultures inoculated with *A. salmonicida.* This induction was shown to be dependent on a functional T3SS, indicating that survival of wild type (w.t.) $T3SS^+$ *A. salmonicida* within the host cell may be facilitated by polarization of macrophages and other leukocytes to an alternative activation state [17]. However, an unequivocal demonstration of an immunomodulatory activity by T3SS *in vivo* is still lacking. In order to start to address this point we aimed to partially characterize the immune response against (i) fully virulent *A. salmonicida*, (ii) T3SS functionally impaired and (iii) T3SS "cured" derivative strains highlighting the potential distinct immune-signatures associated with each of the bacterial strains. We challenged naïve rainbow trout (*Oncorhynchus mykiss*) either with a w.t. strain of *A. salmonicida* carrying the functional T3SS (JF2267) or with a non-functional (Δ*ascV*) derivative thereof that still expresses residual amounts of T3SS toxins and effectors but is unable to secrete and translocate them (JF2747), or finally with a cured strain devoid of the plasmid encoding for the T3SS and associated molecules (JF2397). We then monitored the host immune response by assessing the transcriptional levels of relevant markers known to be associated in mammals with the main pathways of the immune response including Th-1, Th-2, T-regulatory or cell-mediated response. Although it is not conclusively demonstrated that these markers have homologous physiological functions in fish to those they have in mammals, we considered them a *bona fide* representative of the fish immune response. Furthermore, increasing evidence suggests the existence of immune pathways shared between all classes of jawed vertebrates, including mammals and fish [31]. Here we show that the presence of a functional T3SS along with its associated toxins and effector molecules translates into an impairing immunological signature that provides new grounds to better characterize the complex host-pathogen interface in lower vertebrates and that should be considered when designing new vaccines against furunculosis.

2. Material and methods

2.1. Animals and experimental design

A total of 114 rainbow trout (*Oncorhynchus mykiss*) ranging from 8 to 50 g of weight were used in this study. Briefly, the fish in the experiment were divided into four groups, one for each treatment including, i) controls, injected with sterile phosphate buffer saline (PBS) (n = 24), ii) challenged with the fully virulent, w.t. strain JF2267 (n = 18), iii) challenged with the secretion deficient derivative *ΔascV* mutant strain JF2747 (n = 18) iv) challenged with the "cured" strain JF 2397 that is devoid of the complete T3SS (n = 18). The challenged fish were then divided into three additional groups that were sampled at different times following challenge, i.e, 1, 2 and 5 days after challenge. The head kidney was the selected tissue for sampling because its primary relevance in immunopoiesis and immunity in fish. Each sampling time comprised three replicates for each of the challenged fish. Each replicate consisted of 2 (pooled head kidney) fish. Control fish comprised 12 replicates, 6 collected at day one post challenge and 3 at day 2 and day 5, respectively. Finally, two groups comprising 18 fish each, which were inoculated either with the fully virulent JF2267 or the *ΔascV* mutant JF2747 strain, were also included in the study to assess the mortality associated with either one or the other treatment. Mortality was monitored for a total of 14 days. No mortality assessment was set up with the "cured" strain JF2397 because of well-known complete avirulence of this strain [11,15,16]. Experiments were performed according to the Swiss guidelines for animal experimentation under the permission number 66/09.

2.2. Housing

Fish were obtained from a commercial fish farm without any history of furunculosis. For the experiment, fish were stocked in 30-L glass tanks supplied with a constant tap water flow through at a rate of 0.5 l/min. All tanks were constantly aerated. The animals were fed a commercial diet (Hokovit, Bützberg) at a rate of 2% of the body weight once per day.

2.3. Bacterial strains and growth conditions

Aeromonas salmonicida subsp. *salmonicida* w.t. strain JF2267 was originally isolated from an Arctic char (*Savelinus alpinus*) and subsequently re-isolated from a trout (*Oncorhynchus mykiss*) after experimental infection to ensure full virulence of the strain [5]. The deletion mutant *ΔascV* strain JF2747 was derived from the w.t. JF2267 by allelic exchange of the *ascV* gene with a Kanamycin resistance cassette [16]. The w.t. is characterized by a significant virulence as shown by intraperitoneal inoculation of 500 colony-forming units (CFU) per fish inducing 70–80% of mortality in challenge assays. The isogenic *ΔascV* mutant strain JF2267, by contrast, was shown to be non-virulent since 10^5 CFU per fish induced no mortality [16]. JF2267 (*ΔascV*) was shown to be unable to translocate the AexT toxin to host cells [32] and also showed strongly reduced expression of the remaining structural T3SS proteins as well as of the T3SS effector proteins [33]. The cured strain JF2397 was shown to have lost the entire 150 kb T3SS plasmid and thus is devoid of T3SS functions and to be completely avirulent [10]. Each strain was grown in TSB medium or onto TSA plates at 18 °C.

2.4. Challenge

Three groups of 18 fish were challenged with a dose of 5×10^2 CFU of either *A. salmonicida* w.t. strain JF2267 or the *ΔascV* mutant thereof JF2747 or of the cured strain JF2397. Inoculation was carried out by manual syringe injection of 50 μl into the coelomic cavity on each individual fish previously sedated by immersion in water containing 50 mg/l buffered 3-aminobenzoic acid ethyl ester (MS 222®, Argent Chemical Laboratories). Following the challenge, the fish were immediately placed back in their respective tanks. The two additional groups of fish comprising 18 individuals each, which were included in the study to assess the associated mortality, were inoculated either with the fully virulent JF2267 or the *ΔascV* mutant JF2747 strain, similarly as described above. Fish were monitored daily to assess if any death or clinical sign consistent with disease might have occurred. A total of 24 fish injected with sterile PBS served as uninfected controls.

2.4.1. Experimental fish culling

At the end of the experiment all the fish still alive were humanely euthanized with an overdose of MS222 (150 mg/l buffered 3-aminobenzoic acid ethyl ester (MS 222®, Argent Chemical Laboratories)). The procedure was carried out according to the Swiss guidelines for animal experimentation.

2.5. Tissue sampling

Following each sampling, necropsy was performed and the head kidney of each fish was collected. Pools of 2 fish samples each (head kidneys) were carried out from all the sampling groups (2 fish = 1 replicate). Pooling of the head kidneys was carried out because of their small sizes and consequently low amount of tissue available. Furthermore, pooling was also considered to reduce individual variability. Tissues were snap-frozen in liquid nitrogen and then stored at −80 °C until processing.

2.6. RNA extraction and reverse transcription

Total RNA extraction was carried out from each tissue pool consisting of the head kidney of two fish from the same group (=1 replicate). RNA extraction was carried out with the Direct-Zol kit (Zymogen, Irvine, CA, USA) according to the manufacturer's instructions and the extraction product was immediately processed for reverse transcription that was carried out with Superscript III reverse transcriptase (Invitrogen, Foster City, CA, USA) and random primers according to the manufacturer's instructions. Quality assessment of the RNA samples was carried out with the RNA 6000 Nano kit (Agilent, S. Clara-CA, USA) according to the manufacturer's instructions. RNA and their correspondent cDNA samples were stored at −80 °C.

2.7. Markers

In order to partially characterize the immune response of rainbow trout to *A. salmonicida*, we selected a panel of markers that are considered suitable for dissecting the fish immune response and in particular the Th-1, Th-2, T-regulatory-like and cell-mediated immune response. More specifically, forkhead box P3-2 (FOXP3-2) and CD28 were considered relevant markers for assessing the T-regulatory response in the fish in consideration of the role of FOXP3-2 as master transcription factor for T-regulatory cells and of that of CD28 for T-regulatory cells homeostasis [34–37]. Interferon gamma 2 (INFγ), T-box 21 (TBx21) and IL2 expression were evaluated to assess the Th-1 response. The rationale of this choice derived from the tight functional association of these parameters within the Th-1 pathway. Briefly, INFγ is a critical cytokine (together with IL12) that initiates the signaling pathway leading to Th-1 cell development. TBx21 is the master regulator for Th-1 differentiation and transactivates the INFγ gene in Th-1 cells, whereas IL2 promotes naïve CD4+T cells differentiation into Th-1 (and Th-2) cells [37]. GATA-binding protein 3 (GATA3) and IL4/13 were selected to highlight the Th-2 response given the well-known role of GATA3 as master regulator for Th-2 differentiation, and IL4/13 as critical factor for Th-2 differentiation and regulation of GATA3 expression [37,38]. Finally, CD4 and CD8a were selected as markers for the cell-mediated immune response given the role of CD4+T cells as master regulators of the whole adaptive immune response and of CD8+T cells as main T cell effectors. Additional markers selected as internal controls were the elongation factor 1 alpha (EF1α) and the 18SrRNA gene. Primer sequences used for reverse-transcription generated cDNA PCR were obtained with the help of "primer express" software (Thermo Fischer Scientific, Waltham,

Table 1
Names and nucleotide sequences of PCR primers used for the detection of the transcripts of the selected markers.

Primer name	Primer sequence (5′-3′)	Marker
EF1α_FW[a]	AGGTACTACGTCACAATCAT	EF1α (224 nt)[b]
EF1α_RV	AGTAGAGTCCATCTTGTTGA	
18S_FW	AAGGATTGACAGATTGATAG	18SrRNA (169 nt)
18S_RV	CTTGTCCCTCTAAGAAGTT	
INFγ_FW	ATTGAGGACTATTGAGCTT	INFγ (177 nt)
INFγ_RV	ATAGTGGACTTTCAGTTTGT	
IL2_FW	GGACACTGTGAAAGTAGAAT	IL 2 (205 nt)
IL2_RV	GAGCTTGAGTTAAATGTCTT	
TBx21_FW	GAATCATTTCTGTCTAATCC	TBx21 (244 nt)
TBx21_RV	TGTGTTGTTCTAAATGACAG	
IL4/13_FW	CTTCTCTGAAGTACAAGGAC	IL 4 (397 nt)
IL4/13_RV	GAGTAATGTTTCCAGAAGTT	
GATA3_FW	ATAGTATGGAGATACGTGGT	GATA-3 (219 nt)
GATA3_RV	GTGTATATGAACGTTTGCTA	
FOXP3_FW	AGCAGGAGATACAGAGTAAG	FOXP3-2 (194 nt)
FOXP3_RV	TCGTGTTACTTTTGTATGAG	
CD28_FW	AGAGTGACTACATGGACATC	CD28 (135 nt)
CD28_RV	TCGTGTTACTTTTGTATGAG	
CD4_FW	AGAAAGTACAAAATGAAGTC	CD4 (192 nt)
CD4_RV	ATATCAAAGGTTGTGGTG	
CD8_FW	GTCAAGGTAAATCAGTCTTT	CD8a (80 nt)
CD8_RV	GACGACTTTAAAACTATCCT	

[a] FW designates forward and RV reverse primers.
[b] In parenthesis nucleotides of the amplified fragment of the respective gene.

MA, USA) and are provided in Table 1.

2.8. qRT-PCR

A quantitative real time polymerase chain reaction (qPCR) was set up to evaluate the expression levels of the immune markers described above. Each cDNA sample was brought to a final concentration of 20 ng/μl. Briefly a final reaction solution comprising 10 μl of SYBR mix (2×) (Invitrogen, Foster City, CA, USA), 0.5 μl of cDNA (20ng/μl), 1 μl of each primer at a concentration of 10 μm and 7.5 μl of double distilled water was prepared for each sample. The reaction mixtures were placed in the thermocycler (7500-Fast Real Time PCR System-Applied Biosystem Foster City, CA, USA) and underwent initial denaturation at 95 °C for 2 min, followed by 42 cycles comprising a denaturing step at 95 °C for 3 s and annealing/extension step at 57 °C for 30 s. The melting curve stage comprised the following steps: 95 °C for 15 s, 60 °C for 1 min ramping to 95 °C step for acquisition. All the samples were analyzed in duplicate.

Primer efficiency was determined for each of the primer pair used in this study. More specifically a standard curve was prepared for each of the selected markers and the slope was determined for each of them. The efficiency values were then obtained using the following equation: $E = 10(-1/slope)-1$ [39].

Normalization of the obtained results was carried out with the $2^{\Delta\Delta}$CT method [39]. Briefly, for each of the samples to be analyzed, the mean of the replicates was calculated and subtracted from each of the values of the internal controls (18sRNA and EF1α) to obtain the Delta Ct18s and Delta Ct EF1α, respectively. The values obtained were then used in the following calculations according to the $2^{\Delta\Delta}$CT method [40]. In brief, the mean of the two "delta Cts" was calculated and used as an exponent of the base "2" to obtain an estimate of the expression of the immune markers considered. Finally, the values obtained for the challenged fish were normalized to the values obtained from the untreated fish, whose expression level was arbitrarily considered equal to 1. Finally, the mean and the standard deviation were calculated.

Statistical analysis was carried out with the software package InStat V2.05 (Graphpad software, La Jolla, CA, USA). We assumed that our sample data were not normally distributed and the Kruskall Wallis test was selected for the analysis. Post-test analysis was carried out only when statistical differences between the different treatments were significant ($p \leq 0.05$). Post-test analysis was carried out with Dunn's test comparing all pairs of columns. Statistical analysis was carried out on the mean of the $^{\Delta}$CT values.

3. Results

3.1. Challenge study

Following challenge with the fully virulent strain JF2267, 35% of the fish died, whereas none of the fish challenged with the *ΔascV* mutant strain JF2747 did. All deaths were observed later than 5 days post infection. After the challenge there was no change in weight. Only fish challenged with the virulent strain JF2267 showed clinical signs consistent with furunculosis such as hemorrhages in inner organs and muscle tissue, while animals challenged with the *ΔascV* mutant strain JF2747 did not show any sign of disease during the experiment or at necropsy at the end of the experiment. None of the fish challenged with the cured strain JF2397 showed any sign of disease during the study. All the unchallenged naïve fish were still alive at the end of the study and never developed any lesion consistent with furunculosis.

3.2. qRT-PCR assays

Each of the PCR product was run on a gel which revealed the expected sizes for the amplicons corresponding to the markers of the 18s (169bp), FOXP3-2 (194bp), INFγ (177bp), IL2 (205 bp), CD8a (80bp), CD28 (135bp), CD4 (192bp), TBx21 (244bp), IL4/13 (397bp), GATA3 (219bp), and EF1α (224bp). Dissociation curves were examined for all the markers evaluated in the study and showed a uniform peak for CD28, FOXP3-2, TBx21, CD8a, 18s, whereas minor pre-peaks were observed for INFγ, IL2, IL4/13, GATA3, CD4 and EF1α. RNA samples showed RIN (RNA integrity number) values ranging from 2.5 up to 8.2 with an average RIN of 5. Primer efficiency ranged from 92 to 114% for all the markers with the only exception of FOXP3, whose primers showed an efficiency of 150%. Primer efficiency values are summarized in Table 2.

4. Gene expression analysis

4.1. T-regulatory cell-markers

4.1.1. FOXP3-2

FOXP3-2 expression in fish challenged with the w.t. *A. salmonicida* strain JF2267 resulted in slight increase at one day after challenge and then a progressive mild decrease at 2 and 5 days post challenge, respectively compared to the control group. In fish infected with the *ΔascV* mutant that is characterized by an impaired T3SS, induction of FOXP3-2 was about half of the controls' values during the entire time of the treatment. Differently, the fish challenged with the cured strain JF2397 showed values slightly higher than the controls. Statistical analysis did not reveal any significant differences between the treatment groups at 1 ($P = 0.0570$) and 5 days ($P = 0.2525$) post challenge, respectively. However, a statistical difference between the fish infected with the *ΔascV* mutant and those infected with the cured strain was observed at 2 days post challenge ($P < 0.05$) (Fig. 1 A).

4.1.2. CD28

The expression pattern of CD28 showed obvious expression differences between the fish infected either with the w.t. or the *ΔascV* mutant on one side and those infected with the cured strain JF2397 on the other. Both fish infected with the w.t. or the *ΔascV* mutant showed a dramatic reduction of the expression of CD28 that was almost shut down at 2 and 5 days post challenge in fish infected with the w.t. strain and at 1 and 2 days post challenge in those infected with the *ΔascV* mutant, respectively. Differently, fish infected with the cured JF2397 strain had values very close to those of the controls. Overall, statistical analysis revealed significant differences between the treatment groups at any sampling time ($P = 0.0088$ at 1 day, $P = 0.0018$ at 2 days, $P = 0.001$ at 5 days post

Table 2
Primer efficiency of selected markers.

Marker	Dilution curve slope	Primer efficiency[a]
CD4	−3.085186	110.93%
CD8a	−3.100657	110.14%
CD28	−3.059230	112.27%
FOXP3-2	−2.429091	150.03%
IL2	−3.502810	92.97%
GATA3	−3.100689	110.14%
IL4/13	−3.153023	107.57%
TBX21	−3.527662	92.08%
INF-γ	−3.013920	114.68%
18s	−3.387458	97.34%
EF1α	−3.067266	111.85%

[a] Efficiency calculated according to Pfaff et al., 2001 [39].

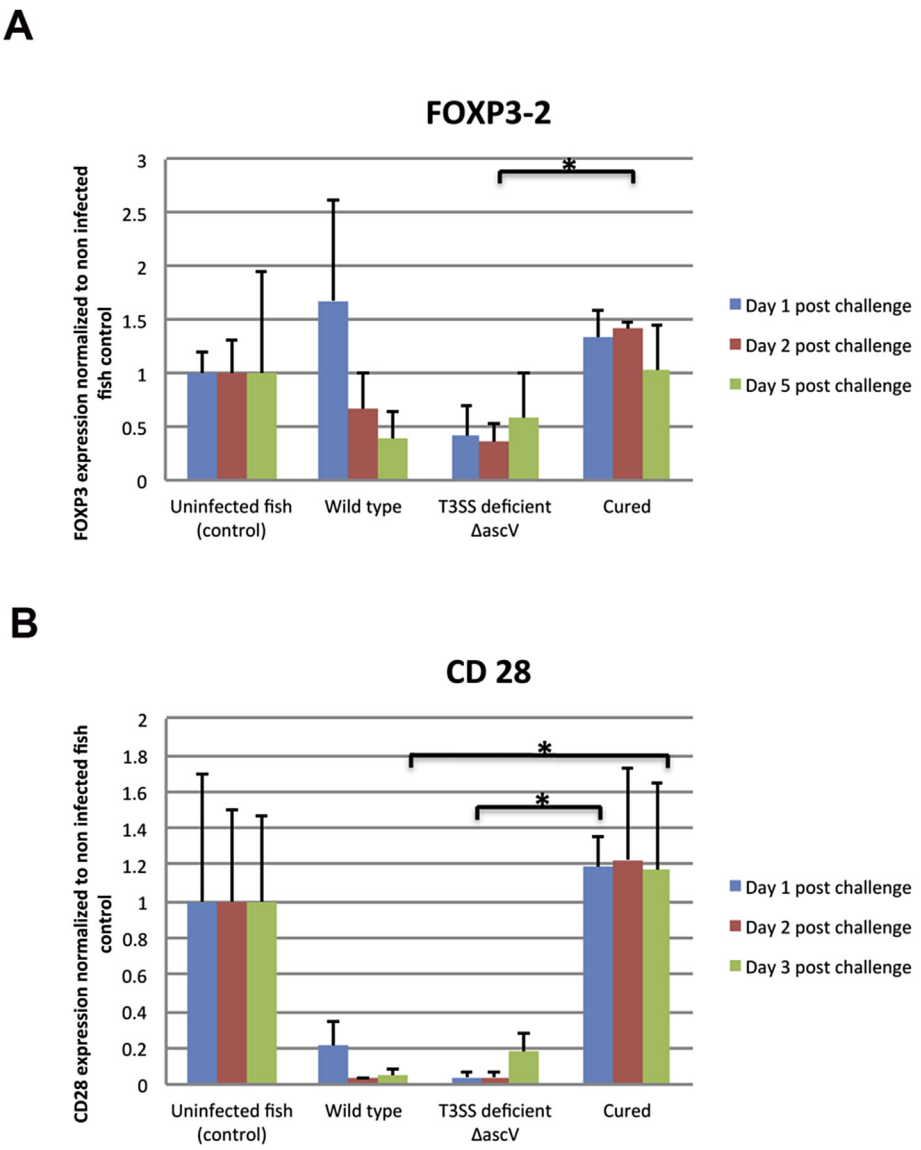

Fig. 1. T Regulatory cell response. Expression levels of FOXP3-2 (A) and CD28 (B) in distinct groups of fish either uninfected or infected with a wild type (JF2267) or mutant (JF2747) or cured (JF2397) strain of *Aeromonas salmonicida*. The expression levels are normalized to those of the uninfected control group of fish that have been arbitrarily considered equal to 1. Bars of different color refer to different sampling times. More specifically, the blue bar refers to the sampling carried out one day post infection, the red bar to two days post infection and the green bar to five days post infection, respectively. Statistically significant differences are highlighted by black bars connecting the specific treatment groups. An asterisk indicates a P value < than 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

challenge, respectively). Post-test analysis revealed a statistical significant difference between the fish infected with the *ΔascV* mutant and those infected with the cured strain at 1 day post challenge ($P < 0.05$) and between those infected with the w.t. and those infected with the cured strain ($P < 0.05$) at 5 days post challenge (Fig. 1B).

4.2. TH-1 markers

4.2.1. IL2

Infection of fish with the virulent w.t. and *ΔascV* mutant strains of *A. salmonicida* resulted in a virtually immediate total shut off of IL2 expression in trout that lasted at least until 5 days post challenge compared to the non-infected control group (Fig. 2A). Differently, the fish infected with the cured strain devoid of the T3SS and the associated toxins and effector molecules showed a progressive increase of IL2 expression that was more than double that of the controls at 5 days post challenge. Statistical analysis revealed significant differences between the treatment groups at 1 ($P = 0.0069$), 2 ($P = 0.006$) and 5 ($P = 0.0001$) days post challenge. Post-test analysis revealed statistical significant differences between the fish infected with the *ΔascV* mutant and those infected with the cured strain ($P < 0.05$) and between those infected with the w.t. and the cured strain ($P < 0.05$) at 5 days post challenge (Fig. 2A).

4.2.2. INFγ

Interferon gamma expression was overlapping that of IL2 described above with a complete or nearly complete shut down in fish infected with the w.t. strain or the *ΔascV* mutant strain. Differently, the fish infected with the cured strain showed very similar values of INFγ expression at 1 and 2 days post challenge to those of the controls. At day 5 after the challenge with the cured strain, INFγ levels raised to more than double of that of the non-

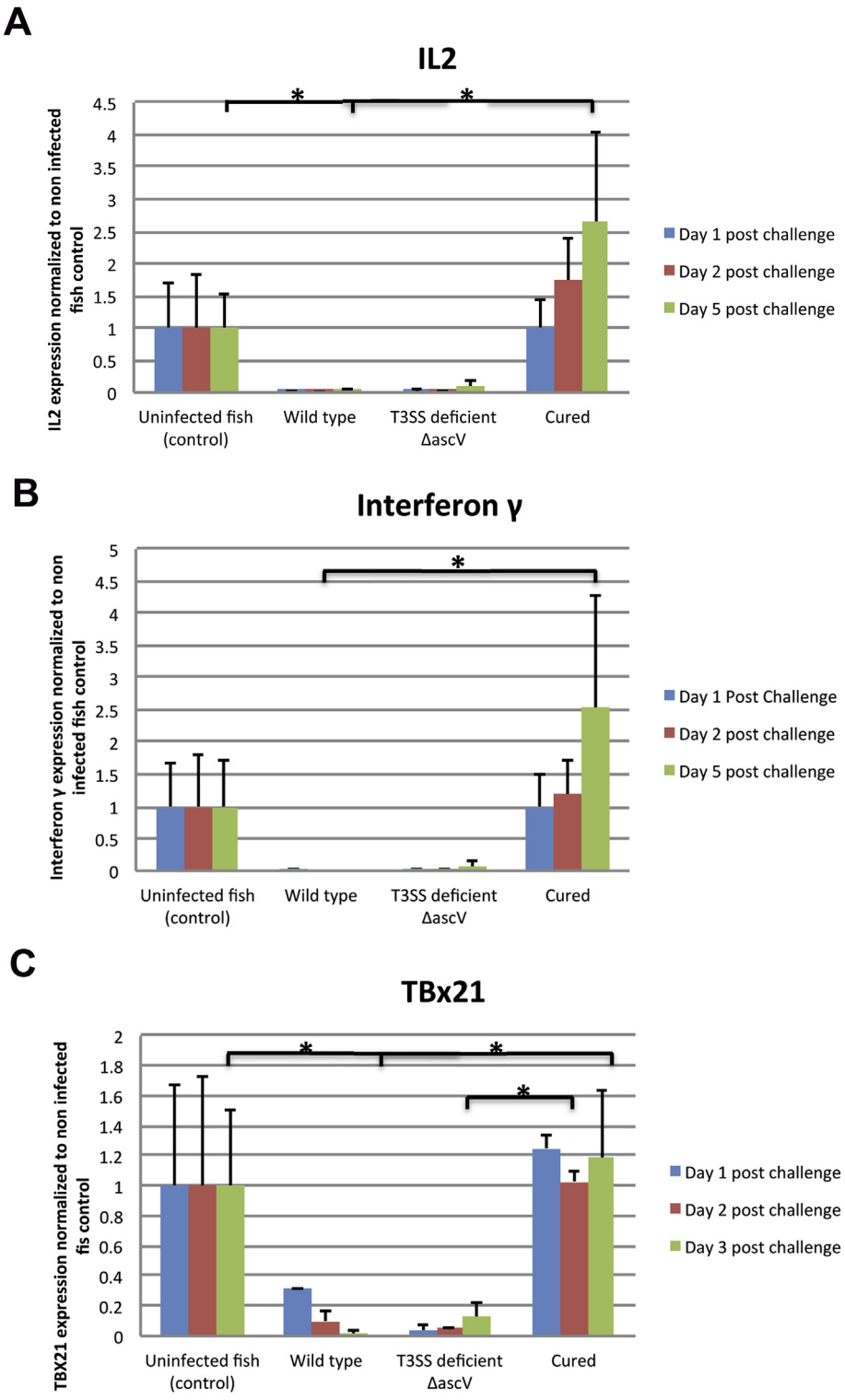

Fig. 2. Th-1 Response. Expression levels of IL2 (A), Interferon γ (B) and TBx21 (c) in distinct groups of fish either uninfected or infected with a wild type (JF2267) or mutant (JF2747) or cured (JF2397) strain of *Aeromonas salmonicida*. The expression levels are normalized to those of the uninfected control group of fish that have has been arbitrarily considered equal to 1. Bars of different color refer to different sampling times. More specifically, the blue bar refers to the sampling carried out one day post infection, the red bar to two days post infection and the green bar to five days post infection, respectively. Statistically significant differences are highlighted by black bars connecting the specific treatment groups. An asterisk indicates a P value < than 0.05. Specifically for TBx21, one replicate out of three was available for the fish infected with the wild type strain JF5055 at 1 day post-infection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

infected individuals. Significant statistical differences were observed between the treatment groups at 1 ($P = 0.0064$), 2 ($P = 0.0016$), and 5 ($P = 0.0002$) days post challenge. Post-test analysis revealed statistical significant differences between the fish infected with the w.t. and the cured strain at 5 days post challenge ($P < 0.05$) (Fig. 2B).

4.2.3. TBx21

TBx21 expression in the fish challenged with the w.t. strain showed a progressive downregulation ranging from approximately a third of the values of the controls at one day post challenge to becoming virtually undetectable at 5 days post challenge ($P < 0.05$). Interestingly, in the fish infected with the *ΔascV* mutant, the stronger downregulation of TBx21 was observed at 1 day post challenge, however, minimally increased at 5 days post infection (Fig. 2C). Similarly to what observed for the other markers described above, the fish infected with the cured strain did not show any downregulation, but showed values very close to those of the controls for the entire duration of the experiment. A statistical significant difference was observed among all the treatment groups at 1 ($P = 0.0112$), 2 ($P = 0.001$) and 5 ($P = 0.0006$) days post infection. Furthermore, post-test analysis showed statistical significant differences between the fish infected with the *ΔascV* mutant and those infected with the cured strain at 2 days post infection ($P < 0.05$). Additionally, differences were also observed between the fish infected with the w.t and those infected with the cured strain ($P < 0.05$) and the control fish ($P < 0.05$), respectively at 5 days post infection (Fig. 2C).

4.3. TH-2 markers

4.3.1. GATA 3

The fish infected with the w.t. strain showed an immediate drop of GATA3 expression already at 1 day post infection and the expression of this marker progressively decreased to become virtually undetectable at 5 days post infection. Similar magnitude of decrease of expression was observed in the fish infected with the *ΔascV* mutant strain. Differently, the fish infected with the cured strain had overlapping GATA3 expression values to the controls up to 2 days post infection, whereas at 5 days post infection the expression level was double of the controls (Fig. 3B). Statistical analysis showed significant differences of expression between the different treatment groups at 1 ($P = 0.0043$), 2 ($P = 0.0017$) and 5 ($P = 0.0002$) days post infection. Post-test analysis revealed a statistical significant difference between the fish infected with the *ΔascV* mutant and those injected with PBS (controls) at 1 day post infection ($P < 0.05$) and between the fish infected with the w.t. and those infected with the cured strain at 5 days post infection ($P < 0.05$) (Fig. 3B).

4.3.2. IL4/13

IL4/13 expression in fish challenged with either the w.t. or the *ΔascV* mutant strain was nearly completely shut down already at day 1 post infection remaining virtually undetectable up to 5 days post challenge. Differently, fish infected with the cured strain maintained expression values close to the controls during the first two days post infection and then rose up to almost three times the control values at 5 days post infection (Fig. 3A). Statistical analysis showed significant differences among all the treatment groups at 1 ($P = 0.0156$), 2 ($P = 0.0105$) and 5 ($P = 0.0006$) post infection. Post-test analysis revealed statistical significant differences between the IL4/13 expression of the fish infected with the w.t. and those infected with the cured strain at 5 days post infection ($P < 0.05$) (Fig. 3A).

4.4. Cell-mediated adaptive immunity

4.4.1. CD4

Similarly for several of the markers described above, CD4 expression in the fish challenged either with the w.t. or the *ΔascV* mutant strain was strongly downregulated compared to the controls during the entire duration of the treatment. The fish infected with the cured strain showed a slight increase in CD4 expression compared to the controls. Significant differences were observed between the treatment groups at 1 ($p = 0.003$), 2 ($P = 0.0002$), and 5 ($P = 0.0003$) days post challenge, respectively (Fig. 4A). Furthermore, post-test analysis revealed a statistical significant difference between the fish infected with the *ΔascV* mutant and those infected with the cured strain ($P < 0.05$) at 2 days post infection. Similarly, a significant difference was observed between the fish infected with the *ΔascV* mutant and the cured strain and between those infected with the w.t. and those with the cured strain at 5 days post infection, respectively (Fig. 4A).

4.4.2. CD8a

CD8a expression was almost undetectable in fish infected with the w.t. strain until 2 days post challenge and then minimally increased at 5 days post challenge. In fish infected with the *ΔascV* mutant strain the expression remained very low for the entire duration of the treatment. Fish infected with the cured strain had expression values that increased up to 4 times higher than the controls. However, these results showed higher experimental errors (Fig. 4B). Statistical analysis revealed significant differences between the treatment groups at day 1 ($p = 0.0242$), 2 ($P = 0.0007$), and 5 ($P = 0.0336$) post challenge, respectively. Post-test analysis revealed a statistical significant difference between the fish infected with the w.t. and those infected with the cured strain at 2 days post infection (Fig. 4B).

5. Discussion

The main goal of this investigation was to assess the immune response of rainbow trout challenged either with a (i) fully virulent *A. salmonicida* w.t. strain or (ii) a *ΔascV* deletion mutant thereof carrying an impaired T3SS, or (iii) a cured strain devoid of the plasmid encoding for the T3SS and its associated toxins and effector molecules. We selected a group of immune markers which are known to be relevant for highlighting specific pathways of the mammalian immune response, including Th-1, Th-2, T-regulatory and cell-mediated, which are also found in fish and possibly share the same principles [31], being fully aware of the differences between fish and mammal immune physiology.

Our findings revealed the existence of unambiguous and distinct immunological signatures associated with the infection of the different strains of *A. salmonicida* almost independently of the investigated markers.

T-regulatory expression was assessed by the investigation of the expression of FOPX3-2 and CD28. FOXP3-2 is a pivotal transcriptional regulator critical for the development and function of the T-regulatory cells, whereas CD28 appears to be important for T-regulatory cells homeostasis [34–36]. The expression of FOXP3-2 did not show major variations between the different treatment groups with the exception of a statistical significant difference between the fish infected with the *ΔascV* mutant and those infected with the cured strain at 2 days post challenge. Differently, CD28 expression showed remarkable differences between the fish infected with the cured strains and those infected either with the fully functional or defective T3SS (Fig. 1A). Overall, it appears that T-regulatory cells are unaffected by the presence of bacteria devoid of T3SS and its associated molecules. Differently, both the functional and the secretion defective *ΔascV* mutant in association with its effector molecules appear to contribute to the downregulation of T-regulatory cells, which would occur immediately after the infection, with the exception of FOXP3-2, which undergoes a slight transitory upregulation at 1 day post infection with the w.t. strain.

The activation of the T regulatory cells in a T3SS dependent manner was proposed among the possible mechanisms mediating

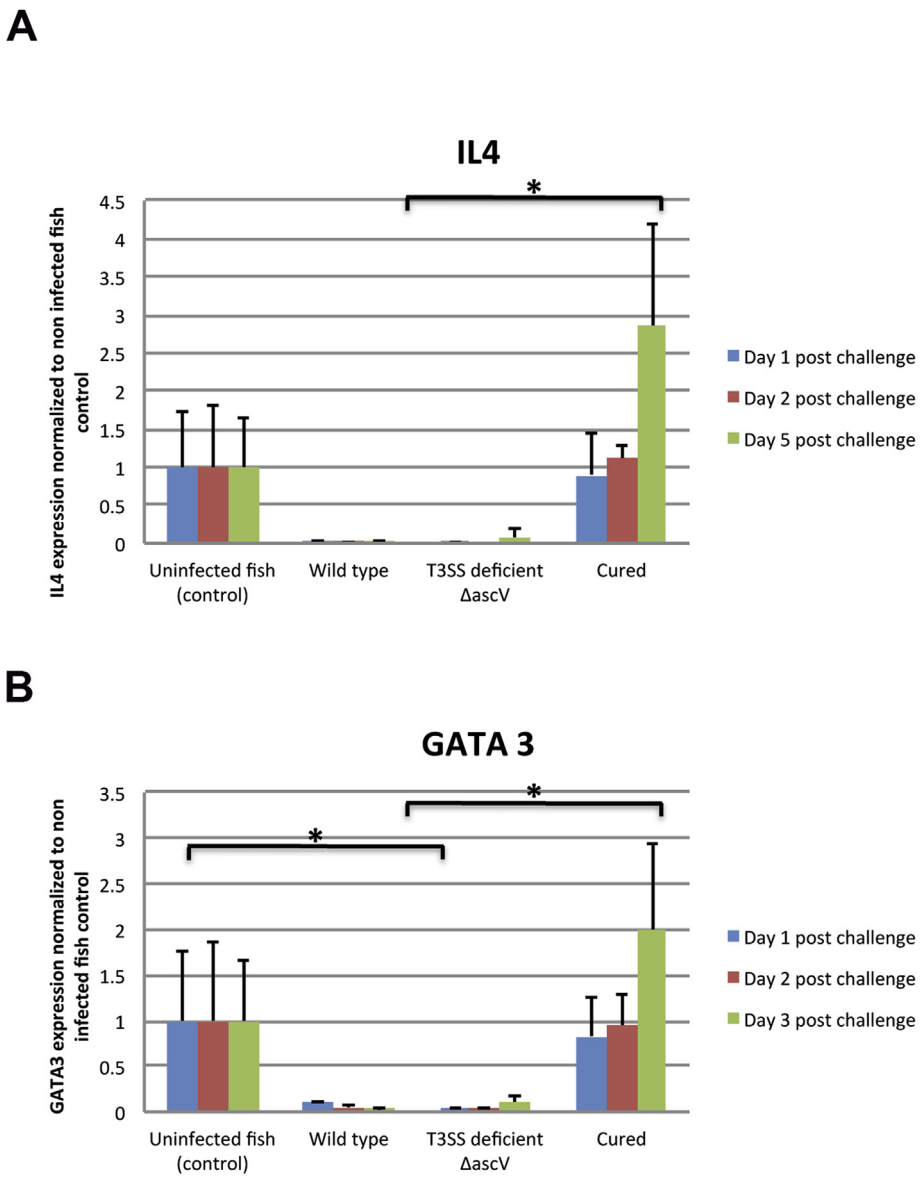

Fig. 3. Th-2 response. Expression levels of IL4/13 (A) and GATA3 (B) in distinct groups of fish either uninfected or infected with a wild type (JF2267) or mutant (JF2747) or cured (JF2397) strain of *Aeromonas salmonicida*. The expression levels are normalized to those of the uninfected control group of fish that have been arbitrarily considered equal to 1. Bars of different color refer to different sampling times. More specifically, the blue bar refers to the sampling carried out one day post infection, the red bar to two days post infection and the green bar to five days post infection, respectively. Statistically significant differences are highlighted by black bars connecting the specific treatment groups. An asterisk indicates a P value < than 0.05. Specifically for IL4/13, one replicate out of three was available for the fish infected with the mutant strain JF2747 at 2 days post-infection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the immunosuppression that would follow *A. salmonicida* infection in fish [11]. Our results are more consistent with a relative down-regulation of both FOXP3-2 and CD28, although an initial upregulation does occur in fish infected with the w.t. strain. The downregulation pattern, that is particularly severe for CD28, was observed in all the other markers examined, as we will discuss below and consequently, the immunosuppression that follows *A. salmonicida* infection might be not necessarily dependent essentially on a T-regulatory cell-mediated effect, but differently, on a more global effect on a larger numbers of immune players.

The Th-1 response in the challenged fish was assessed by measuring the expression of some critical markers including IL2, INFγ and TBx21. IL2 is a key cytokine important for the survival, expansion and differentiation of activated T cells and NK cells [41] together with CD28, which provides a co-stimulatory signal. Interferon gamma is a molecule which is involved in several pathways of both innate and adaptive immune response, whereas TBx21 is a master transcriptional regulator for Th-1 differentiation and interferon gamma production [42]. Similarly to what was observed for CD28, the challenge with the fully virulent w.t. strain or the *ΔascV* mutant was invariably associated with a strong and immediate downregulation, whereas the infection with the cured strain was associated with a variable upregulation of the tested markers. These data are suggestive of a severe downregulation of INFγ expression together with that of TBx21 and IL2, by components of the T3SS or by its effectors or by both of them. Interestingly, in a recent publication [43], it was reported that Atlantic salmon (*Salmo salar*) infected with a w.t. of *A. salmonicida* showed an upregulation of both INFγ and TBx21 expression within 5 days after challenge partially resembling that observed for the cured

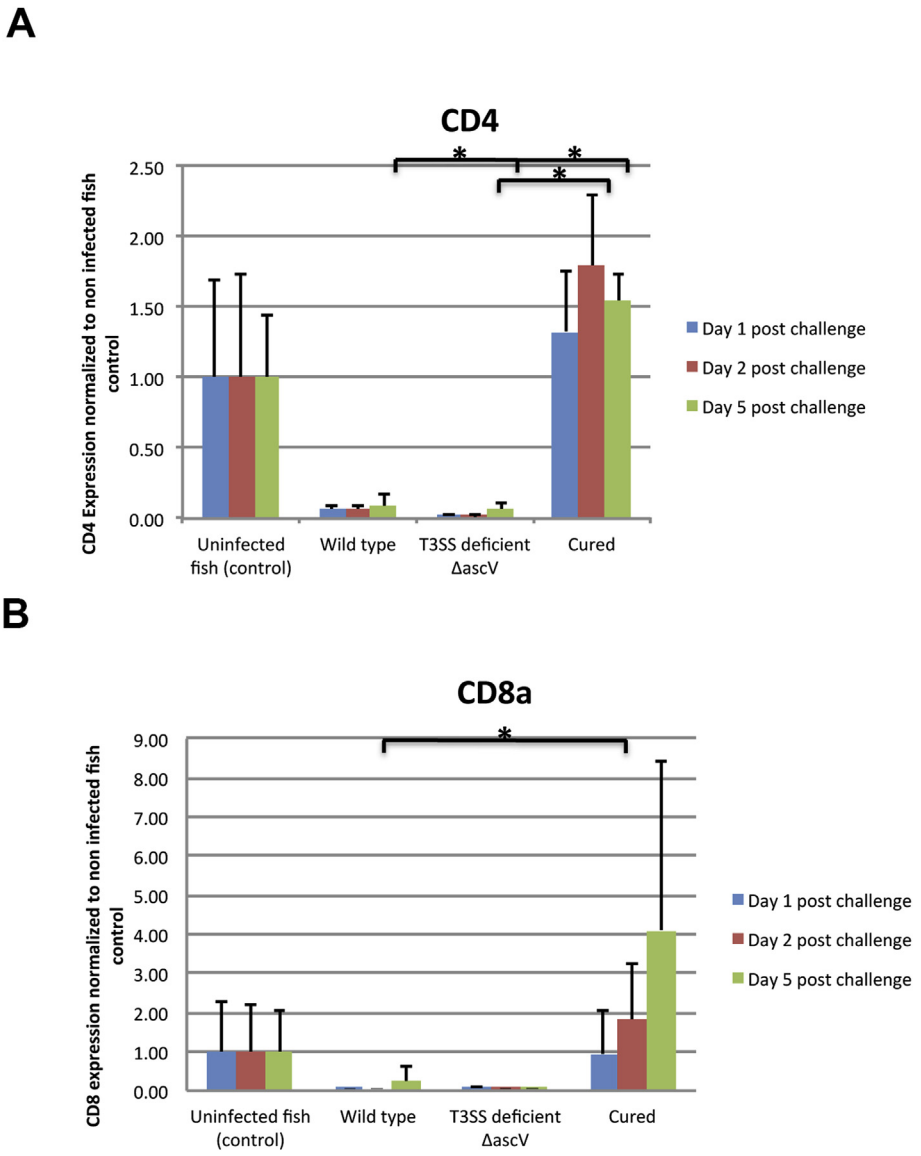

Fig. 4. Cell-mediated response. Expression levels of CD4 (A), CD8a (B) in distinct groups of fish either uninfected or infected with a wild type (JF2267) or mutant (JF2747) or cured (JF2397) strain of *Aeromonas salmonicida*. The expression levels are normalized to those of the uninfected control group of fish that have been arbitrarily considered equal to 1. Bars of different color refer to different sampling times. More specifically, the blue bar refers to the sampling carried out one day post infection, the red bar to two days post infection and the green bar to five days post infection, respectively. Statistically significant differences are highlighted by black bars connecting the specific treatment groups. An asterisk indicates a P value < than 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

strain in our study. However, no information concerning the growth conditions of the bacteria was provided and consequently, it is not possible to assess whether in this experiment the T3SS genes were still present in the bacteria or might have been lost by the w.t. bacteria during their *in vitro* expansion. Furthermore, in the study of Kumari et al., 2015 fish were infected with a dose of organisms 500,000 times higher than that used in our experiment (5×10^2 Vs 1×10^8) which indicates for a partially attenuated strain possibly by loss of certain or all T3SS functions [10,12]. Increase of INFγ expression was also observed in the gills of rainbow trout inoculated with a high dose of inactivated *A. salmonicida* [44]. In Arctic charr (*Salvelinus alpinus*) inoculated with *Aeromonas salmonicida* subsp. *achromogenes* INFγ gamma expression showed no significant differences in comparison with uninfected control fish [45]. This is in agreement with our observations, as analysis of the entire genome sequence of *A. salmonicida* subsp. *achromogenes* (Genbank accession number NZ_AMQG02000015-1) reveals that this species is devoid of T3SS genes and previous studies had shown that pathogenicity of *A. salmonicida* subsp. *achromogenes* is driven by other virulence factors than T3SS [46]. The persistence of the downregulation of key molecules such as INFγ, IL2 and TBx21 for at least 5 days following the infection with a w.t. *A. salmonicida* subsp. *salmonicida* leaves the host with basically no defensive tools against the pathogen during the most critical time of the infection, i.e. the incubation period, occurring within the first 3–4 days [8,11]. Considering the major role of INFγ both towards the innate and adaptive immunity, such impairment is likely to provide the host with a severe immune deficit. In particular it is interesting to observe how the blockade of INFγ expression might play a synergistic role with some of the previously characterized functions of the effector molecules produced by *A. salmonicida* and delivered to the host leading finally to very high mortality in fish [5,16]. In fact, the T3SS dependent *A. salmonicida* toxin AexT, as well as AopH, and potentially Ati2, and AopO damage the cytoskeleton of the host cell

[11]. The cytoskeleton plays a major role in the cell physiology including phagocytosis [47]. Hence it is intuitive to postulate that a defective phagocytosis associated with the reduced killing rate of the phagocytosed bacteria due to the virtual shutdown of the production of INFγ, IL2 and TBx21 is a severe double inhibitory step that reduces the bacterial clearance of the host. Furthermore the T3SS effector AopS is known to indirectly prevent the NLRC4 inflammosome activation, a multiprotein complex necessary for caspase-1 activation, leading to the secretion of pro-inflammatory cytokines [48]. In addition, AopP is known to impair the NF-kB pathway leading to cellular apoptosis [11,14].

The Th-2 response in the challenged fish was assessed by measuring GATA3 and IL4/13 expression. GATA3 is a key transcription factor which regulates T-cell development, Th-1/Th-2 balance and Th-2 differentiation by IL4/13 secretion, which promotes the skewing of Th-0 to Th-2, [49,50]. Both GATA3 and IL4/13 expressions were influenced by the infection either with the w.t. or *ΔascV* mutant strain by an immediate and marked downregulation similarly to what was observed for the other markers described above, indicating that T3SS effectors have an impact on also the host's Th-2 response to infection and most likely on other aspects of the T cell physiology given the broad spectrum of activities that GATA3 is involved with [49]. A wide array of T-cell related immune functions critical for an anti-*Aeromonas* response might be then compromised by *Aeromonas salmonicida*. By contrast, the infection with the cured strain showed once again very similar values to the controls or upregulation during the later stage of the experiment. Interestingly, both for GATA3 and IL4/13 expression, but also for the other factors examined before there is an opposite trend of expression between the fish infected with the w.t. and those infected with the *ΔascV* mutant, although within the limits of their low expression values. More specifically, the infection with the w.t was invariably associated with a progressive downregulation, whereas the infection with the *ΔascV* mutant was associated with an initial strong downregulation, which minimally, but progressively recovered later on during the experiment. The biological meaning and impact of these subtle changes are not clear, but are suggestive of different baseline dynamics associated with the different bacterial strains. Finally, in our investigation we assessed the cell-mediated immune response by measuring the expression of CD4 and CD8a. CD4 is a well-known marker of a critical subgroup of T-cells, involved in modulating several aspects of the adaptive immune response, whereas CD8a is the marker of effector T-cells. Overall, CD4 and CD8 expression patterns were overlapping with those observed for the other markers examined consistently showing a marked downregulation in the fish infected either with the w.t or the *ΔascV* mutant strain, whereas the fish infected with the cured strain showed higher values than the controls. The overall CD4 and CD8 downregulation observed in the fish infected either with the w.t. or the *ΔascV* mutant strains could be interpreted either as a downregulation of their specific cluster of differentiation markers (CD) or as a net loss of CD4 and CD8 T cells. The latter scenario might occur secondary either to cell death or emigration of CD4 and CD8 T cells from the head kidney into other organs. Interestingly, a recent publication suggests that following *A. salmonicida* infection T cells would immigrate into the head kidney starting 12 h post infection. Alternatively the net cell increase might be secondary to T cell multiplication in the head kidney [51]. Both these hypotheses are in conflict with our results. However, Brietzke and collagues [51] used as T cell marker, the T cell receptor (detected with a specific antibody by FACS), whereas we used as T cell markers CD4 and CD8a, detected by RT-PCR (gene expression). It is possible that part of the differences in the experiments might be due to technical aspects, however, this discrepancy warrants further future investigations.

The very similar transcription pattern observed in the immune markers described above is strongly suggestive of a concerted detrimental effect on the host's immune response played by the secreted effector molecules by *A. salmonicida.* However, the almost overlapping effect observed both in fish infected either with the w.t. or the *ΔascV* mutant might appear as a surprising finding given that the defective T3SS in the *ΔascV* mutant impairs the translocation into the host cells of the effector factors synthetized by *A. salmonicida*. However, these very factors are still synthetized by the *ΔascV* mutant although at a lower level compared to the fully virulent strain [33]. Yet, they apparently impact the host's immune functions if the bacteria are administered experimentally into the coelomic cavity of the host fish. Nevertheless, the absence of mortality in the fish infected with the *ΔascV* mutant despite the very similar downregulation of the immune factors described above is also relatively surprising. However, this might be secondary to the highly cytopathic effects that these factors would have on the host only when injected intracellularly by the w.t. with a functional T3SS, but not with the secretion-defective *ΔascV* mutant. In the former case, the factors synthetized by the bacteria would act on the cells host only extracellularly, possibly acting on cell receptors likely priming intracellular pathways leading to immune suppression but not necessarily to death.

Finally, our measurements were carried out within an interval of five days. It is intuitive that within such a short time span not all the adaptive immune armory would be deployed, however it is has been described that B- and T-cell activity can be detected in rainbow trout infected with w.t. *A. salmonicida* already after 12 h from infection [51] suggesting that *A. salmonicida* might concur to impair critical events affecting adaptive immune cells at the beginning of their activation following the infection. Furthermore, in consideration of the major role that innate immunity has in counteracting *A. salmonicida* resulting in a very early massive migration of myeloid cells including monocytes/macrophages in the coelomic cavity following infection [51], it is clear that downregulation of the secretion of powerful factors including interferon gamma would inevitably have a negative significant impact on the capacity of the phagocytes to clear the infection.

None of the known effector factors detected in *A. salmonicida*, including AexT, AopH, Ati2, AopP, AopO, AopN and ExsE [11] has been shown to directly or indirectly affect specific immune pathways [11]. However, our data indirectly reveal the existence of such an activity. Interestingly, this effect appears to be very broad and to occur very early upon infection, likely impacting some major cell signaling pathway. Among those partially characterized, AopP inhibiting the NF-κB pathway preventing the translocation of the p50/65 complex into the nucleus of the target cells might be a candidate agent worth to investigate further in this direction. However the broad activity required to affect all the markers investigated is suggesting that also other factors are likely to be involved [11]. The rapidity of the occurrence of this blockade would promote the rapid spread of the bacterium in the host in the absence of virtually any effective immune response already at just few hours from the infection, the most critical for the outcome of the disease [8].

Our data are supported by a qPCR protocol with sets of primers showing satisfactory efficiency (Table 2). The only exception concerns the primer set amplifying the FOXP3-2 transcripts, whose associated results might need to be interpreted more cautiously. Finally, the average RNA quality was also satisfactory. The relatively long size of the IL4/13 transcript (397 nt) might raise some concerns for samples showing moderate degradation, however, the consistency of the results across all the markers evaluated suggest the yield of reliable results also for this marker.

In conclusion, our results show that infection of rainbow trout

with a fully virulent *A. salmonicida* that harbors a functional T3SS was invariably associated with a remarkable downregulation or even complete shut off of the expression of specific immune markers affecting both the innate and the adaptive immune response and caused mortality of the infected fish. Similarly, the challenge with the non-virulent *ΔascV* mutant that has no functional T3SS but yet produces a low level of all known effector molecules, was associated with a parallel downregulation of the same immune-relevant genes but in absence of mortality. Hence, translocation, but also extracellular secretion of T3SS effectors of *A. salmonicida* translates into a concerted blockade of relevant immune players similarly to the T3SS-associated effect in *Yersinia* species [52]. Finally, a cured strain, devoid of the plasmid encoding for the T3SS and of all its associated toxins and effector molecules did not negatively affected the expression of any of the immune relevant genes examined and frequently was associated with mild to moderate upregulation of the same genes. These findings are strongly suggesting that the absence of T3SS and its associated toxins and effector molecules might allow the immune system of the trout to efficiently and rapidly clear the infection and to mount an efficient adaptive immune response protecting the trout later on during a successive infection. These findings might have profound implication in the concept and design of vaccine against *A. salmonicida* and theoretically against other pathogens characterized by similar virulence factors and pathogenesis. Furthermore, these data highlight the complex interaction of T3SS effectors that not only impair the host's cytoskeleton thus damaging cell physiology and phagocytosis, but also directly shut down the host's alarm system preventing it from recognizing the infection and inducing an immune response.

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