INTRODUCTION

Piscine novirhabdovirus belonging to the family Rhabdoviridae, originally called Egtved virus and commonly known as “viral haemorrhagic septicaemia virus (VHSV),” is a highly pathogenic virus causing overwhelming harmful effects in salmonids, specifically in Europe, resulting in great economic losses. This virus severely affects not only hatchery-reared rainbow trout but also cultured brown trout (Salmo trutta), leading to disease outbreaks (Warren, 2002). In farmed brown trout, VHSV has also been reported, but this disease has more devastating effects in rainbow trout (Skall et al., 2005). In another study, the virus tends to be less invasive following infection (Eruznannx & Konnao, 1985). Brown trout, a much-valued European sport fish among anglers, is also preferred for the table and has high economic importance in Europe. Nevertheless, VHSV is one of the most important pathogens affecting the aquaculture industry. According to European legislation (2006/88/EC), it is a notifiable disease. There are little data regarding the CD4-related immune response of brown trout against this virus. Challenging the fish immune system with a virus is suggested to be a valuable to investigate the immune defence mechanisms in host (Kennedy & Farrell, 2008).

In fish, there are two types of CD4-like molecules: CD4-1 and CD4-2 (or CD4REL). In teleost, four Ig-like domains: (D1-D3 and D2-D4) are included in CD4-1 molecules. However, CD4-2 molecules may contain two or three Ig-like domains (D1 and D3) (Castro et al., 2011). CD4 homologues have already been reported in fish, that is sea bass (Buonocore et al., 2008), rainbow trout (Dijkstra et al., 2006; Laing et al., 2006), Atlantic salmon (Moore et al., 2009), channel catfish (Edholm et al., 2007) and common carp (Sun et al., 2007). In a previous study, we have successfully characterized...
three genes (CD4-1, CD4-2a and CD4-2b) in brown trout (Ashfaq et al., 2020). The lack of antibodies for both molecules in other teleosts has been the limited factor to study the expansion pattern of CD4+ cells during environmental challenges of biological or chemical nature.

Activated CD4+ cells are responsible for controlling the antigen-specific immunity by mediating cytotoxic T-cell and B-cell immune responses through the cytokine cascade (Zhu & Paul, 2008). The direct impact of CD4+ cells in both humoral and cell-mediated responses has been demonstrated in fish after a viral infection (Somamoto et al., 2014). Similarly, the role of CD4-1+ in the adaptive immunity was explored using polyclonal antibodies against zebrafish CD4 (Yoon et al., 2015). Moreover, CD4-1+ cells have demonstrated a protective effect against viral diseases in ginbuna crucian carp (Somamoto et al., 2014). Furthermore, DNA vaccines have displayed favourable outcome in protection against VHSV in fish (Sepúlveda & Lorenzen, 2016), suggesting the involvement of the adaptive immune response. Akin to mammals, IFN-γ produced by CD4+ cells coordinates cell-mediated immune response, which ultimately enhances the response of cytotoxic T lymphocytes and macrophages against viral threats (Yamasaki et al., 2014).

This study has been designed to investigate the involvement of brown trout CD4+ cells in protection against VHSV using polyclonal antibodies to screen CD4+ cells in certain cellular functional studies. Furthermore, for the first time, a polyclonal antibody against a brown trout CD4-1 has been used to screen CD4+ cells during a VHSV infection. Besides, the gene expression of CD4-1 and CD4-2 transcripts has been monitored in response to VHSV infection in brown trout.

2 | MATERIALS AND METHODS

2.1 | Fish

A total of 65 brown trout (Salmo trutta) juveniles (average length 10–15 cm ± 0.5) were obtained from a certified local fish farm and maintained in the wet laboratory in the Clinical Division of Fish Medicine, University of Veterinary Medicine, Vienna, Austria. The fish were acclimated in a 1000-L capacity tank with a continuous flow-through system at 15 ± 1°C for 15 days with sufficient aeration and feeding at the rate of 1% of body weight per day. All experiments were performed following guidelines and regulations with approval from the ethical committee of the University of Veterinary Medicine, Vienna, and the national authority, according to §26 of the Austrian Law for Animal Experiments, Tierschutzgesetz 2012, under approval number 68.205/0208-V/3b/2019.

2.2 | Infection experiment

For viral infection, a rainbow trout highly virulent VHSV isolate (A-389/16) was used. The isolate has been isolated at the Austrian National Reference Laboratory for fish diseases from fish perished during an outbreak of VHS disease at the end of 2016. By means of RT-PCR, it has been identified as VHSV genotype Ia. The BF-2 (Bluegill fry) and EPC (Epithelioma papulosum cyprini) cell lines were used for propagation of the virus. In addition, before the experimental infection, the virus was quantified by end-point titration using BF cells.

The experimental design of this study is shown in Figure 1. After acclimation, fish were randomly divided into two experimental groups: infected (n = 30) and control (n = 30) groups. Each group represented in triplicates of 10 fish. Five extra fish were added to the infected group to cope with any mortality. Each fish in the infected group was intraperitoneally injected with 0.2 ml containing 4 × 10^5 TCID50/ml VHSV. On the other hand, each fish in the control group was intraperitoneally injected with 0.2 ml of sterile MEM. At different time points (1, 2, 3, 5, 7, 9, 11, 13, 22 and 28 days) 3 fish/group were collected and killed. Head kidney, spleen, gills and intestine were sampled and used to screen for the cellular immune response and for gene expression against VHSV. In case of gill samples, the primary and secondary filaments of the

![FIGURE 1 Experimental design for the in vivo experiments. This scheme depicts schematically the studies conducted with brown trout in the present work. The infected group (n = 35) was injected with VHSV intraperitoneally (represented by yellow colour). The untreated control group (n = 30) was injected with sterile virus-free MEM (represented by light yellow colour). The sampling time points are represented by pink colour.](image-url)
four-gill arches including interbranchial lymphoid tissues (ILT) of one side of each fish were sampled and homogenized using liquid nitrogen and then used for the subsequent analysis. Similarly, in intestinal samples, the second segment of mid-intestine and the posterior segment of the intestine from each fish were pooled and homogenized using liquid nitrogen and then used for the subsequent analysis.

2.3 | Production of polyclonal antibodies against CD4-1 of brown trout

For cellular functional studies, a signal peptide sequence (27–41: CELKDFRPQPTSNVT) was carefully selected from brown trout CD4-1 gene, subjected to BLAST search analysis against other CD4 sequences and used to commercially produce polyclonal antibodies (Biosyntan GmbH). The peptide was exceptionally unique as compared to other CD4 molecules; CD4-2a and CD4-2b. Briefly, the synthesized peptide (15–20 mg) was conjugated to keyhole limpet haemocyanin with glutaraldehyde and inoculated intraperitoneally into two rabbits. The rabbits were given booster doses of 5 mg of the conjugates every two weeks. Subsequently, 28 days after each immunization, a blood sample was taken from the ear vein for immunoassay. The protocol included prebleeding–post-bleeding phases. The preimmune sera were collected before inoculation and stored at −20°C. An ELISA test was conducted to test the collected antisera for its reactivity and specificity.

2.4 | Leucocyte isolation

To prepare single-cell suspensions (leucocytes) from fish organs using the density gradient method, spleen, head kidney, gills and intestinal tissues from each dissected fish samples (n = 3 fish/group/time point) were immediately kept in Dulbecco’s phosphate-buffered saline (DPBS; Sigma-Aldrich) already placed on ice. Then, the tissues were aseptically removed from the DPBS and pressed through 100-μm sterile nylon mesh placed in a cell strainer (Corning Life Sciences), for intestine and gills, a digestion solution, RPMI + 37.5 mg collagenase II + NCS was used before pressing through the strainer. The homogenates were resuspended in 4 ml of phosphate-buffered saline (PBS) containing 2% NCS and centrifuged at 400 g for 40 min at 4°C. The leucocytes were recovered as a white band at the interface between Percoll and the PBS; this band was aspirated carefully and was washed twice with PBS. Subsequently, the cells were washed with 8 ml of PBS containing 2% NCS and centrifuged at 400 g for 6 min at 4°C. Lastly, after discarding the supernatant, the samples were resuspended in 1 ml of PBS containing 2% NCS and counted in a Neubauer chamber. All the procedures were carried out at 4°C or were kept on ice until further use.

2.5 | SDS-PAGE and Western blotting

To confirm the specificity of the prepared polyclonal antibodies, we used leucocytes isolated from head kidney, which expectedly contains more CD4 cells. The cell lysate from head kidney was placed separately in 250 μl of 4x Laemmli protein sample buffer (Bio-Rad). Following the incubation at room temperature for 5 min, the whole protein extract (excluding tissue remains) was incubated at 95°C for 5 min to be analysed by SDS-PAGE. For loading of samples to 12% polyacrylamide gel and separation by electrophoresis (SDS-PAGE), Mini-PROTEAN® 3 cell apparatus (Bio-Rad) was configured, including making of reagents, running gel, stacking Gel and running buffer. The reagents and chemicals, that is SDS, TEMED, ammonium persulphate and Precision Plus Protein Dual Color standards, were purchased from Bio-Rad, and dithiothreitol (DTT), glycine, Tris base, bovine serum albumin, methanol, acrylamide/bisacrylamide (40%), skim milk and isopropanol were purchased from Sigma-Aldrich. The gels were run at 100 volts for 120 min. Following electrophoresis, the gels were transferred onto 0.2 μm nitrocellulose membrane, as per the manufacturer’s guidelines. After proper transferring of protein, the membrane was transferred to 100 ml of blocking buffer for 15 min on a rocking platform at room temperature. After washing, the membrane was placed at room temperature for 20 min with the prepared rabbit anti-brown trout CD4-1 (dilution 1:100), and the preimmune serum from rabbits was used as a negative control. Lastly, the membrane was washed and incubated with the alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibodies diluted at 1:500 for 30 min. The membrane was washed 3 times for 10 min each with the washing buffer and then incubated with the colorimetric detection substrate for 30 min.

2.6 | Immunofluorescence

For the immunofluorescence experiment, the single-cell suspension, prepared in Section 2.4, and sorted CD4- cell population were fixed with 4% paraformaldehyde on a microscopic slide. Subsequently, the slides were blocked with 5% skim milk in PBS-T (PBS +0.1% Tween-20) for 30 min, and the cells were then incubated with the primary antibody, rabbit anti-brown trout CD4-1 (dilution 1:200) for 3 hr. Control slides were incubated with rabbit preimmune sera as a negative control. Afterwards, the slides were washed 5 times for 10 min each with PBS-T and then incubated with anti-rabbit IgG conjugated with Alexa Fluor 488 at a concentration of 1:1,000. The slides were washed again for 5 times for 10 min each and counterstained with DAPI (Biolegend). Finally, the slides were examined under a fluorescence microscope (Olympus).

2.7 | Flow cytometry

The leucocytes (1 × 10^6 cells/ml) from HK, spleen, intestine and gills were isolated from each sampled fish (n = 3 fish/group/time point) as
given in Section 2.4. The identification of cells bearing CD4-1 was carried out by staining the leucocytes for 20 min with the rabbit anti-brown trout CD4-1 primary antibodies (dilution 1:100). The cells were washed twice with PBS and centrifuged at 510 g for 6 min at 4°C. The supernatant was discarded, and the cells were incubated with anti-rabbit fluorochrome-labelled (Alexa Fluor 488) secondary antibodies (Thermo Fisher) for 20 min at 4°C. The cells were again washed twice with PBS and centrifuged at 510 g for 6 min at 4°C. Controls (autofluorescence, preimmune sera and secondary antibodies) were also included. To eliminate the debris and identify cell populations based on size and granularity, forward scatter (cell size) and side scatter (granularity) gating strategies were used. Cells located by FSClow or SSClow were identified as lymphocytes of head kidney (b), spleen (c), gills (d) and intestine (e) (Figure 5). Flow cytometry was performed using a FACSCanto II flow cytometer and analysed by BD FACSDiva software (BD Biosciences).

Furthermore, stained cells were sorted for gene expression analyses with BD FACSAria III flow cytometer (BD Biosciences). The analysis was carried out with BD FACSDiva™ software.

### 2.8 | Gene expression analysis

Leucocytes extracted from HK and spleen (n = 3 fish/group/time point) were stained for CD4-1 and CD4+ populations. CD4+ cells (40,000) were subjected to RNA extraction using RNeasy Micro Kit (Qiagen) as per the manufacturer's instructions. The quantity and quality of the genomic RNA was assessed using Bioanalyzer 2100 (Agilent). Using the iScript™ cDNA Synthesis Kit (Bio-Rad), the total RNA was reverse-transcribed into cDNA. Quantitative real-time PCR primers were designed (Table 1), and the protocol was optimized for the screening of T-cell-related genes, using the QuantiTect SYBR Green PCR Kit (Qiagen).

Expression of the brown trout CD4-1, CD4-2a and CD4-2b genes was assessed in the RNA extracted from each organs from the sampled fish (n = 3 fish/group/time point) at the following time points (1, 5, 9, 13 and 20 dpi) using brown trout EF-1α as a reference gene according to Ashfaq et al., (2020). All the samples were run in triplicates.

### 2.9 | Statistical analysis

A non-parametric Mann-Whitney U test was used to analyse the data generated after flow cytometry, depicted by asterisk above the histograms. Each sampling point was analysed by Duncan's multiple range tests and represented using lowercase letters above the histogram bars. Furthermore, one-way analysis of variance (ANOVA) was performed using the SPSS version 17.0 program (SPSS, Inc.) to investigate the average fold change between uninfected and infected fish after gene expression analysis. p-Values <.05 were considered significant. Values were expressed as mean ± standard error of mean (SEM).

### 3 | RESULTS

#### 3.1 | Specificity of the generated brown trout CD4-1 antibodies through Western blot and immunofluorescence assay

Western blot analysis revealed that the antibody recognized a ~54 kDa protein in head kidney, which was the predictable size for CD4-1 in salmonids (Figure 2a).

In addition, results of immunofluorescence analysis verified the presence of CD4 on the cell membrane as a phenotypic identification (Figure 2b-d). No significant staining could be detected in the control slide.

#### 3.2 | Specificity of the generated brown trout CD4-1 antibodies through cell sorting

To establish whether sorted CD4+ brown trout cell population exclusively expressed CD4-1 gene, the analysed sorted CD4-1+ cells for the expression profiles of IgM, TCR, CD8 and CD4-1, gene transcripts using the CD4-1-negative cells as control revealed that the sorted CD4+ population highly expressed CD4+ and TCR, while a negligible amount of CD8 gene expression was recorded (Figure 3).
Gene transcripts of CD4-1+ lymphocytes were only amplified by the primers expressing T-cell transcripts and not B-cell transcripts.

3.3 | Involvement of CD4-1-positive lymphocytes in brown trout tissues in response to VHSV infection

The presence of the VHSV was confirmed in exposed fish by qPCR-based diagnostic assay.

3.3.1 | Head kidney

Lymphocytes were identified based on their low granularity and small size (FSClow; SSClow) (Figure 4). Analysing the flow cytometric data at 10 distinct time points revealed enhanced high percentage of CD4-1+ cells in HK in the infected group at 1, 5, 7 and 13 dpi (Figure 5a) reaching the maximum level of 39 ± 3.65 on dpi 7. In contrast, at 20 dpi, significantly lower (p-values <.05) numbers (14.46 ± 2.4) of CD4+ cells were present in the infected group. Overall, in the control group, CD4+ cells ranged from 13.1 ± 1.68 to 29.36 ± 4.71. Furthermore, we found that within the infected group, the number of CD4+ cells increased (p-values <.05) during the infection until 7 dpi in HK, while a gradual decline was observed from 7 dpi until 28 dpi.

3.3.2 | Spleen

Infection resulted in significantly higher percentage (p-values <.05) of CD4+ cells in the spleen at 2, 5, 7 and 13 dpi in the infected group reaching the peak (32.46 ± 6.12) at 2 dpi (Figure 5b). Again, at 20 dpi, the value (8.36 ± 1.12) was significantly lower (p-values <.05) than that in the control group. The average number of CD4-1+ stained cells ranged from 13.48 ± 0.47 to 18.133 ± 2.03 in the control group. Moreover, within the infected group, an irregular trend was observed from 1 to 7 dpi, followed by a steady increase in CD4-1+ cells until 13 dpi. By the end, a decline in CD4-1+ cells were witnessed at 20 and 28 dpi.

3.3.3 | Gills

Flow cytometry of the gill leucocytes showed a significant increase (p-values <.05) in CD4-1 T cells at nearly all time points (2, 3, 5, 7, 9, 11 and 13 dpi) in the infected group (Figure 5c). A prominent peak of CD4-1+ cells was observed at 2 dpi (18.96 ± 1.26) and 3 dpi (20.86 ± 1.34). Within the control group, the number of CD4-1+ cells ranged from 3.96 ± 4.89 to 11.1 ± 9.769. Additionally, CD4-1+ cell percentage increased in the infected group at 2 and 3 dpi, and further decreased until 9 dpi with sudden hype at 11 and 13 dpi, when analysed.

3.3.4 | Intestine

The CD4-1+ cell number was elevated in the intestine; it was significantly higher (p-values <.05) at 3 and 5 dpi as compared to that in the control group (Figure 5d). A conspicuous rise in CD4-1+ cells was observed at 3 dpi (26.16 ± 3.36) and 5 dpi (22.86 ± 1.58). The
number of CD4-1+ cells ranged from $7.4 \pm 2.59$ to $17.26 \pm 3.95$ in the control group throughout the experiment. Furthermore, an increase in CD4-1+ cell number was observed from 1 to 9 dpi, and then, a sudden decline was observed at 11 dpi, which continued till 28 dpi within the infected group.

3.4 | Gene expression analysis of brown trout CD4-1, CD4-2a and CD4-2b in response to VHSV infection

3.4.1 | CD4-1 expression

Normalized gene expression of the brown trout CD4-1 gene in the spleen showed upregulation with a significant difference at 5, 13 and 20 dpi with fold increases of $67.93 \pm 53.3$, $15.82 \pm 29.9$ and $14.6 \pm 23.6$, respectively (Figure 6a). In HK and gills, the expression of CD4-1 gene increased with significant differences (p-values <.05) at 5 and 13 dpi with fold increases of $78.30 \pm 68.34$, $20.1 \pm 16.3$, $56.11 \pm 89.2$ and $12.54 \pm 32.3$, respectively. However, in the intestine the expression of CD4-1 gene was significantly upregulated (p-values <.05) at 13 dpi ($13.92 \pm 18.92$).

3.4.2 | CD4-2a

CD4-2a expression significantly (p-values <.05) peaked at 5 dpi in the VHSV-infected group relative to the control groups (p < .05) in HK ($36.06 \pm 45$), gills ($55.34 \pm 31.3$) and intestine ($26.54 \pm 16.8$). CD4-2a transcripts were significantly downregulated in spleen at 5, 13 and 20 dpi with values ($-16.06 \pm 0.05$), ($-22.9 \pm 0.09$) and ($-25.56 \pm 1.2$), respectively (Figure 6b).

3.4.3 | CD4-2b

An upregulation of CD4-2b was observed in all investigated tissues of the infected group. This gene exhibited a significantly high (p-values <.05) constitute expression level up to 24.2-fold in the spleen ($57.98 \pm 58.9$) and HK ($20.20 \pm 23.7$) at 5 and 8 dpi (Figure 6c). However, significant (p-values <.05) downregulation of this gene was observed in the spleen at 20 dpi ($-28.9$ folds). Slight upregulation of CD4-2b gene was observed in gills and intestine at all time points excluding 1 dpi.
DISCUSSION

The fish immune system has been studied extensively and reported to be closely related to the immune system of mammals. The role of CD4+ cells in fish is ill-defined; in teleost, two copies of CD4 genes are found, which is in contrast to mammals that had a single gene (Dijkstra et al., 2006; Moore et al., 2009; and Ashfaq et al., 2020). The dynamics and function of CD4+ lymphocytes in olive flounder has been reported recently (Jung et al., 2020) with a comparable function to that of higher vertebrates. The lack of appropriate antibodies is a major hurdle in the immunological studies in fish (Moore et al., 2009; Somamoto et al., 2014). In teleosts, cytotoxic activities of leucocytes have been reported (Fischer et al., 2006). Polyclonal antibodies consist of a combination of antibodies recognizing different epitopes of the same antigen. Polyclonal antibodies allow for detection of cell surface marker by binding to several regions on an antigen. CD4 molecules interact with MHC II, cause T-cell activation and initiate TCR signalling cascade by engaging the co-receptor-bound cytoplasmic protein tyrosine kinase (lck) (Swain et al., 2012). CD4+ cells have been reported to contribute to adaptive immunity after stimulation with mitogens and PAMPs, and following antigen exposure (Kitao et al., 2009).

In this study, we inspected the expansion of CD4+ T cells in brown trout by developing polyclonal antibodies against it. Using flow cytometric analysis, the dynamics, distribution and expansion of CD4+ cells in different lymphoid and non-lymphoid tissues were investigated in brown trout after VHSV infection. We found elevated levels of CD4+ T-cell percentage during the course of infection with VHSV, which is indicative for CD4+ T-cell pivotal role in immune response against the VHSV virus in brown trout.

The morphology of cells, after immunofluorescence analysis, was comparable to that of lymphocytes. Although thrombocytes and lymphocytes are similar in granulation and size (Miyazawa et al., 2018), the stained cells can be regarded as lymphocytes as very few thrombocytes are present in the head kidney in fish. Furthermore, CD4+ cells sorted after FACS were subjected to gene expression analysis for several cell marker genes by RT-PCR. The sorted cells exhibited increased expression of both CD4+ and TCR genes, and insignificant expression levels of CD8 and B-cell marker (IgM) genes, indicative of CD4+ T cells as suggested in earlier studies (Jung et al., 2020; Yoon et al., 2015).

The detailed investigation on CD4+ T cells was performed by incorporating flow cytometry using polyclonal antibodies against CD4-1 protein of brown trout and gene expression analysis (Kono & Korenaga, 2013; Miyazawa et al., 2018; Toda et al., 2011). Similarly,

FIGURE 5 Flow cytometric analysis of percentages of CD4-1-positive lymphocyte in tissues; head kidney (a), spleen (b), gills (c) and intestine (d), in response to viral haemorrhagic septicemia (VHS) virus in brown trout at 1, 2, 3, 5, 7, 9, 11, 13, 20 and 28 days post-infection. The leucocytes were stained with the brown trout anti-CD4-1 polyclonal antibody followed by fluorescence-conjugated rabbit IgG produced in goat. Each figure (a-d) is representative of three analyses, and the data are depicted by mean ± SD with statistically significant difference at <0.05 after ANOVA. Diverse little letters show significant differences at p < 0.05 in different groups of each sampling point with Duncan’s multiple range test. Red bar indicates the infected group, whereas blue indicates the control group.
in catfish, production and further use of IgM mAb through flow cytometry were successfully documented (Bunnoy et al., 2020). In a fluorescence-activated cell sorting study, the catfish lymphocytes were gated and the targeted T-lymphocyte population was screened based on low forward scatter (FSC) and side scatter (SSC) scores. In a previous study, Koppang et al., (2010) recorded moderate percentages of T-cell+ lymphocytes in spleen and head kidney without induction of an infection. In the current study, the expansion of CD4+ T cells after the infection is an indicator of Th cell activation; CD4+ T-cell percentage was found to be considerably higher on 2, 3, 5 and 7 dpi in spleen and HK. Furthermore, the CD4-1 gene expression was found to be the highest at 5 dpi in gills, HK and spleen as compared to that in the control groups, in line with the preceding observations suggesting the start of cell-mediated responses against viruses at 5 to 7 days (Guidotti & Chisari, 2001). Besides, the current study is in agreement with a study in olive flounder, in which proliferation of CD4-1 lymphocytes was recorded highest at 7 dpi in head kidney against NNV infection, signifying the involvement of CD4-positive T cells in a virus-specific immune response (Jung et al., 2020). Similarly, a study conducted on CD4+ T cells, after a bacterial infection, recorded the highest activity of the CD4+ cells was at 8 dpi (Yamasaki et al., 2014). The results propose that CD4+ cells play a significant role in the immune response against viral infection by activating CD4-mediated cascade. It is attractive to speculate that this could underlie the reduced susceptibility of brown trout to VHSV infection. Expression levels of CD4-1, CD4-2a and CD4-2b were found to be differentially regulated in the same tissue during infection in agreement with earlier studies in rainbow trout (Kato et al., 2013).

The polyclonal antibodies against CD4-1 enabled us to compare the expansion of these cells in different tissues. The HK and spleen are among the major lymphoid organs in teleosts (Laing et al., 2006), the highest expression level of CD4-1+ cells was observed in lymphoid organs. Gills showed significant amounts of CD4-1+ cells at 3, 5 and 7 dpi, this may be explained by the fact that the gill samples include number of interbranchial lymphoid tissues (ILT), which is considered the greatest source for T cells in the gills (Haugarvoll et al., 2008; Koppang et al., 2010; Salinas et al., 2011). Likewise, T-cell+ lymphocytes were detected in higher level in intestinal and gill leucocytes of Atlantic salmon (Koppang et al., 2010), although reasonable percentages were detected in in splenocytes and in cells of the head kidney. In other studies, CD4-1+ T cells in head kidney, spleen and gut of rainbow trout (Maisey et al., 2016) and olive flounder (Jung et al., 2020) were reported using double staining against T cells and CD4-1 cells. Our findings oppose other studies, involving tissue-specific expression of CD4 genes and flow cytometric studies. A comparatively lower number of CD4+ cells were observed in non-lymphoid organs than in lymphoid organs (Buonocore et al., 2008; Jung et al., 2020). In addition, the expression of CD4-1 in the intestine was elevated at the same time point as in the spleen. However, CD4-2a and CD4-2b levels showed a different pattern than CD4-1 did during the infection, which is comparable to the findings of a previous study (Kato et al., 2013), where different CD4 genes were differentially regulated after infection. Our results propose a diversified function of CD4 homologues in brown trout and that CD4-1 and CD4-2 are expressed in the different cell types in a different way because the
tissue distributions of these two transcripts were different. A rapid response of CD4+1-cells was observed during the earlier phase of VHSV infection in contrary to the findings by Kato et al., (2013), but in line with other findings, where virus-specific cell-mediated responses were generally observed in the first 5 days after infection.

5 | CONCLUSION

Polyclonal antibodies recognizing the CD4-1 T lymphocytes in brown trout were generated and used to show dynamics of CD4-1 T cells upon VHSV infection. Cell morphology, tissue distribution and gene expression of brown trout CD4 T lymphocytes were comparable to those in other fish species. The specificity of polyclonal antibodies was validated, and CD4-1+ cells expand in the immune response against viral infections.

ACKNOWLEDGEMENTS

The first authors thank the Higher Education Commission of Pakistan for providing Ph.D. scholarship, and the University of Veterinary and Animal Sciences (Pakistan) to allow him to study abroad. Special thanks go to the members of the Clinical Unit for Poultry for kindly providing flow cytometric machine for this experiment.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

MEL and HS designed the experiments. HA performed the laboratory tests. SF helped in sorting the leucocytes with BD FACSAria III flow cytometer, analysed the data and drafted the manuscript. HS and MS participated in the data analysis and result interpretation and helped to draft the manuscript. HS, VS, MEL and MS revised the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experiments were performed following the guidelines and regulations with approval from the ethical committee of the University of Veterinary Medicine, Vienna, and the national authority, according to §26 of the Austrian Law for Animal Experiments, Tierversuchsgesetz 2012, under approval number 68.205/0208-V/3b/2019.

DATA AVAILABILITY STATEMENT

There are no additional data available.

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