Developmental and Comparative Immunology 157 (2024) 105193

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Virus-specific antibody secreting cells reside in the peritoneal cavity and systemic immune sites of Atlantic salmon (*Salmo salar*) challenged intraperitoneally with salmonid alphavirus

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ARTICLE INFO

Keywords:
Salmo salar
Peritoneal cavity
Antibody secreting cells
Salmonid alphavirus
B cells
ELISpot

ABSTRACT

The development and persistence of antibody secreting cells (ASC) after antigenic challenge remain inadequately understood in teleosts. In this study, intraperitoneal (ip) injection of Atlantic salmon (*Salmo salar*) with salmonid alphavirus (WtSAV3) increased the total ASC response, peaking 3–6 weeks post injection (wpi) locally in the peritoneal cavity (PerC) and in systemic lymphoid tissues, while at 13 wpi the response was only elevated in PerC. At the same time point a specific ASC response was induced by WtSAV3 in PerC and systemic tissues, with the highest frequency in PerC, suggesting a local role. Inactivated SAV (InSAV1) induced comparatively lower ASC responses in all sites, and specific serum antibodies were only induced by WtSAV3 and not by InSAV1. An InSAV1 boost did not increase these responses. Expression of immune marker genes implies a role for PerC adipose tissue in the PerC immune response. Overall, the study suggests the Atlantic salmon PerC as a secondary immune site and an ASC survival niche.

1. Introduction

Teleosts mount antigen-specific antibody responses upon immune challenge such as infection or vaccination (Bromage et al., 2004; Kaattari et al., 2002; Ma et al., 2013; Picard-Sánchez et al., 2019; Wu et al., 2019). Unlike mammals, however, they rely exclusively on unswitched tetrameric IgM as the major systemic immunoglobulin both during primary and secondary immune responses (Kaattari et al., 1998; Scapigliati et al., 2018). B cells that have differentiated into antigen-specific antibody-secreting cells (ASC) mediate the specific antibody response. In higher vertebrates, the generation of ASC to a T-dependent antigen follows either the follicular or the extrafollicular pathway (Baumgarth, 2021; Elsner and Shlomchik, 2020). The mechanisms of how and where B cell responses are induced in teleosts are still poorly understood and an extrafollicular pathway has been assumed to dominate due to the lack of classical germinal centers (GC) and follicular structures (Abós et al., 2016). More recently data has emerged with the identification of GC-analogous lymphoid microstructures in teleosts. These structures are suggested to have important functions in induction and support of teleost B cell responses (Shibasaki et al., 2023; Waly et al., 2022). Differentiation of B cells via any of these pathways is accompanied by changes in transcription factors and surface molecules that define the resulting distinct B cell subsets. Due to lack of markers, B cell responses in teleosts have not been adequately characterized, and thus, knowledge about subpopulations and stages of B cell differentiation is limited (Peñaranda et al., 2019).

Induction of a durable protective B cell response is a hallmark of a successful humoral immune response to vaccination. Intraperitoneal (ip) injection is the most common route of vaccine administration in today's Atlantic salmon (*Salmo salar*) aquaculture, substantiating the importance of characterizing the local B cell response in the peritoneal cavity (PerC). In Atlantic salmon and other teleost species, the resident PerC leukocyte population includes IgM^+ B cells and ASC (Granja and Tafalla, 2019; Jenberie et al., 2020; Korytář et al., 2013; Shi et al., 2022; van der Wal et al., 2021). Following ip antigen stimulation of rainbow trout (*Oncorhynchus mykiss*) these PerC B cell populations expand and local PerC IgM antibody (ab) secretion is increased (Castro et al., 2017; Granja and Tafalla, 2019; Pignatelli et al., 2014; Simón et al., 2022). There is however insufficient information on how the local PerC ASC response

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https://doi.org/10.1016/j.dci.2024.105193
Received 9 February 2024; Received in revised form 30 April 2024; Accepted 7 May 2024
Available online 8 May 2024

Abbreviations

Ab (Abs)	antibody (antibodies)
ASC	Antibody/IgM-secreting cell(s)
Dpi	days post infection
HI	heat inactivation
HK	head kidney
InSAV1	Inactivated salmonid alphavirus1
IP	intraperitoneal
NC	negative control
PerC	peritoneal cavity
SAV	Salmonid alphavirus
TLRs	Toll-like receptors
Wpi	weeks post infection
WtSAV3	Wild type (infectious) salmonid alphavirus 3

correlates with measures of systemic immunity and protection after ip vaccination of fish. In mammals, antibodies produced by PerC B cells can neutralize ip vaccine antigens and render it unavailable for further optimal stimulation of the immune system (Gautam et al., 2019). A recent study in Atlantic salmon reported strong specific and non-specific IgM ASC responses in PerC after ip challenge with *Piscirickettsia salmonis* (PS), and found a positive correlation between the PS-specific ASC response in PerC and the anti-PS antibody response in serum, indicating a contribution of IgM secreting cells in PerC to the systemic antibody response (van der Wal et al., 2021). Consequently, these findings highlight the need for a comprehensive understanding of PerC immune responses during development of new vaccines.

Salmonid alphavirus (SAV) is the causative agent of pancreas disease (PD) and SAV subtype 3 (SAV3) affects Atlantic salmon in Norway (Jansen et al., 2017). We have previously reported an increased total IgM ASC response in the PerC of Atlantic salmon for up to 9 weeks following ip infection with SAV3 (Jenberie et al., 2020). As a follow up, we have here examined the local virus-specific ASC response in the PerC and systemic sites following ip injection with either infectious SAV3 (WtSAV3) or inactivated SAV (InSAV1). The SAV E2 protein, which is one of the viral capsid proteins, was utilized as an antigen to analyze specific B cell responses. Our findings showed that WtSAV3 induced a significantly higher E2-specific ASC response compared to InSAV1 in the PerC, HK, and spleen at 13 wpi, with PerC exhibiting the highest frequency, suggesting its local long-term significance. In the same period, the total ASC response declined, indicating a selective mechanism that favors the E2-specific ASC response over time. The extended presence of E2-specific ASC in the PerC supports earlier findings suggesting that fish PerC plays an active immunological role against ip antigens (Jenberie et al., 2020; Pignatelli et al., 2014; Simón et al., 2022; van der Wal et al., 2021; Veenstra et al., 2018). However, it remains unclear whether these PerC responses influence the overall systemic immune response to ip antigens or only facilitate a local clearance/neutralization of virus.

2. Materials and methods

2.1. Virus

Salmonid alphavirus subtype 3, herein designated as WtSAV3, originally isolated from heart tissue of Atlantic salmon with clinical signs of PD, was obtained from Øystein Evensen (Norwegian University of Life Sciences, Norway). The isolation and characterization of this virus has been described elsewhere (Xu et al., 2010, 2012). The virus was propagated and titrated in CHH-1 cells derived from the heart tissue of chum salmon (*Oncorhynchus keta*) (Lannan et al., 1984)(Sigma) as previously reported (Jenberie et al., 2020). In addition, formalin inactivated SAV subtype 1, isolate F93-125, herein designated InSAV1, was obtained from MSD Animal Health Innovation AS, Bergen Norway. Prior to inactivation, InSAV1 was titrated as described above, exhibiting a titer of 4.9×10^9 $TCID_{50}$/mL. Both WtSAV3 and InSAV1 were subsequently diluted in PBS to the required concentration before injection. The E2 sequence of SAV3 (Accession number: JQ799139) and InSAV1 (Accession number: CAC87722) used herein has 95.43% amino acid identity.

2.2. In vivo immunization and infection experiment

Atlantic salmon (*Salmo salar*), strain Aquagen standard, were hatched and raised at Tromsø Aquaculture Research Station (Tromsø, Norway). Pre-smolts were supplied with fresh water at 10 °C, 24 h of daylight and fed ad libitum with commercial dry feed (Skretting, Norway). After an initial sampling (n = 12), fish (average weight 57.3 g) were randomly allocated into two tanks. Fish kept in one of the tanks were injected ip with 200 μl of either PBS (control; n = 96) or InSAV1 (4.9×10^8 $TCID_{50}$ n = 96), while fish in the other tank were injected with 200 μl of WtSAV3 (10^5 $TCID_{50}$; n = 36). The InSAV1-injected fish were marked with Alcian blue tattooing for identification. At 6 weeks post-injection (wpi), 36 fish from the InSAV1 group were re-injected with a quarter of the original InSAV1 dose (boost InSAV1; 1.2×10^8 $TCID_{50}$ InSAV1) and transferred to a separate tank with the same water temperature, lighting and feeding regimen, while 36 control fish were injected with the same dose of InSAV1 and kept together with the boost group as controls (boost control). Prior to each sampling, feed was withheld for 24 h and then, fish were overdosed (0.008%) with benzocaine (ACD Pharmaceuticals, Norway). Sampling was performed at 2, 3, 6, and 13 wpi for all groups, while an additional sampling at 21 wpi was included for the InSAV1, boost InSAV1 and boost control groups (n = 6 to 10). The experiment setup and sampling plan are presented in Fig. 1. Blood, PerC wash, head kidney (HK), spleen, posterior kidney, heart, pancreas and PerC adipose tissue were collected for various analyses as described below. The experiment was approved by the Norwegian Food Safety Authority in accordance with the European Union directive 2010/63/EU for animal experiments (ID: 16409).

2.3. Cell isolation

Fish were bled from the caudal vein before sampling. PerC cells were collected by a procedure described previously (Jenberie et al., 2020). Additionally, HK, spleen, and posterior kidney leukocytes were isolated from the same fish as described elsewhere (Jørgensen et al., 2001). Briefly, PerC washes and tissue homogenates were layered on 25/54% discontinuous Percoll gradients (GE Healthcare) followed by centrifugation at 400×*g* for 40 min at 4 °C. Leukocytes were collected from the gradient interface, washed twice in L-15 medium (400×*g* for 10 min at 4 °C), counted on Countess II (Invitrogen) and kept on ice until seeded for ELISpot assay.

2.4. Enumeration of total IgM ASC using ELISpot

A recently developed ELISpot assay (Jenberie et al., 2020) with a modification on the number of cells seeded per well was used to enumerate the total *ex vivo* ASC from PerC, HK, spleen, and posterior kidney. ELISpot plate wells (MSIPS4510, Merck Millipore) were briefly activated with 35% ethanol, washed four times with PBS and coated overnight at 4 ^{O}C with an anti-trout IgM mAb (15 μg/mL, IgF1-18 (6-1-18) (Hedfors et al., 2012);). Plates were washed with PBS and blocked with L-15 with 2% bovine serum albumin (BSA) for 90 min at room temperature (RT). After four washes, 12500 leukocytes in 100 μl L-15 with 10 U/mL penicillin, 10 μg/mL streptomycin and 5% FBS were seeded in duplicate wells. Following 48 h of incubation at 14 ^{O}C, leukocytes were removed by 5x washing with PBS/0.1% Tween 20. A detection antibody (biotinylated anti-trout IgM mAb; 1.5 μg/mL) was added and incubated for 60 min at RT. This was followed by sequential

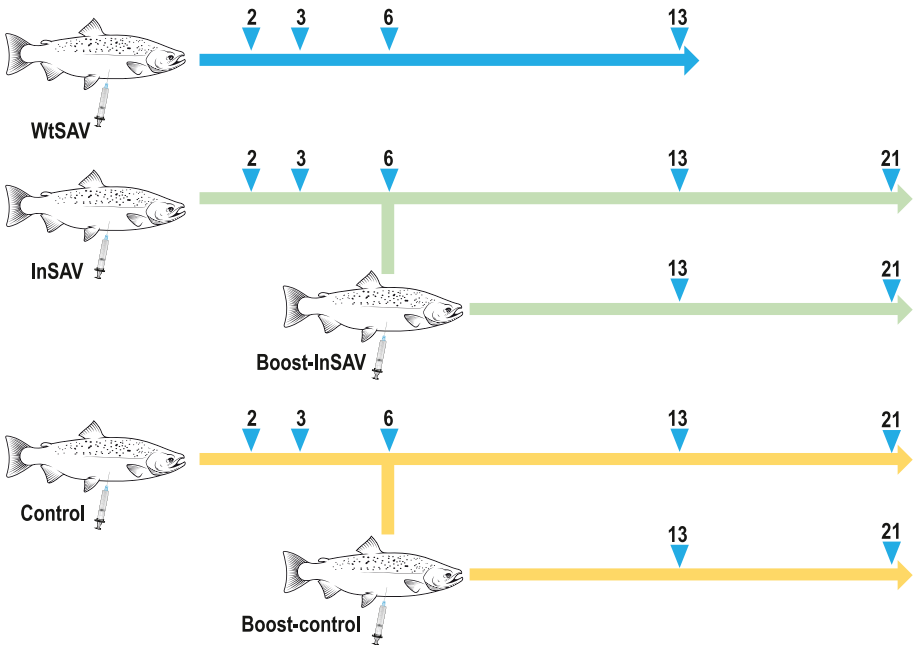

Fig. 1. Schematic illustration of experiment design and sampling plan. Atlantic salmon in freshwater at 10°were injected intraperitoneally (ip.) with either PBS (Control, n = 96), InSAV1 (n = 96), or WtSAV3 (n = 36). At 6 wpi, 36 fish that had been injected with InSAV1 were given a booster injection ip. with InSAV1 (Boost-InSAV1), while 36 control fish were injected ip. with the same dose of InSAV1 as the Boost-InSAV1 group (Boost-control). Sampling (indicated by blue arrows) was performed at 2, 3, 6 and 13 wpi for the control, InSAV1 and WtSAV3 groups, while the boost groups were sampled at 13 wpi (or 7 weeks after boost) and 21 wpi (or 15 weeks after boost) (n = 6 to 10). The control and InSAV1 groups were also included in the last sampling at 21 wpi (n = 6 to 10). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

incubations with streptavidin-HRP (1:500) and TMB substrate solution (Mabtech). Spots were developed for 10 min in the dark before the reaction was stopped by excessive washing with tap water. After overnight air-drying, plates were scanned digitally, and spots were counted automatically using the smart count wizard of an ImmunoSpot version 7 software (CTL ImmunoSpot S6 Ultra-V analyzer). IgM ASC frequency was defined as number of spots per 12500 cells. Controls were included on all plates, including wells with all reagents without cells (NCC-no cell control) or without the biotinylated detection antibody (DAC-detection antibody control).

2.5. Enumeration of SAV E2-specific ASC using ELISpot

E2-specific ASC were enumerated using a modified version of the ELISpot assay for total IgM ASC (Jenberie et al., 2020). The SAV3 E2 protein used for coating the plates was custom made by MRC PPU Reagents and Services (University of Dundee, Scotland) as described in supplementary figure 1. Following activation with 35% ethanol, ELISpot plate wells (MSIPS4510) were coated with 3 μg/well E2 protein overnight at 4 °C. After 4x washes and blocking with L-15 with 2% BSA for 90 min at RT, 250000 leukocytes/well were seeded in duplicate. Plates were incubated and further processed and analyzed as described for the total IgM ASC ELISpot. Gates for spot counting were set based on background signal from the control fish. The background balance and sensitivity parameters available with the basic count wizard were fine-tuned based on control wells to minimize noise, but without affecting signal from other wells. Residual background spots from the control fish were subtracted from InSAV1, WtSAV3, boost control and boost InSAV1 groups before E2-specific ASC were presented as frequency per 250000 leukocytes. All plates included wells with NCC and DAC as controls.

2.6. Detection of anti-E2 antibody response in serum using ELISA

The ELISA assay was optimized prior to use by testing of 2-fold serum dilutions ranging from 1:5 to 1:400. Briefly, ELISA wells (Maxisorp plates; Thermo Scientific) were coated (200 ng/well) overnight at 4 °C with the same recombinant E2 protein as used in the ELISpot assay. Wells were washed (PBS), blocked with protein free blocking buffer (Thermo Scientific), and incubated overnight at 4 °C with sera samples diluted 1:160. After washing (PBS/0.05% Tween-20), anti-trout IgM mAb (IgF1-18 (6-1-18), 1:500) and HRP conjugated goat anti-mouse (Bio-Rad, 1:1500) were sequentially added and incubated at RT for 1 h each. Plates were developed with TMB substrate (Invitrogen) for 20 min in the dark. Optical density (OD) was immediately measured at 450 nm on a VersaMax microplate reader (Molecular devices).

2.7. Measuring SAV neutralizing activity in serum

An immunoperoxidase-based virus neutralization assay (Graham et al., 2003) was used to measure virus neutralizing activity in serum. The assay was performed by Agri-Food and Biosciences Institute, Belfast, Ireland. Briefly, sera samples collected at 0, 3, 6, 13 and 21 wpi were diluted 1/10 and 1/20, and preincubated with equal volume of virus for 2 h at RT (15 μl, 100 $TCID_{50}$ salmon pancreas disease virus strain F93-125). Then, CHSE-214 cells were added to a final volume of 150 μl. After three days of incubation, cells were fixed and stained with mAb 2D9 (1:8000), raised against the SPDV strain F93-125 (Todd et al., 2001; Welsh et al., 2000). Plates were examined microscopically, and wells with the absence of viral staining were scored as positive for virus neutralizing activity. Sera samples that tested positive at 1:20 dilution were re-tested at further dilutions to determine the endpoint titer. Assays were considered valid when a back titration of input virus was within defined limits (30–300 $TCID_{50}$/15 μl), and positive and negative control sera results were correct. To test complement-mediated virus neutralization activity, virus neutralizing positive sera were retested after heat inactivation at 56 ^{O}C for 30 min.

2.8. RT-qPCR analysis

SAV3 nsP1 and immune related genes were analyzed in PerC adipose tissue, heart and pancreas using RT-qPCR. Tissues were homogenized in RLT buffer using TissueLyser II (Qiagen) and hearts were further treated with proteinase K (Applied Biosystems) as previously described (Jenberie et al., 2020). Total RNA was isolated from heart and pancreas using RNeasy Min kit, and from PerC adipose tissue using RNeasy Lipid Tissue Mini Kit, according to the manufacturer's protocol (Qiagen). cDNA synthesis was performed using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacture's protocol with a 600 ng total RNA input. PCR was run on a 7500 Fast Real-Time PCR system using Fast SYBR green master mix in 15 μl reactions with 5 μl of 1:10 diluted cDNA with an initial denaturation of 20 s at 95 °C and 40 cycles of 3 s at 60 °C (all from Applied Biosystems). The EF1aB gene has a stable expression across tissues and time points and was used as an endogenous control. Relative gene expression was calculated using the $2^{-\Delta Ct}$ method (Schmittgen and Livak, 2008). Primers used in this study are listed in Supplementary Table 1.

2.9. Statistical analysis

Data analyses and graphical presentations were done on GraphPad Prism version 9. A two-tailed one-way ANOVA followed by Bonferroni multiple comparison post hoc test was used to assess the presence of statistical significance in ASC frequencies or relative gene expression in adipose tissue. However, Kruskal-Wallis test was used when the Brown-Forsythe test indicated a significant difference in standard deviations ($p < 0.05$). Mann-Whitney *U* test was used to analyze differences in relative gene expression from heart and pancreas. Differences were statistically significant at $p < 0.05$ and strength of significance is indicated by number of asterisks (*); where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Results

3.1. Total IgM-secreting cells increased in the PerC and systemic immune sites upon intraperitoneal injection of WtSAV3, while the response to InSAV1 was modest

Using ELISpot assay, the total IgM ASC response was analyzed in the WtSAV3 and InSAV1 injected fish and compared with the control fish. WtSAV3 infection increased the frequency of total IgM ASC at 3 and 6 wpi in PerC, HK and spleen; at 6 wpi a 2.9-, 1.8- and 2.6-fold increase was found in the above tissues, respectively, compared to the control group (Fig. 2a, b, c), largely consistent with our previous findings (Jenberie et al., 2020). In posterior kidney, a less studied tissue of Atlantic salmon, WtSAV3 infection induced a 3.5-fold higher frequency of total IgM ASC already at 2 wpi, which remained elevated (2.4-fold) until 6 wpi, compared with the control group (Fig. 2d). At 13 wpi, the final sampling for the WtSAV3 group, only PerC showed a significantly higher (1.8-fold) total ASC frequency, while all other tissues were similar to the control group. This result emphasizes the role of the local PerC environment in sustaining the long-term total ASC response in Atlantic salmon and are in line with earlier reports from our group (Jenberie et al., 2020; van der Wal et al., 2021).

In general, fish in the InSAV1 group showed a lower total ASC response than fish infected with the WtSAV3. Although the ASC response to WtSAV3 reached its highest point at 3 to 6 wpi, the most prominent ASC response in the InSAV1 group was observed at 2 wpi (Fig. 2). This early peak at 2 wpi could be attributed to the higher dosage of InSAV1 particles injected, which was approximately 300 times greater than that of WtSAV3, possibly leading to an early polyclonal stimulation of B cells. However, in the spleen, the frequency of total ASC in the InSAV1 group was significantly increased at 6 wpi, and then declined. At 13 and 21 wpi, the total ASC response in the InSAV1 group

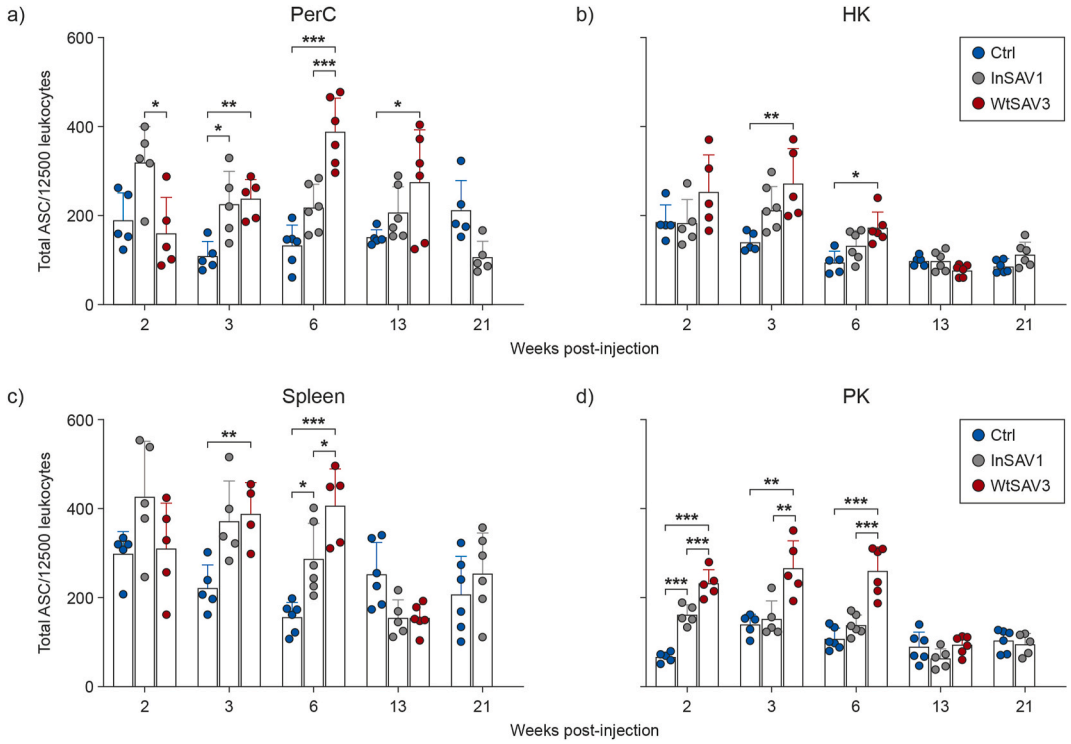

Fig. 2. WtSAV3 induces stronger total antibody secreting cell (ASC) responses than inactivated SAV (InSAV1). Total IgM ASC frequencies in the PerC (a), HK (b), spleen (c) and posterior kidney (PK) (d) of control, InSAV1 and WtSAV3 groups at 2, 3, 6, and 13wpi were determined by the IgM ELISpot assay. At 21 wpi, only control and InSAV1 groups were analyzed. Data present total IgM ASC per 12500 leukocytes shown as scatter plots of individual values with SD (n = 5 or 6). Statistically significant comparisons between control, InSAV1 and WtSAV3 groups within each time point and site are indicated by asterisk '*' and number of asterisks indicates strength of significance.

was comparable to that of the control group across the sites (Fig. 2).

3.2. E2-specific ASC reside in the PerC and systemic immune sites after ip. injection with WtSAV3 or InSAV1

In a previous study (Jenberie et al., 2020), we established a positive correlation between the SAV3-induced total IgM ASC response in PerC and the anti-E2 antibody response in serum. To further investigate this, we established a SAV3-specific ELISpot assay to determine the frequency of SAV E2-specific ASC in the PerC, HK, and spleen. E2-specific ASC were present in the PerC and HK at 6 and 13 wpi in WtSAV3 group (Fig. 3a). The spleen was not analyzed at 6 wpi, but at 13 wpi, E2-specific ASC were present in spleen of the WtSAV3 group (Fig. 3a). The frequency of E2-specific ASC was significantly higher in all three sites in the WtSAV3 group compared to the InSAV1 group at 13 wpi, accounting for 0.55%, 0.40%, and 0.25% of the total IgM ASC

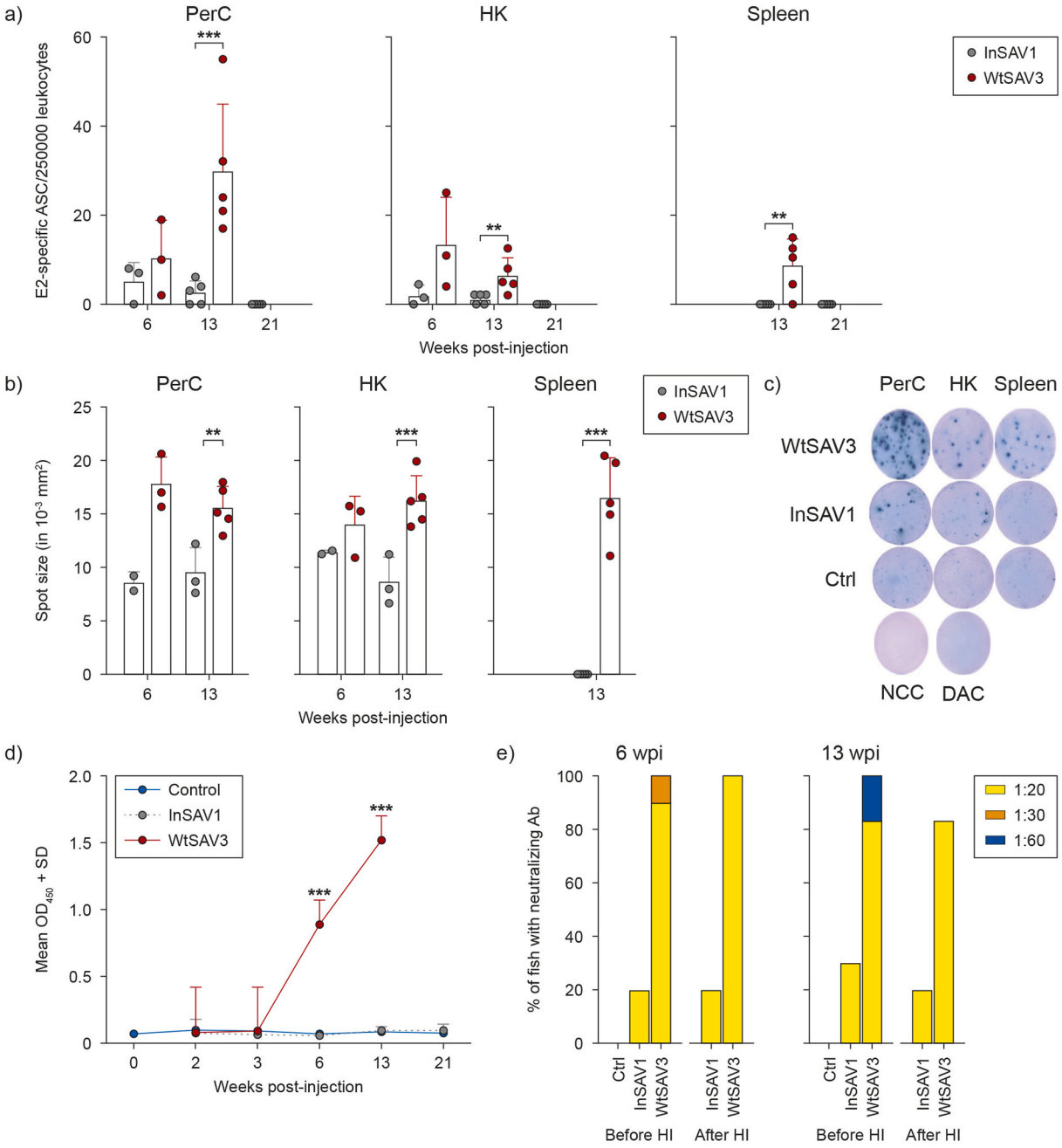

Fig. 3. WtSAV3 induces stronger E2-specific ASC responses than InSAV1 in the PerC and systemic sites. a) E2-specific ASC frequencies in the PerC, HK, and spleen were determined by the SAV E2-specific ELISpot assay. Data is presented as E2-specific ASC per 250000 leukocytes after subtraction of residual spots in the control group (spleen was not analyzed at 6 wpi). Data present scatter plots of individual values with SD (n = 3 at 6 wpi and 5 at 13 wpi). **b)** Average spot size of E2-specific ASC as an estimate of IgM secretion rate presented as scatter plot of individual values with SD (n = 3 at 6 wpi or 5 at 13 wpi). **c)** Representative scanned ELISpot wells showing spots generated by E2-specific ASC from the WtSAV3, InSAV1 and control group at 13 wpi or assay negative control wells (NCC- no cell control; DAC- no detection antibody control). **d)** Anti-SAV3 E2 antibody response in serum was determined by ELISA and is presented as mean OD_{450} with SD (n = 5 for control, 10 for InSAV1 and 8 for WtSAV3). e) Percent of fish with virus neutralizing (VN) antibody before and after heat inactivation (HI) (n = 5 for controls at all sampling points; 10 at 6 wpi (both WtSAV3 and InSAV1); 10 (InSAV1) or 6 (WtSAV3) at 13 wpi). Colors show at which serum dilution the sample was positive as indicated in the legend. Statistically significant comparisons between control, InSAV1 and WtSAV3 groups are indicated by asterisk '*' and number of asterisks indicates strength of significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

population in the PerC, HK, and spleen, respectively (Fig. 3a). Notably, the total cell count in HK and spleen was higher than in PerC (supplementary figure 2), resulting in a 23- and 10-fold higher number of E2-specific ASC per whole tissue in HK and spleen at 13 wpi, respectively, compared to the PerC. The E2-specific ASC were also present in the InSAV1 group at 13 wpi, but at lower frequencies than in WtSAV3 infected fish, accounting for only 0.05% and 0.06% of the total IgM ASC in the PerC and HK, respectively. At 21 wpi, E2-specific ASC were undetectable at the three sites of InSAV1-injected fish (Fig. 3a). Only WtSAV3-infected fish showed a detectable anti-E2 Ab response in serum as measured by ELISA (Fig. 3d). This may be attributed to a higher antibody secretion rate per cell in the WtSAV3 group than in the InSAV1 group, as demonstrated by spot sizes in the ELISpot assay (Fig. 3b and c). In the WtSAV3 group, we found a positive correlation between the frequency of E2-specific ASC in the PerC and the serum anti-E2 antibody response as measured by ELISA ($r = 0.74$, $p < 0.05$). Virus neutralization titers of 1:20 or higher were detected in all sera from WtSAV3-infected fish at both 6 ($n = 10$) and 13 ($n = 6$) wpi, while only 2/10 and 3/10 individuals in the InSAV1 group showed virus neutralization titers of 1:20 at 6 and 13 wpi, respectively (Fig. 3e). Heat inactivation at 56 ^{O}C for 30 min eliminated the virus neutralizing activity of 11 of the total 53 positive sera. In addition, heat inactivation reduced the titers of 2 sera with a titer of 1:30 and 1:60 to 1:20. At 21 wpi, all sera from the InSAV1 group were negative for virus neutralizing activity ($n = 10$).

3.3. Booster dose of InSAV1 is not accompanied by recall ASC or virus specific ab responses

The administration of a booster injection of InSAV1 did not result in an increase in total ASC (Fig. 4a) or E2-specific ASC frequency (supplementary figure 3) compared to the control or the boost control groups. This suggests that there was no recall or secondary immune response against InSAV1. When analysing sera using ELISA, no anti-E2 antibody response was detected from fish which received the booster injection (supplementary figure 3). At 13 wpi (or 7 weeks after boost), virus neutralizing activity was detected in 8 out of 10 sera tested in both the boost control and boost InSAV1 groups, with titers of 1:15 or 1:20 (Fig. 4b). However, at 21 wpi (or 15 weeks after boost), the number of sera with VN activity decreased to 1 out of 10 in the boost control group with a titer of 1:10 and 4 out of 10 in the boost InSAV1 group with titers of 1:15 or 1:20 (Fig. 4b). Notably, all sera with a titer of 1:10 or 1:15, and some with a titer of 1:20, lost virus neutralizing activity after HI.

3.4. Ip. SAV3 infection induces peak transcription of sIgM at 6 wpi in target tissues

The pancreas and heart are the primary target tissues for SAV3 replication in Atlantic salmon (McLoughlin and Graham, 2007), and by analysing the presence of SAV *nsP1* transcripts in these organs, we investigated virus dynamics during the 13-week ip. infection experiment. SAV *nsP1* transcripts were present in the heart of the WtSAV3 group from 2 to 13 wpi., peaking at 2 to 3 wpi, followed by a marked decline thereafter (Fig. 5a). In contrast, a peak *nsP1* level was found at 2 wpi in the pancreas, where the level was 7.23 times lower than that of the heart, and it continued to decrease until 3 wpi, becoming undetectable in 5 out of 6 fish at both 6 and 13 wpi (Fig. 5b). In both tissues, the expression of *mIgM* steadily increased in the WtSAV3 group from 2 wpi and peaked at 3 to 6 wpi. Additionally, *sIgM* expression was upregulated by about 10-fold in both tissues of infected fish at 6 wpi and remained elevated at 13 wpi (Fig. 5a and b). Notably, the peak of *sIgM* levels coincided with the decrease or undetectable levels of SAV *nsP1*, indicating that the IgM response likely plays a role in the host's defense against SAV infection.

3.5. Immunological role of PerC adipose tissue as indicated by upregulation of immune genes

The persistent local presence of both total IgM and E2-specifc ASC in PerC of the WtSAV3-group raised the question of how these responses are initiated and maintained. As previous studies suggest the involvement of PerC adipose tissue in local B cell responses we analyzed the levels of SAV *nsp1* and immune gene transcripts in PerC adipose tissue at 6 and 13 wpi using RT-qPCR. The gene expression profile of the control group indicated that steady state PerC adipose tissue harboured a mixture of immune cells, including myeloid cells, B cells, IgM secreting cells, CD4 and CD8 T cells, and antigen presenting cells (Fig. 6). The highest basal transcript levels were found for *sIgM, MHCI*, and *MHCII* genes (Fig. 6c and d). In the WtSAV3 group, all analyzed genes exhibited significantly higher expression compared to the control group at 6 wpi, suggesting an ongoing multifaceted immune response in the PerC adipose tissue of the infected fish (Fig. 6b, c, d). The genes were upregulated by 2 to 4-fold compared to the control group, except for *CD8* and *sIgM*, which showed a 17.8 and 11.6-fold increase, respectively. This indicates the importance of both humoral and cellular immunity in the PerC adipose tissue response to SAV3. The simultaneous upregulation of *mIgM* and *sIgM* transcripts in the WtSAV3 group at 6 wpi suggests an increased generation and/or trafficking of IgM^{+} B cells in the PerC adipose tissue.

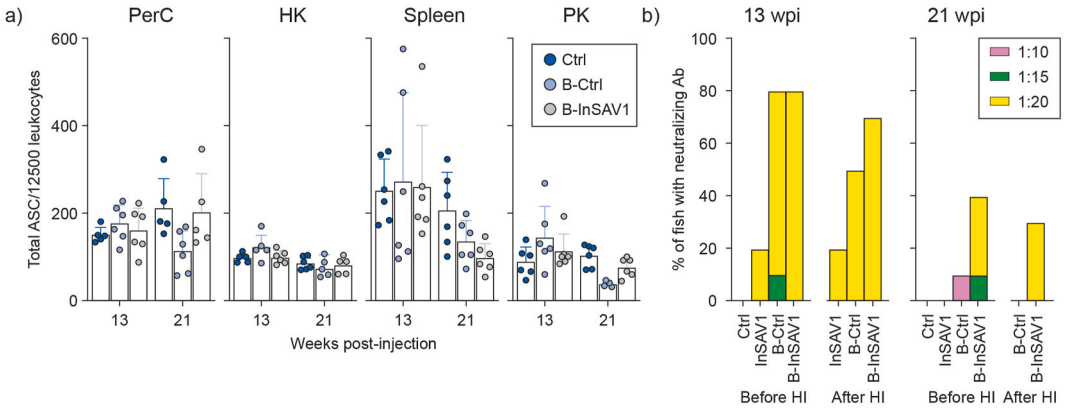

Fig. 4. Booster injection of InSAV1 does not induce recall ASC or virus neutralizing antibody responses. Six weeks after the initial injection an ip. booster of InSAV1 was administered (B-InSAV1). At the same time one group of fish received a primary ip. injection with the same dose of InSAV1 to serve as a control for the boost group (B-Ctrl). **a**) Frequency of total IgM ASC was determined by IgM ELISpot and is presented per 12500 leukocytes in the PerC, HK, spleen and posterior kidney (PK) of boost control and boost InSAV1 groups at 13 and 21 wpi. Data present scatter plots of individual values with SD ($n = 5$ or 6). **b**) Percent of fish with VN antibody before and after heat inactivation (HI) ($n = 5$ for controls or 10 for boost control and boost InSAV1 groups at both sampling points). Ctrl: Control, B-Ctrl: Boost control, B-InSAV1: Boost InSAV1.

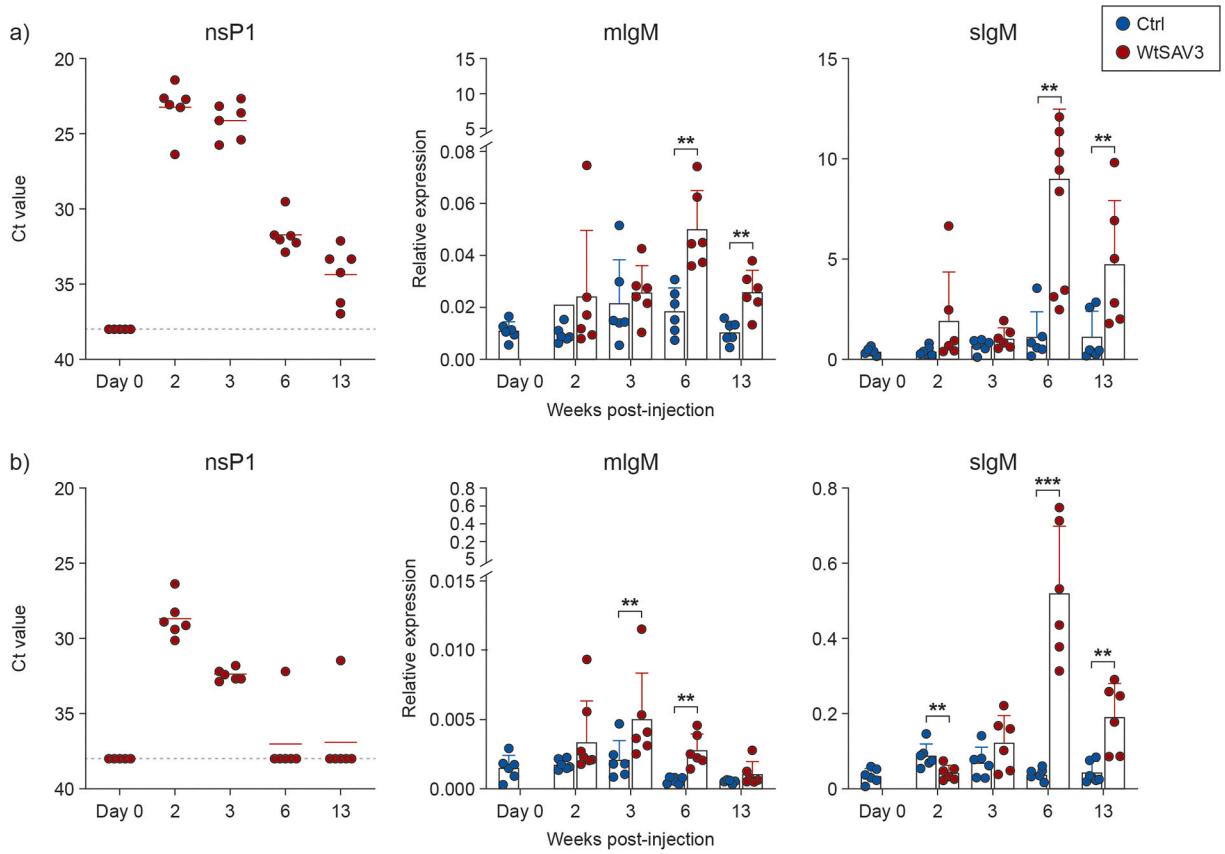

Fig. 5. The B cell response to ip WtSAV3 infection peaks at six wpi in heart and pancreas. *SAV3 nsP1*, *sIgM* and *mIgM* transcript levels were measured by RT-qPCR in the heart (**a**) and pancreas (**b**). Data present scatter plots of individual Ct (*nsP1*) and relative gene expression values (*sIgM* and *mIgM*) with SD (n = 6). *EF-1aB* gene was used as an endogenous control and relative gene expression was calculated using the $2^{-\Delta Ct}$ method (Schmittgen and Livak, 2008). The Ct cut-off value for the *nsP1* gene qPCR assay was 38 (shown with a broken line).

The upregulation of *sIgM* coincided with the peak total ASC response at 6 wpi, as measured by ELISpot assay (Fig. 2). However, the E2-specific ASC response peaked later at 13 wpi (Fig. 3a), indicating a local selection mechanism in the PerC that favors E2-specific ASC over time. *IgD* and *IgT* transcript levels were also upregulated in the infected fish at 6 wpi (Fig. 5c) suggesting that different B cell subsets are taking part in the PerC adipose tissue immune response. At 13 wpi, the PerC adipose tissue response to SAV3 had decreased, as most transcripts had returned to control levels. SAV detection followed a similar pattern as the expression of immune genes, as traces of viral *nsP1* transcripts were detected in the PerC adipose tissue of all six individuals in the WtSAV3 group at 6 wpi (mean Ct = 35.9), while the *nsP1* transcript was undetectable in five of the six individuals at 13 wpi (Fig. 6a). In the InSAV1 group no significant changes in gene expression were found at either 6 or 13 wpi for any of the genes analyzed (Fig. 6), consistent with the low or absent responses found in this group in other analyses (Figs. 2 and 3).

4. Discussion

The formation, dynamics and tissue trafficking of ASC after infection or vaccination are inadequately understood in teleosts in general, and also in Atlantic salmon. In the current study, we analyzed the presence and persistence of total IgM and SAV3 E2 specific ASC in Atlantic salmon after ip injection of WtSAV3 or InSAV1 and after InSAV1 boost. Ip delivery was chosen since most vaccines are administered to Atlantic salmon by this route, making it relevant to study how ASC responses are initiated and maintained in PerC compared to systemic lymphoid tissues. Although recent findings suggest that lymphoid microstructures have a GC-like role in induction of teleost B cell responses (Shibasaki et al., 2023; Waly et al., 2022), the anatomy of the teleost immune system is different from that in mammals with the absence of lymph nodes and classical GCs. This points to whether teleosts employ peripheral sites, such as the PerC, to a larger extent in the formation of ASC responses. The present study demonstrated increased numbers of SAV3 E2-specific ASC in the PerC and upregulation of IgM transcripts in the PerC adipose tissue and SAV target tissues, heart and pancreas, of the virus infected fish, implying their local role. Along the same line, several other studies in fish have shown differentiation and localization of B cells or ASC at sites of infection or immunization (Bakke et al., 2020; Ballesteros et al., 2013; Castro et al., 2017; Erkinharju et al., 2019; Jenberie et al., 2020; Sobhkhez et al., 2018; van der Wal et al., 2021). A recent study in Atlantic salmon using Ig-seq and analysis of co-occurrence of clonotypes indicated trafficking of B cells from spleen to heart after a SAV3 challenge and proposed that ASC at the site of infection may increase the efficacy of the humoral response (Bakke et al., 2020). As has been reported in higher vertebrates, local antibodies secreted by PerC ASC may play a vital role in virus neutralization and maintaining homeostasis, as circulatory IgM does not guarantee efficacy at the peripheral sites of infection due to the large molecular weight of IgM (Jackson-Jones and Bénézech, 2018).

In the current study, the finding of a higher frequency of E2-specific ASC in PerC of infected fish at 13 wpi (Fig. 3a) compared to the systemic lymphoid tissues, HK and spleen, suggests on mechanisms that sequester or support their prolonged presence in PerC. This increased E2-specific ASC frequency was found simultaneously as total ASC frequency decreased (Figs. 2 and 3a) pointing at an independent regulation of the E2-specific and total IgM ASC populations. This could be due to antigen (Ag)-driven selection of ASC, where the diminishing concentration of Ag

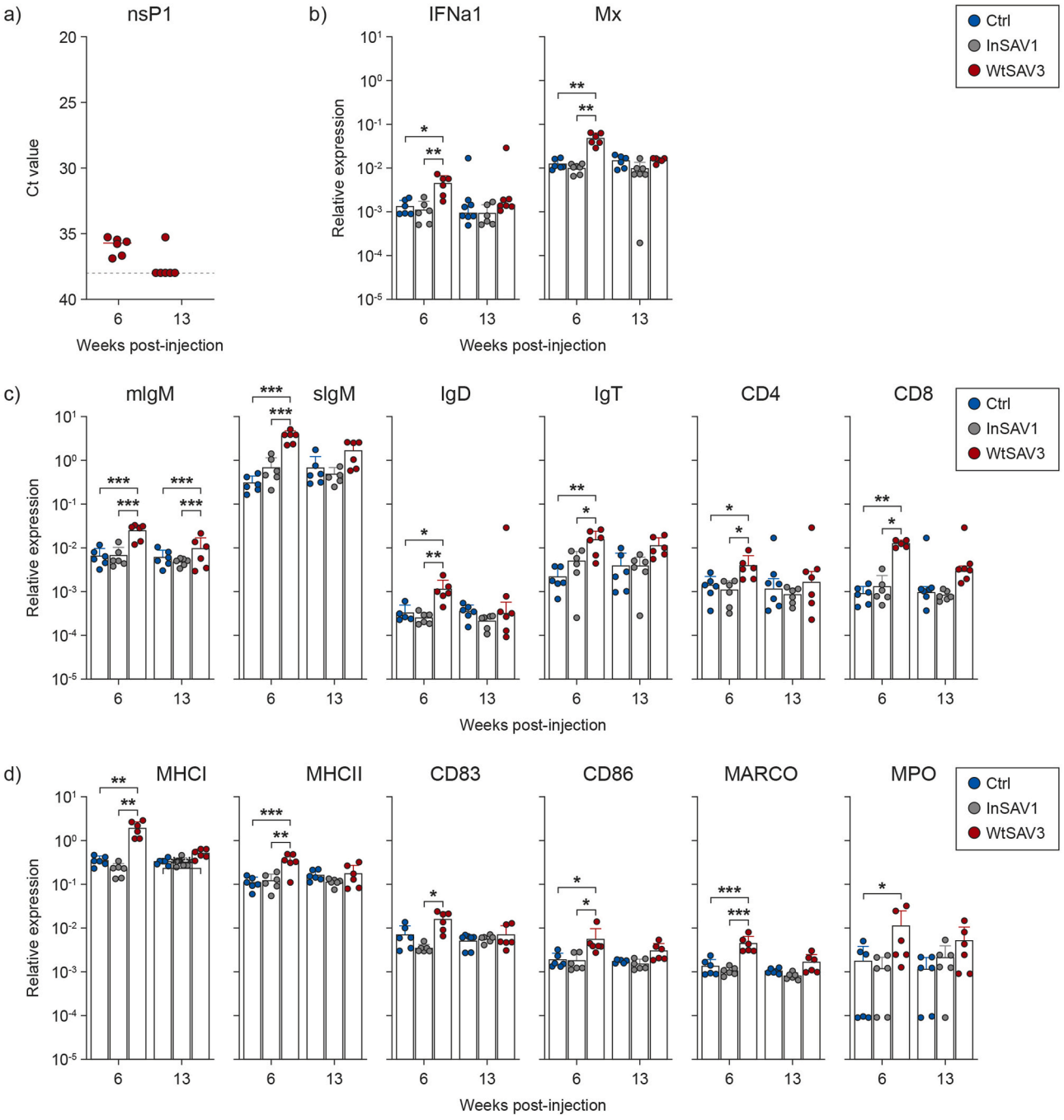

Fig. 6. Transcript profile of immune genes in PerC adipose tissue. Expression of SAV3 *nsP1* (**a**), antiviral (**b**), B cell and T cell (**c**), and antigen presentation and myeloid cell (**d**) marker genes in PerC AT of control, WtSAV3 and InSAV1 injected fish at 6 and 13 wpi. Data present scatter plots of individual Ct values (*nsP1*) or relative expression values (immune genes) with SD (n = 6). *EF-1aB* gene was used as an endogenous control and relative gene expression was calculated using the $2^{-\Delta Ct}$ method (Schmittgen and Livak, 2008). The Ct cut-off value for the *nsP1* gene qPCR assay was 38 (shown with a broken line in panel 'a').

late in the immune response favors the emergence of high affinity long-lived ASC as suggested previously by others (Kaattari et al., 2002; Wu et al., 2019). To the best of our knowledge, antigen-specific ASC responses lasting for such a long duration has not been previously reported in a peripheral site such as PerC of a teleost species. It remains unclear whether PerC ASC produce IgM that remains local or contributes to the E2-specific antibody response in serum that was detected from 6 wpi (Fig. 3d). However, there was a positive correlation between PerC E2-specific ASC frequency and anti-E2 antibody response in serum at 13 wpi. A previous study in rainbow trout immunized ip found antigen-specific ASC in blood, spleen, and HK, and from 10 weeks post immunization and onwards, the specific ASC response was localized only to the HK (Bromage et al., 2004). Similarly, we found E2-specific ASC in HK and spleen, and although the frequency of E2-specific ASC was higher in PerC, total numbers were much higher in the systemic sites. Whether PerC provides a niche for long-term survival of salmonid antigen-specific ASC in parallel to HK warrants further study.

The current study showed that the total ASC response in the SAV-infected fish was most pronounced in posterior kidney during the early time points of infection (2, 3, 6 wpi). Notably, only posterior kidney exhibited an elevated total ASC response at 2 wpi (Fig. 2). This observation is consistent with a previous study that reported the

presence of IgM ASC in posterior kidney and suggested this tissue as a site for B cell activation in rainbow trout (Zwollo et al., 2005). Additionally, posterior kidney cells were shown to possess the highest capacity to produce plasmablasts or plasma cells after *in vitro* stimulation (Zwollo et al., 2005). Despite these initial findings, posterior kidney remains a less explored lymphoid tissue compared to HK and spleen, and hence, how B cell differentiation and/or trafficking occurs in Atlantic salmon posterior kidney needs further investigation.

In general, the B cell response to the inactivated virus (InSAV1) was comparatively weaker than the response to the infection with the live virus. In accordance with our previous study (Jenberie et al., 2020), a SAV E2 antibody response was clearly present in serum of the WtSAV3 group from 6 wpi and increased onwards (Fig. 3d), while no E2-specific antibody response was detected in the InSAV1 group. However, a few E2-specific ASC were detected by ELISpot in PerC and HK in the InSAV1 group, but these cells generated smaller spots compared to the ASC in the WtSAV3 group (Fig. 3b and c), indicating a lower IgM secretion rate. This discrepancy between the two assays could be explained by the higher sensitivity of the ELISpot assay, which detects ASC at a single cell level, compared to ELISA (Tanguay and Killion, 1994). A certain threshold number of Ag-specific ASC and a certain antibody secretion rate is probably needed to detect a significant level of serum antibody by ELISA, and in the InSAV1 group, these parameters appear to be below the threshold for detection.

SAV is grouped into six genotypes and the InSAV1 used for injection of the fish in the present study belongs to SAV1, while the E2 protein used as coating antigen for ELISA and ELISpot is based on the SAV3 genotype. Given that all the six SAV genotypes cross-neutralize (Graham et al., 2014) and that the SAV E2 represents the protein with most of the neutralizing epitopes (McLoughlin and Graham, 2007), it is unlikely that the use of different variants of SAV has affected the ELISA result of the InSAV1 group. Consistent with the present finding, two other studies using commercial oil-adjuvanted inactivated SAV vaccines did not induce a significant increase in serum antibody levels compared to unvaccinated controls (Chang et al., 2017; Veenstra et al., 2020). In contrast, a DNA vaccine encoding the SAV3 polyprotein (Chang et al., 2017) or SAV3 infection (Veenstra et al., 2020) induced a significant increase in serum antibodies. These findings support our results and suggests that the nature of the SAV antigens and how they are expressed influences the strength and duration of the antibody response.

In addition to the serum antibody response measured by ELISA, the current study also found SAV neutralizing activity in serum of all individuals in the WtSAV3 group. However, in the InSAV1 group, although the ELISA results were negative, two and three fish out of 10 still showed positive neutralizing activity at 6 and 13 wpi, respectively (Fig. 3e). In contrast to the ELISA and ELISpot assays where anti-E2 antibodies were measured, this assay displayed neutralizing activity against the whole intact SAV particle which may explain the discrepancy in results between the assays. Collectively, the results from the E2-specific ELISpot and the neutralization assay implies that fish from the InSAV1 group raised ASC that secreted E2-specific antibodies, but their secretion was below the detection limit by the ELISA. In contrast to an inactivated virus, infection with live SAV is likely to induce production of type I interferons, proinflammatory cytokines together with cell mediated immune responses (Dahle and Jørgensen, 2019). This may trigger a broad presentation of viral antigens to the immune system possibly resulting in increased antibody levels, consistent with the data presented here.

How and where immunological memory in teleost is initiated and maintained is still elusive (Yamaguchi et al., 2019). To further highlight this topic, we included an experimental group of fish which was boosted ip with InSAV1 six weeks after the primary injection with the same antigen. As discussed above, only minor changes in B cell responses were induced by the primary InSAV1 injection, and the booster injection did not result in an increase in the total ASC, E2-specific ASC, or E2-specific serum antibodies 7 or 15 weeks after boost (supplementary figure 3). Only a slight increase in the number and titer of sera positive for virus neutralizing activity at 15 weeks after boost (21 weeks after the primary injection) was found (Fig. 4b). Overall, the primary B cell response to InSAV1 was very weak and it did not catch up with the response induced by WtSAV3 even after the booster injection, suggesting no secondary humoral response. In a recent study in rainbow trout using attenuated viral hemorrhagic septicemia virus (VHSV) as the antigen, similar findings were reported. No increase in neutralizing or serum antibody titres were found after boost, but rather a decrease (Castro et al., 2022).

To understand immune responses to ip injected antigens in fish, the visceral adipose tissue has come into focus. It is known that after ip injection of oil-adjuvanted vaccines in Atlantic salmon, oil droplets with vaccine antigens accumulate in visceral adipose tissue that leads to an inflammatory response characterized by the formation of granulomas (Koppang et al., 2005; Midtlyng, 1997). A recent study in rainbow trout has shown the presence of different B cell subsets in the PerC adipose tissue in steady state (Simón et al., 2022) and others have shown differentiation of B cells in the PerC adipose tissue after antigenic stimulations (Castro et al., 2017; Pignatelli et al., 2014; Veenstra et al., 2018). In the current study, gene expression data of PerC adipose tissue in control fish showed expression of marker genes for B cells, T cells, APCs and costimulation (Fig. 6) suggesting the presence of a cellular niche equipped for antigen presentation and further activation of the immune response. Furthermore, the ip infection with WtSAV3 caused upregulation of a wide range of these genes at 6 wpi (Fig. 6), suggesting active involvement of the adipose tissue in the regulation of PerC immune responses. PerC adipose tissue immune responses of such long duration as 6 wpi have only recently been reported in Atlantic salmon infected ip with *Piscirickettsia salmonis* (van der Wal et al., 2021). Given the presence of the SAV *nsP1* transcripts for at least 6 wpi, upregulation of *IFNa1* and *Mx* genes shows an ongoing antiviral response in the adipose tissue. In fish as well as in higher vertebrates, beyond their innate antiviral effects, type I IFNs play a critical immunoregulatory function by linking the innate and adaptive immune responses (Chang et al., 2015; Lee and Ashkar, 2018), which might promote the long-term PerC and adipose tissue B cell responses. The concurrent upregulation of *MHCI* and *CD8* transcripts at 6 wpi indicates also a canonical antiviral immune response against virus-infected cells in the PerC adipose tissue. Of all genes examined, *CD8* and *sIgM* showed the highest fold induction at 6 wpi, 17.8 and 11.6, respectively, suggesting that the PerC adipose tissue mediates both cell mediated and humoral responses. Consistent with this, an increase in $CD8^+$ cells and IgM^+ cells with a plasmablast phenotype were also observed in rainbow trout adipose tissue after ip virus infection (Pignatelli et al., 2014).

In summary, ip delivery has been the aquaculture industry's standard of vaccination, while indicators of vaccine efficacy have primarily relied on findings in the systemic compartment. This study demonstrates that ip injection with WtSAV3 induces a long-lasting E2-specific ASC response skewing to PerC. The uneven distribution of E2-specific ASC between the PerC and systemic sites, HK and spleen, with PerC having a higher frequency, strongly suggests their local importance. However, whether PerC ASC contribute to the systemic antibody response is currently unknown and represents an interesting area for future study. The extended presence of E2-specific ASC in PerC, even in the absence of the virus as evidenced by the absence of SAV *nsP1* transcript in the PerC adipose tissue, implies the existence of a specialized niche that supports their long-term survival. Whether this corresponds to a secondary lymphoid tissue or to the previously described long-term survival niche in the HK warrants further exploration. Additionally, the upregulation of a wide array of immune genes in the PerC adipose tissue that likely influence the activation and differentiation of B cells indicates its active role in regulating the PerC ASC response. However, the mechanisms controlling the activation of antigen-encountering B cells in the PerC and their fate remain largely unexplored. Future studies on whether PerC ASC contribute to protection induced by ip vaccination may provide insights into developing more effective vaccines or using local

responses as indicators of vaccine efficacy.

Funding

This work was supported by The Research Council of Norway (grant number 254892) and UiT the Arctic University of Norway.

CRediT authorship contribution statement

Shiferaw Jenberie: Writing – original draft, Visualization, Methodology, Investigation. **Henriette Rogstad Nordli:** Investigation. **Guro Strandskog:** Investigation. **Linn Greiner-Tollersrud:** Investigation. **Ma Michelle D. Peñaranda:** Investigation. **Jorunn B. Jørgensen:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Ingvill Jensen:** Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Acknowledgements

The authors acknowledge MSD Animal Health for providing InSAV1, Karsten Skjødt, University of Southern Denmark for providing anti-trout IgM mAb and Øystein Evensen, Norwegian University of Life Sciences for providing WtSAV3. We extend our gratitude to the Tromsø Aquaculture Research Station for technical assistance during the virus challenge experiment. Additionally, the graphical support by Rod Wolstenholme from the graphical services section at the University of Tromsø is greatly appreciated.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2024.105193.

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