Antigen-specific T-cell response of swine against *Salmonella* Typhimurium

Thesis submitted by

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2 Abstract

The gram-negative facultative intracellular bacterium *Salmonella* Typhimurium (STM) continues to be of great concern for both public and animal health with pork being among the major sources of infection in humans. Among recommended measures in affected swine populations is vaccination with the live attenuated vaccine Salmoporc. Although the vaccine was shown to be effective in the field, information on the cellular immune response to vaccination with Salmoporc or STM infection is still scarce. The project at hand aimed to investigate the STM-specific T-cell immune response in pigs vaccinated and/or infected with STM.

In a first experiment, pigs were vaccinated twice with Salmoporc followed by a challenge infection with a virulent STM strain. Lymphocytes isolated from blood and organ tissues were stimulated *in vitro* with heat-inactivated STM antigen and analyzed for the production of IFN-γ, TNF-α, and IL-17A by flow cytometry. STM-specific cytokine-producing $CD4^+$ T cells were induced in vaccinated and infected pigs with highest frequencies in the lamina propria of the gut tissue and predominantly possessed a $CD8\alpha^+CD27^-$ phenotype, indicative of an effector memory differentiation stage.

The second experiment included four different treatment groups: pigs vaccinated with Salmoporc, pigs challenged with the virulent STM strain, pigs both vaccinated and infected as well as an untreated control group. In addition to STM-specific $CD4^+$ T cells, cytokine-producing $CD8^+$ and $CD4^-CD8\beta^-$ T cells were analyzed in response to *in vitro* stimulation with heat-inactivated STM and abundances of cytokine-producing phenotypes for all T-cell subsets were compared across organs and animal treatment groups. Results showed that frequencies of STM-specific cytokine-producing cells were highest in the $CD4^+$ T-cell subset. While low-level induction of STM-specific cytokine-producing $CD4^+$ T cells, especially for the IFN-γ/TNF-α co-producing phenotype, was observed after vaccination, significant contrasts in cytokine production were detected mainly after challenge infection. Interestingly, significantly higher abundances of cytokine phenotypes were detected in only infected pigs in mesenteric lymph nodes and systemic organs when compared to vaccinated and infected animals.

In conclusion, the results obtained in both studies detected a strong $CD4^+$ T-cell response after STM infection, both locally and systemically, indicating that $CD4^+$ T cells play an important role in protective immunity against STM in the pig. Furthermore, vaccine-induced STM-specific cytokine-producing $CD4^+$ T cells seem to support local immunity in the gut by limiting

the spread of STM to lymph nodes and systemic organs. Thus, the present PhD project could elucidate underlying mechanisms of the efficacy of the Salmoporc vaccine and provide a foundation for further studies on the cellular immune response against STM in the pig.

3 Introduction

3.1 *Salmonella* Typhimurium

3.1.1 General introduction to *Salmonella* Typhimurium

Salmonella enterica subspecies *enterica* serovar Typhimurium (STM) is a gram-negative, facultative anaerobe flagellated bacterium belonging to the family of the *Enterobacteriaceae*. The *Salmonella* genus consists of two species, *Salmonella bongori* and *Salmonella enterica* (Brenner et al. 2000). *Salmonella enterica* is further subdivided into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* (Cheng et al. 2019; Guibourdenche et al. 2010), amongst which *Salmonella enterica* subspecies *enterica* has been studied the most extensively (Lamas et al. 2018) due to its relevance for infections in humans. All subspecies contain a great number of serotypes that are differentiated based on O (somatic) and H (flagellar) antigens (Grimont and Weill. 2007).

While some serotypes such as *S*. Typhi, *S*. Choleraesuis and *S*. Gallinarum are mostly restricted to one host (Uzzau et al. 2000), broad host range serovars such as *S*. Enteritidis and STM can infect a wide variety of species (Gal-Mor et al. 2014). These broad host range serovars harbor great zoonotic potential and are frequently the cause of human infections with *Salmonella* through the consumption of food products of animal origin. In fact, salmonellosis still ranks second after campylobacteriosis among the gastrointestinal infections most frequently reported in humans (EFSA. 2019). Apart from eggs and egg products, pork is among the major sources to transmit *Salmonella* to humans through the food chain (EFSA. 2018). Serotypes that are primarily responsible for infections in humans in the European Union are *S*. Enteritidis, STM and its monophasic variant (Campos et al. 2019; EFSA. 2019). This monophasic variant *S*.1,4,[5],12:i:-, expressing the same O antigens and phase 1 H antigens as STM but lacking the second flagellar phase, has become increasingly prevalent in recent years (Switt et al. 2009). While *S*. Enteritidis is frequently related to poultry products, STM and its monophasic variant range among the top most frequent serotypes isolated from pigs (Chlebicz and Slizewska. 2018).

Clinical disease in pigs occurs mainly in the post-weaning period and manifests as diarrhea, lethargy and anorexia albeit with a low mortality rate (Mastroeni and Maskell. 2006). However, most pigs are asymptomatic carriers with *Salmonella* persisting in tonsils, gut and gut-associated lymphoid tissue (Campos et al. 2019) thus making it difficult to control the disease

at the farm level. To complicate matters further, a large number of multi-drug resistant STM strains have been isolated from pigs and pork constituting a major public health risk (Boyen et al. 2008; Gupta et al. 2019; Hauser et al. 2010; Tassinari et al. 2019). Therefore, alternative strategies to antibiotic treatment are necessary to ensure effective control of *Salmonella* in affected pig herds. Apart from management optimizations such as feed intervention (e.g. addition of organic acids, prebiotics), rodent control and strict hygiene measures, vaccination can be a very effective tool (Andres and Davies. 2015).

3.1.2 Control of *Salmonella* infections in pigs by vaccination

While it is probably illusory to produce completely *Salmonella*-free animals, the aim of vaccination as an on-farm measure lies in the reduction of clinical disease, colonization and shedding as well as in preventing the development of long-term carrier-animals (Haesebrouck et al. 2004; Wales and Davies. 2017). A number of both live and killed vaccines have been developed for the use in pigs against STM in the past years although most of these have not been licensed yet. While live attenuated vaccines promise to induce strong stimulation of cell-mediated immunity (Haesebrouck et al. 2004), killed vaccines are appealing as to their higher safety profile for both user and consumer. Studies using inactivated vaccines have met with varying degrees of success (Arguello et al. 2013; Gradassi et al. 2013; Roesler et al. 2006). There have also been some efforts to produce *Salmonella* DIVA vaccines, which offer the advantage that vaccinees can easily be distinguished from naturally infected animals. In two recent studies, a live attenuated *S.* Enteritidis strain has been evaluated regarding its potential for cross-protection against STM in BALB/c mice and piglets with promising results (Gil et al. 2020; Latasa et al. 2016). However, the only currently licensed live vaccine for the protection of pigs against STM in Europe is the attenuated histidine-adenine auxotrophic vaccine Salmoporc (Ceva Santé Animale, formerly IDT Biologika GmbH). Several studies have shown the efficacy of this vaccine to control STM infections in pigs both under laboratory conditions (Eddicks et al. 2009; Leyman et al. 2012; Roesler et al. 2010; Springer et al. 2001) and in field trials (Davies et al. 2016; Peeters et al. 2019a; Ridder et al. 2014; Smith et al. 2018; van der Wolf et al. 2021) by reducing clinical signs, shedding and colonization of host tissues. Furthermore, it also offers cross-protection with monophasic STM strains (Theuß et al. 2017). A number of European countries such as Belgium, Denmark, Germany, Ireland and The Netherlands use serology-based governmental monitoring programs as a standard quality control measure for *Salmonella* reduction in food hygiene. It has been shown that Salmoporc does not to interfere with these as induced IgG levels in vaccinated animals stay below the

threshold of positivity as long as the cut-off value for optical density (OD) values as measured by ELISA is set at OD ≥ 40% (Lindner et al. May 9–11, 2007; Roesler et al. 2010; Theuß et al. 2017). As an alternative to the percentage of optical density, absorbance values can also be expressed as a sample to positive (S/P) ratio. A cut-off value of OD ≥ 40% corresponds to an S/P ratio ≥ 1.00, which is the cut-off specified in guidelines of Germany and The Netherlands. However, Belgium and Denmark define samples as positive at S/P ratios of ≥ 0.6 and ≥ 0.5, respectively (QS Qualität und Sicherheit GmbH. 2020; Peeters. 2019).

3.1.3 *Salmonella* cell invasion and pathogenesis

One of the reasons why the development of a vaccine against STM has proven to be a difficult undertaking is its nature as a facultative intracellular pathogen. Once it has entered the cell, *Salmonella* can evade humoral immunity explaining the often poor performance of inactivated vaccines (Haesebrouck et al. 2004; Wales and Davies. 2017). To enter the cell, *Salmonella* has different mechanisms at its disposal. In the pig, transmission among animals occurs mainly via the fecal-oral route; this can take place from pig to pig, from contaminated environment to pig and from the dam to its offspring (Straw et al. 2013). After oral uptake, the bacteria travel from the stomach to the intestine where they come into contact with the cells forming the intestinal epithelium. *Salmonella* can be directly taken up by phagocytes (Müller et al. 2012; Vazquez-Torres et al. 1999) or traverse the epithelial layer via paracellular penetration (Köhler et al. 2007; Meyerholz et al. 2002).

For the invasion of non-phagocytic cells such as epithelial cells and microfold (M) cells, two main entry mechanism have been identified (Velge et al. 2012). In case of the so-called Trigger mechanism, bacterial effector proteins are injected into the cell via a type III secretion system (T3SS), termed T3SS-1, which is encoded by *Salmonella* pathogenicity island-1 (SPI-1). A total of 23 different SPIs have currently been described; five of which have been identified in STM (Dos Santos et al. 2019). These pathogenicity islands represent chromosomal regions that carry the majority of virulence genes present in *Salmonella* (Marcus et al. 2000). T3SS are highly regulated protein complexes with the capability to inject effector proteins into the host cell cytoplasm. Within *Salmonella*, T3SS-1 and T3SS-2, encoded by SPI-1 and SPI-2 respectively, are the ones that have been characterized in most detail. The effector proteins translocated into the host cell by the T3SS-1 cause extensive cytoskeletal rearrangement leading to an engulfment of the bacteria which are then internalized into membrane-bound vacuoles (McGhie et al. 2009; Velge et al. 2012). These intracellular compartments, termed *Salmonella*-

containing vacuoles (SCV), undergo a maturation process and move toward a perinuclear position (Ibarra and Steele-Mortimer. 2009). Once the SCV is situated in the correct location, bacterial replication takes place. The integrity of the SCV along with intracellular survival and replication of the bacteria within is ensured by effector proteins translocated by the T3SS-2 (Bakowski et al. 2008; Waterman and Holden. 2003). Although *Salmonella* has mainly been categorized as a vacuolar pathogen, work in recent years has shown that the bacteria can also survive in the cytosol, especially in mammalian epithelial cells. While Trigger invasion was reported to often lead to release of the bacteria into the cytosol, this seems to rarely be the case when cell entry is gained by the Zipper mechanism (Röder and Hensel. 2020). During this additional way of invasion that has been described for *Salmonella*, termed Zipper mechanism, entry is generated via the activation of host cell receptors. It can be induced by the *Salmonella* outer membrane protein (OMP) Rck, encoded by the *rck* gene, which is located on the large virulence plasmid (Rotger and Casadesús. 1999). While it is highly conserved in *S.* Enteritidis and STM, not all serotypes that possess a virulence plasmid also carry the gene. Nonetheless, this marks *Salmonella* as one of the few pathogens that has been shown to be able to use both Trigger and Zipper mechanisms (Rosselin et al. 2010).

To exit the epithelial cell, STM travels to the basolateral side of the cell and reaches the lamina propria by exocytosis (Müller et al. 2012). Once the epithelial barrier has thus been overcome, the bacteria can target the great variety of cell types present in the lamina propria like macrophages, dendritic cells and lymphocytes (Di Martino et al. 2019; Müller et al. 2012) allowing transport to the mesenteric lymph nodes via the lymph with potential dissemination to systemic organs such as spleen and liver.

As outlined above, *Salmonella* has developed efficient ways to colonize its host. Despite these mechanisms, protective capacity has been observed in pigs immunized with the Salmoporc vaccine. This may be due to its nature as a live vaccine and concomitant ability to stimulate T cells. However, the effect of immunization with Salmoporc or infection with STM on the T-cell response in the pig has not yet been investigated in great detail.

3.2 T-cell biology in the pig

Knowledge on the porcine immune system is not as comprehensive as on human and mouse immunology but a lot of research has been conducted in recent years to diminish this gap. Notably, considerable effort has gone into the investigation of porcine T cells (Gerner et al. 2015). While the porcine immune system differs anatomically from many other species due to

its inverted lymph node structure, lymphocyte generation and development take place in bone marrow and thymus as it is known from other mammals (Rothkötter. 2009).

In accordance with data from T lymphocytes in humans and mice, porcine T cells express the co-receptor CD3 and can be divided into two subsets based on the composition of their T-cell receptor (TCR). T cells termed αβ T cells carry a TCR consisting of α and β chains, γδ T cells have a TCR with γ and δ chains (Saalmüller and Gerner. 2016). Interestingly, pigs along with other species such as birds and cattle have a higher frequency of γδ T cells in blood and lymphatic tissues than can be found in mice or humans (Mair et al. 2014; Takamatsu et al. 2006). With the currently available tools, αβ T cells cannot be identified directly on the protein level; their two major subsets, CD4 and CD8 T cells, are therefore defined indirectly via their expression of the lineage markers CD3, CD4 and CD8β.

3.2.1 $CD4^+$ T cells

$CD4^+$ T cells express CD4 proteins on their cell surface and in contrast to humans and mice, a large subset of porcine $CD4^+$ T cells in blood and secondary lymphoid organs co-express CD4 and CD8α (Pescovitz et al. 1994; Saalmüller et al. 1987). This population expands in the blood with increasing age of the animals (Talker et al. 2013) and responds to *in vitro* stimulation with numerous antigens by proliferation (Blanco et al. 2000; Lefevre et al. 2012) and the production of cytokines (Ebner et al. 2017; Sassu et al. 2017; Talker et al. 2015). Due to these observations, $CD4^+CD8\alpha^+$ T lymphocytes are widely regarded as activated or memory $CD4^+$ T cells (Gerner et al. 2015). Expression of CD8α along with CD27 can be used for more detailed information on the differentiation stage of $CD4^+$ T cells in swine. While $CD8\alpha^-CD27^+$ $CD4^+$ T cells are considered naïve T cells, $CD8\alpha^+CD27^+$ and $CD8\alpha^+CD27^-$ $CD4^+$ T cells exhibit features of central memory and effector memory subsets (Reutner et al. 2013), respectively, as they have been described in humans (Appay et al. 2008).

Studies on the functional differentiation of $CD4^+$ T cells in mice and humans have led to the identification of T helper (Th) 1, Th2, Th17, regulatory T cells, T follicular helper cells and cytolytic $CD4^+$ T cells (Swain et al. 2012). Currently, it stands to reason that these subsets exist in the pig as well. Indeed, Th1 cells have been identified via their production of interferon-γ (IFN-γ) when encountering viral and bacterial antigens like porcine reproductive and respiratory syndrome virus (PRRSV) (Kick et al. 2019; Kick et al. 2021; Piras et al. 2005), swine influenza A virus (FLUAVsw) (Martini et al. 2021; Talker et al. 2016), *Chlamydia suis* (Amaral et al. 2020) and *Lawsonia intracellularis* (Cordes et al. 2012). A key regulator of Th1

development is the transcription factor T-bet, whose expression on effector $CD4^+$ T cells and ability to induce IFN-γ production has also been confirmed in the pig (Rodríguez-Gómez et al. 2016). While IFN-γ is widely regarded as the signature cytokine for Th1 cells, Th17 cells are characterized by their production of interleukin-17A (IL-17A). First descriptions of porcine $CD4^+$ IL-17A-producing T cells following stimulation with PMA and ionomycin were made possible by the identification of cross-reactive IL-17A mAbs (Elnaggar et al. 2018; Stepanova et al. 2012). Further studies investigating pigs infected with *Actinobacillus pleuropneumoniae* (Sassu et al. 2017) or immunized with a *Mycoplasma hyopneumoniae* bacterin vaccine (Matthijs et al. 2019) could demonstrate the existence of Th17 cells producing IL-17A in an antigen-specific manner.

T cells capable of producing two or more cytokines simultaneously, so-called multifunctional T cells, have been associated with enhanced effector function and increased protection (Seder et al. 2008). Indeed, multifunctional $CD4^+$ T cells have been shown to serve as a correlate of protection in many studies on mice involving intracellular pathogens (Darrah et al. 2007; Lindenstrøm et al. 2009) and are of great interest for vaccine research (Seder et al. 2008). First studies in the pigs could demonstrate the existence of $CD4^+$ T cells with the capacity to produce several cytokines thus showing their capacity for multifunctionality. Immunization of pigs with a porcine circovirus type 2 vaccine induced antigen-specific IFN-γ/tumor necrosis factor-α (TNF-α) co-producing $CD4^+$ T cells in the blood (Koinig et al. 2015) which were presumably associated with the protection seen in these pigs after subsequent challenge infection. Other studies have demonstrated the generation of IFN-γ/TNF-α/IL-2 multifunctional $CD4^+$ T cells in the blood and bronchoalveolar lavage of FLUAVsw-infected pigs (Edmans et al. 2020; Martini et al. 2021; Talker et al. 2015), which possessed an antigen-experienced phenotype (Talker et al. 2015).

3.2.2 $CD8^+$ T cells

In general, $CD8^+$ T cells are characterized by their expression of the CD8 heterodimer, consisting of an α and a β-chain. This also applies to pigs. However, many porcine lymphocytes such as $CD4^+$ T cells, γδ T cells and natural killer (NK) cells also express CD8, but as an αα-homodimer (Gerner et al. 2009). More in detail, porcine $CD8^+$ T cells have been described to have a $CD4^-CD8\alpha^{high}CD8\beta^+$ phenotype (Yang and Parkhouse. 1996). Additionally, the expression of perforin, a marker for cytotoxic activity, has been demonstrated on porcine

cytolytic T cells (Denyer et al. 2006) and the percentage of perforin$^+$ CD8$^+$ T cells increases with age of the animals (Talker et al. 2013).

In contrast to CD4$^+$ T cells, which recognize peptides presented via major histocompatibility complex (MHC) class II molecules, CD8$^+$ T cells require MHC class I presentation and have long been established as key players in the immune response against intracellular pathogens. In the field of veterinary medicine, CD8$^+$ T cells have been shown to play a role in various viral infections such as foot-and-mouth disease (Patch et al. 2011), classical swine fever (Franzoni et al. 2013) and influenza A (Edmans et al. 2020; Tungatt et al. 2018) to name but a few.

However, reports about the involvement of CD8$^+$ T cells in bacterial infections in swine are scarce. A study where female pigs were re-infected with *Chlamydia suis*, an obligate intracellular pathogen, has reported an increase in the frequency of CD8$^+$ T cells alongside elevated production of IFN-γ in peripheral blood mononuclear cells (PBMC) (Clercq et al. 2014). Studies using another obligate intracellular bacterium, *Lawsonia intracellularis*, revealed that protection against re-infection in pigs was associated with the development of antigen-specific IFN-γ producing CD8$^+$ T cells and CD4$^+$CD8$^+$ T cells (Cordes et al. 2012; Riber et al. 2015). Furthermore, the infection of pigs in a *Helicobacter pylori* animal model detected the induction of a cytotoxic CD8$^+$ T cell response (Kronsteiner et al. 2014).

3.2.3 γδ T cells

Similar to CD8$^+$ T cells, porcine γδ T cells are located within the CD3$^+$CD4$^-$ subset but they can be identified directly with antibodies directed against the δ-chain of the porcine γδ TCR (Davis et al. 1998) or binding to CD3 molecules exclusively associated with it (Yang et al. 2005). CD8α in connection with CD2, which has been suggested as a lineage marker for γδ T cells in swine (Stepanova and Sinkora. 2013), can be employed to discriminate between three subpopulations. Whereas the CD2$^-$CD8α$^-$ subset is supposed to consist of naïve T cells, the CD2$^+$ subset is made up of CD8α$^-$ γδ T cells with effector or memory characteristics and CD8α$^+$ γδ T cells with a more terminally differentiated phenotype (Sedlak et al. 2014; Stepanova and Sinkora. 2012). While CD2$^-$ γδ T cells are dominating in blood and liver, CD2$^+$ γδ T cells can mainly be found in lymphoid tissue (Sedlak et al. 2014; Yang and Parkhouse. 1996).

A large number of functions have been discussed for γδ T cells in murine and human immunology (Bonneville et al. 2010), demonstrating the great range and plasticity of their response. In swine, both the CD2$^+$ and CD2$^-$ subset of γδ T cells have been described to produce

cytokines upon stimulation. $CD2^-$ γδ T cells produced IL-17A, often in co-production with TNF-α after polyclonal stimulation of porcine PBMC and splenocytes, whereas $CD2^+$ γδ T cells contained mostly IFN-γ single-producing and IFN-γ/TNF-α co-producing cells (Sedlak et al. 2014). In another study, blood-derived γδ T cells from gilts that had previously been infected with PRRSV proliferated and produced IFN-γ at a larger scale than γδ T cells isolated from their non-infected counterparts (Olin et al. 2005). Even the formation of memory-like responses have been discussed in swine (Lee et al. 2004) and cattle (Blumerman et al. 2007) after vaccination of the animals with bacterial antigen. Additionally, $CD2^+$ γδ T cells express surface molecules such as MHC class II and CD80/86 suggesting that they are capable of antigen-presentation (Takamatsu et al. 2002).

3.3 The immune response to *Salmonella*

Most of the information about the immune response to *Salmonella* has been collected from studies in humans and mice. Like swine, humans mostly develop self-limiting gastroenteritis upon infection with non-typhoidal *Salmonella* (NTS) serovars like STM. More severe disease progression with bloodstream infection can occur in immunocompromised individuals and young children caused by multi-drug resistant or invasive NTS strains in parts of Africa and Asia (Arya et al. 2017; Feasey et al. 2012). When mice are infected with STM, however, they invariably develop a systemic typhoid-like disease making it a suitable model for *S.* Typhi infection in humans (Santos et al. 2001). In order to reproduce an infection with STM characterized by enterocolitis and diarrhea, mice need to be pre-treated with an antibiotic before infection (Kaiser et al. 2012). Usually streptomycin is used for this purpose, which eliminates the gut microbiota of the mouse thus allowing STM to efficiently colonize the intestine (Barthel et al. 2003).

Once it has reached the intestine, *Salmonella* comes into contact with the intestinal epithelial cells which subsequently launch an inflammatory response (Pham and McSorley. 2015b). Epithelial cells are equipped to identify bacterial flagellin and lipopolysaccharides via toll-like receptor (TLR) 5 and TLR4, respectively, leading to a release of cytokines and chemokines and the recruitment of phagocytes (Kaiser and Hardt. 2011; Keestra-Gounder et al. 2015). Particularly neutrophils are important in this scenario which appear to be major producers of IFN-γ following STM infection (Spees et al. 2014) and it has been suggested that IFN-γ is a key factor in this early immune response in the intestinal mucosa in keeping *Salmonella* from systemic dissemination (Conlan. 1996).

The role of the humoral immune response in protection against STM has been the subject of much discussion. Antibodies secreted by B cells can contribute to antimicrobial defense by opsonizing free bacteria and activating the complement system (MacLennan et al. 2008). In a study, mice without peripheral B cells could clear a primary infection with an attenuated *Salmonella* strain but were not protected when later challenged infected with a virulent *Salmonella* strain (Mittrücker et al. 2000). However, this result is not solely explained by a lack of antibody-mediated protection in these mice. Rather, B cells have also been shown to produce inflammatory cytokines in response to STM infection and act as antigen-presenting cells, thus constituting an indispensable factor in driving T-cell differentiation and the development of CD4 T-cell memory (Barr et al. 2010; Ugrinovic et al. 2003). Interestingly, it seems that *Salmonella* is capable of suppressing humoral immunity by impairing B-cell lymphopoiesis and hindering niche occupation and survival of IgG-secreting plasma cells in the bone marrow (Takaya et al. 2019) which might have caused an undervaluation of B-cell responses overall in the context of *Salmonella* infections. In swine, studies on the humoral immune response have largely focused on the analysis of serum antibody titers, showing the induction of STM-specific IgM, IgA and IgG in pigs vaccinated and/or infected with STM (Matiasovic et al. 2014; Roesler et al. 2004; Szabó et al. 2008; Theuß et al. 2017; Trepnau et al. 2008). Additionally, a more recent work identified T cells with a T follicular helper (Tfh) cell phenotype in lymph node and spleen of Salmoporc-vaccinated piglets (Ugolini et al. 2018) suggesting a role of this cell subset, known for providing B-cell help in secondary lymphoid organs, during the porcine immune response against *Salmonella*.

In general, the importance of T cells in combatting *Salmonella* infection has long been established and especially $CD4^+$ T cells have been shown to play a central role in primary bacterial clearance (Kurtz et al. 2017). A depletion of $CD4^+$ cells with anti-CD4 monoclonal antibodies (mAb) in mice led to increases in the bacterial load in various organs (Johanns et al. 2010; Nauciel. 1990). This can most likely be attributed to a lack of IFN-γ production as outlined in a work where chronically STM-infected mice that were treated with IFN-γ neutralizing antibody suffered from higher tissue colonization and shedding levels than the placebo-treated group (Monack et al. 2004). Th1 cells have been shown to be a most vital source of IFN-γ during *Salmonella* infection (Lee et al. 2012b; Nelson et al. 2013). In fact, mice lacking the transcription factor T-bet, therefore impaired in their development of Th1 cells, were unable to resolve *Salmonella* infection (Ravindran et al. 2005). IFN-γ and TNF-α produced by Th1 cells induces the activation of macrophages and enhances their killing capacity (Tubo and Jenkins. 2014), facilitating the destruction of intracellular bacteria such as *Salmonella*.

However, neither adoptive transfer of Th1 cells from immunized to naïve mice (Mastroeni et al. 1993) nor a shared blood circulation between such mice (Benoun et al. 2018b) is enough to confer complete protection against *Salmonella* infection. Indeed, a recent study could show that it is in fact non-circulating tissue-resident memory Th1 cells, which constitute an essential element of protective immunity against *Salmonella* (Benoun et al. 2018b), a finding consistent with data obtained from studies with other intracellular pathogens (Iijima and Iwasaki. 2014; Stary et al. 2015).

In addition to Th1 cells, Th17 cells and their production of IL-17 seem to have an important additional protective role in the control of *Salmonella* infection (Schulz et al. 2008). IL-17 contributes to keep the mucosal barrier intact by regulating tight junction proteins such as claudin and occludin, inducing the production of antimicrobial peptides by epithelial cells and recruiting neutrophils to the intestine (Abusleme and Moutsopoulos. 2017). Impairment of these IL-17 responses in rhesus macaques infected with simian immunodeficiency virus led to increased dissemination of STM to internal organs (Raffatellu et al. 2008). A study where mice were orally infected with STM observed the generation of Th17 cells in the intestinal mucosa that recognized bacterial flagellin (Lee et al. 2012b). It therefore seems plausible that Th17 cells are engaged in the early phase of *Salmonella* infection before cell entry and subsequent downregulation of flagellin expression (McGeachy and McSorley. 2012). Apart from Th17 cells, γδ T cells have also been identified as contributors to IL-17 production in mice infected with STM (Godinez et al. 2009).

Of note, in addition to classical stimulation through their TCR, non-cognate stimulation has also been described for $CD4^+$ T cells during *Salmonella* infection (Pham and McSorley. 2015b). As the host organism recognizes the presence of the pathogen by identifying pathogen-associated molecular patterns (PAMPs) via TLRs, inflammatory cytokines are secreted leading to an inflammatory environment where bystander $CD4^+$ T cells that lack specificity for the specific pathogen can be stimulated in a TCR-independent manner by the presence of certain cytokines (O'Donnell and McSorley. 2014). These bystander responses have mostly been described for previously activated or memory $CD4^+$ T cells (Bangs et al. 2009; Di Genova et al. 2010) and, interestingly, effector $CD4^+$ T cells induced by immunization with a *Salmonella* subunit vaccine seem to have a higher threshold for non-cognate stimulation than those generated by live *Salmonella* infection (Pham et al. 2017). Though the contribution of non-cognate $CD4^+$ stimulation to the overall immune response against STM is not known, it is likely that this alternative way of $CD4^+$ T-cell activation is beneficial as *Salmonella* has been known

to be able to lower the expression of MHC class II in order to escape recognition by the adaptive immune system (Gogoi et al. 2018).

$CD8^+$ T cells are generally not considered an integral part of the cellular immunity towards *Salmonella* (Kurtz et al. 2017). No significant changes in bacterial burden were observed in any organ except for the spleen in mice treated with anti-CD8 mAb when comparing combined $CD4^+$ and $CD8^+$ T cell depletion to $CD4^+$ T cell depletion alone (Johanns et al. 2010). A study using MHC class I deficient mice or mice without $CD8^+$ T cell cytotoxic granules, however, could show a very moderate protective capacity for $CD8^+$ T cells in primary infection with an attenuated STM strain (Lee et al. 2012a). It has further been shown in the mouse model that STM employs the PD-1:PD-L axis to impair the $CD8^+$ effector T-cell response during chronic infection (López-Medina et al. 2015), possibly explaining the limited involvement of $CD8^+$ T cells reported in other studies. In the context of *S.* Typhi in humans however, robust $CD8^+$ T-cell responses have been elicited by both oral immunization with the live attenuated *S.* Typhi Ty21 vaccine (Salerno-Goncalves et al. 2002) and infection with wild-type *S.* Typhi (Fresnay et al. 2017). Moreover, these antigen-specific $CD8^+$ responses were found to be multifunctional (Fresnay et al. 2017; McArthur and Sztein. 2012), present at both local and systemic sites (Booth et al. 2017; Booth et al. 2019b) and associated with protection from typhoid fever (Fresnay et al. 2016).

Although there are a few reports showing potential involvement of γδ T cells in protection against *Salmonella* infection in mice (Edelblum et al. 2015; Mixter et al. 1994), γδ T cells have been much more extensively researched in STM immunology of avian species. Studies in chicken have revealed an increase of $CD8\alpha\alpha^{hi+}$ γδ T cells, a subset of avian γδ T cells, in peripheral blood after immunization of young chicks with *S.* Enteritidis (Berndt et al. 2006). This cell subset furthermore showed a strong increase in the expression of the activation marker CD25, suggesting an activated phenotype, when avian whole blood cells where stimulated *ex vivo* with a live *S.* Enteritidis vaccine (Braukmann et al. 2015a). In an *in vivo* infection model, $CD8\alpha\alpha^{hi+}$ γδ T cells increased in frequency in blood, spleen and cecum of one day old chicks and displayed elevated levels of IFN-γ messenger RNA (mRNA) after infection with STM (Pieper et al. 2011). This suggests a more important role of γδ T cells in *Salmonella* infection in γδ-high species such as chickens, cattle and pigs.

Previous works on the cellular immune response against *Salmonella* in swine have mostly focused on gene expression analysis on mRNA level. Corresponding with data from murine studies, an upregulation of proinflammatory molecules and induction of immune-related

markers were detected in the porcine intestine, gut-associated lymphoid tissue and peripheral blood of piglets orally infected with STM (Bescucci et al. 2020; Collado-Romero et al. 2010; Knetter et al. 2015; Martins et al. 2013; Meurens et al. 2009; Skjolaas et al. 2006), largely pointing towards a Th1-associated immune response. While different transcriptional profiles have been observed in STM versus *S.* Choleraesuis-infected pigs (Uthe et al. 2007), immunization of pigs with both an STM and *S.* Choleraesuis vaccine induced IFN-γ secreting cells in the blood as measured by ELISpot assay (Foss et al. 2013). Furthermore, analysis of CD25 expression on protein level by flow cytometry and immunohistochemistry revealed presence of $CD25^+$ lymphocytes in blood, mesenteric lymph nodes and ileal Peyer's Patches in pigs two days after STM infection (Kreuzer et al. 2014).

Taken together, the current knowledge drawn from species such as mice and humans, as well as pigs and chickens, as elucidated above, suggests a central role for $CD4^+$ T cells in host defense against *Salmonella* infection, with B cells, γδ T cells and $CD8^+$ T cells as possible supporting players. In the PhD project at hand, I aimed to examine the cellular immune response against STM in the pig in more detail with the tools currently available in porcine immunology.

4 Aims and hypotheses

As stated above, little is known about the immune response to STM vaccination or infection in swine. Information on the cellular immune response in particular is very limited. Therefore, the aim of this project was the detailed characterization of the STM-specific T-cell response in the pig, comprising a phenotypic and functional characterization of the involved T-cell subsets such as $CD4^+$ T cells, $CD8^+$ T cells as well as potentially γδ T cells. This included the analysis of the production of IFN-γ, TNF-α and IL-17A by the aforementioned T-cell subsets in both local and systemic tissues at different time points after STM vaccination and/or infection.

The following main hypotheses were addressed in this project:

- Infection of swine with STM or vaccination with live attenuated STM elicits a measurable antigen-specific cellular immune response

- STM-specific IFN-γ, TNF-α and/or IL-17A producing $CD4^+$ T cells are induced by STM vaccination or infection

- There are differences in the local and systemic STM-specific T-cell immune response following contact with STM

5 Publications

- Schmidt S, Sassu EL, Vatzia E, Pierron A, Lagler J, Mair KH, Stadler M, Knecht C, Spergser J, Dolezal M, Springer S, Theuß T, Fachinger V, Ladinig A, Saalmüller A, Gerner W (2021) Vaccination and Infection of Swine with *Salmonella* Typhimurium Induces a Systemic and Local Multifunctional $CD4^+$ T-Cell Response. Front. Immunol. 11:603089. doi: 10.3389/fimmu.2020.603089

- Schmidt S, Kreutzmann H, Stadler M, Mair KH, Stas MR, Koch M, Vatzia E, Dürlinger S, Knecht C, Spergser J, Dolezal M, Springer S, Theuß T, Fachinger V, Ladinig A, Saalmüller A, Gerner W (2021) T-Cell Cytokine Response in *Salmonella* Typhimurium-Vaccinated versus Infected Pigs. *Vaccines*. 9(8):845. doi: 10.3390/vaccines9080845

Additional publications not included in this thesis:

- Lagler J, Mitra T, Schmidt S, Pierron A, Vatzia E, Stadler M, Hammer SE, Mair KH, Grafl B, Wernsdorf P, Rauw F, Lambrecht B, Liebhart D, Gerner W (2019) Cytokine production and phenotype of *Histomonas meleagridis*-specific T cells in the chicken. Veterinary Research 50 (1), 1-15. doi: 10.1186/s13567-019-0726-z

- Lagler J, Schmidt S, Mitra T, Stadler M, Wernsdorf P, Grafl B, Hatfaludi T, Hess M, Gerner W, Liebhart D (2021) Comparative investigation of IFN-γ-producing T cells in chickens and turkeys following vaccination and infection with the extracellular parasite *Histomonas meleagridis*. Developmental & Comparative Immunology 116. doi: 10.1016/j.dci.2020.103949.

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Vaccination and Infection of Swine With *Salmonella* Typhimurium Induces a Systemic and Local Multifunctional CD4$^+$ T-Cell Response

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The gram-negative facultative intracellular bacteria *Salmonella* Typhimurium (STM) often leads to subclinical infections in pigs, but can also cause severe enterocolitis in this species. Due to its high zoonotic potential, the pathogen is likewise dangerous for humans. Vaccination with a live attenuated STM strain (Salmoporc) is regarded as an effective method to control STM infections in affected pig herds. However, information on the cellular immune response of swine against STM is still scarce. In this study, we investigated the T-cell immune response in pigs that were vaccinated twice with Salmoporc followed by a challenge infection with a virulent STM strain. Blood- and organ-derived lymphocytes (spleen, tonsils, jejunal and ileocolic lymph nodes, jejunum, ileum) were stimulated *in vitro* with heat-inactivated STM. Subsequently, CD4$^+$ T cells present in these cell preparations were analyzed for the production of IFN-γ, TNF-α, and IL-17A by flow cytometry and Boolean gating. Highest frequencies of STM-specific cytokine-producing CD4$^+$ T cells were found in lamina propria lymphocytes of jejunum and ileum. Significant differences of the relative abundance of cytokine-producing phenotypes between control group and vaccinated + infected animals were detected in most organs, but dominated in gut and lymph node-residing CD4$^+$ T cells. IL-17A producing CD4$^+$ T cells dominated in gut and gut-draining lymph nodes, whereas IFN-γ/TNF-α co-producing CD4$^+$ T cells were present in all locations. Additionally, the majority of cytokine-producing CD4$^+$ T cells had a CD8α$^+$CD27$^-$ phenotype, indicative of a late effector or effector memory stage of differentiation. In summary, we show that *Salmonella*-specific multifunctional CD4$^+$ T cells exist in vaccinated and infected pigs, dominate in the gut and most likely contribute to protective immunity against STM in the pig.

Keywords: interferon-γ, interleukin-17A, pig, *Salmonella* Typhimurium, multifunctional T cells, lamina propria lymphocytes, tumor necrosis factor-α

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INTRODUCTION

Salmonella Typhimurium (STM) is a gram-negative facultative intracellular bacterium that belongs to the family of the *Enterobacteriaceae* and is able to infect a broad range of hosts. Non-typhoidal *Salmonella* serovars such as STM frequently cause food-borne gastroenteritis in humans due to their zoonotic properties and pose a permanent risk for food safety (1, 2). Apart from eggs and egg products, pigs and pork are among the top transmission vectors of STM to humans *via* the food chain (3, 4).

Salmonellosis in the pig can manifest as diarrhea and lethargy in weaned pigs; however, in many cases, pigs are infected subclinically and often become carrier animals with *Salmonella* persisting in tonsils, gut and gut-associated lymphoid tissues (5). In addition to hygiene measures and feed intervention strategies, vaccination is considered an effective tool in controlling *Salmonella* in affected pig farms (6). The live attenuated histidine-adenine auxotrophic vaccine Salmoporc (Ceva Santé Animale, Libourne, France, formerly IDT Biologika GmbH) is commercially available in Europe, and has already been proven to reduce clinical signs, shedding and tissue colonization in pigs in several studies (7–13).

So far, studies covering the immune response against STM in the pig have focused on detecting signs of antibody-mediated immunity. Vaccination of pigs with Salmoporc has been shown to induce *Salmonella*-specific IgM, IgG and IgA antibodies (11, 14). Studies in which pigs were infected with a virulent STM strain observed an increase of *Salmonella*-specific IgM antibodies with IgM-IgG class switch occurring 14 days post infection, as well as the induction of *Salmonella*-specific IgA antibodies (15, 16). In contrast to the humoral immune response, the cell-mediated immune response to STM in swine has not been investigated in detail so far. Existing studies have mainly focused on measuring mRNA expression of cytokines in STM-infected piglets (17–20).

STM infection has been more intensively studied in mice where $CD4^+$ T cells were shown to have a dominant role in primary clearance of infection (21, 22). Especially the development of Th1 cells and their production of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), leading to the activation of macrophages, is required for successful bacterial killing (23–26). Additionally, interleukin-17A (IL-17A) produced by Th17 cells contributes to protection by recruiting neutrophils to the intestine and regulating the expression of epithelial tight junction proteins (27, 28). In fact, *Salmonella*-specific Th1 and Th17 cells have been shown to develop simultaneously in spleen and intestine of orally infected mice (29). More recent studies in mice have also demonstrated the importance of non-circulating tissue-resident memory Th1 cells which are able to react immediately in case of a re-infection (30). In contrast to mice in which STM leads to septicemia, STM colonization in the pig is mainly focused on the intestinal tract. The local cellular immune response mounted in gut and mesenteric lymph nodes as well as the generation of memory T cells within these tissues is therefore of great interest. Although the identification of tissue-resident T cells (Trm) in swine is not yet possible due to a lack of antibodies specific for Trm associated markers, differentiation of $CD4^+$ T cells in the pig can be analyzed by the expression of CD8α and CD27 (31). In this way three major subsets within porcine $CD4^+$ T helper cells can be identified: the subset of $CD8\alpha^-CD27^+$ $CD4^+$ T cells constitutes naïve cells, while the $CD8\alpha^+CD27^+$ and $CD8\alpha^+CD27^-$ subsets represent central and effector memory $CD4^+$ T cells, respectively (32).

Due to the lack of knowledge on T-cell mediated immunity in pigs in response to STM infections, we studied the antigen-specific $CD4^+$ T-cell immune response of swine, both locally and systemically. $CD4^+$ T cells and their production of IFN-γ, TNF-α and IL-17A were analyzed in various organs by intracellular cytokine staining (ICS) after *in vitro* stimulation with *Salmonella* antigen. Our results show the induction of STM-specific multifunctional $CD4^+$ T cells after vaccination and infection with STM that predominantly possessed an effector memory phenotype and dominated in the porcine intestine.

MATERIALS AND METHODS

Animal Vaccination and Infection Experiment

Prior to the study, sows (Large White x Landrace) from a University-owned pig farm in Lower Austria were tested for *Salmonella*-specific antibodies by the IDEXX Swine *Salmonella* Ab Test (IDEXX Europe, Hoofddorp, The Netherlands). The five sows with the lowest sample to positive (S/P) ratios were selected and sixteen four-week-old male castrated pigs (Large White x Landrace x Pietrain) from those sows were included in the study. Piglets at this farm are routinely vaccinated against PCV-2 (Ingelvac CircoFLEX®, Boehringer-Ingelheim, Ingelheim am Rhein, Germany) at three weeks of age and *Mycoplasma hyopneumoniae* (M^+PAC®, MSD Animal Health, Kenilworth, USA) in the first and third week of life. After arrival, the animals were weighed and the data was used to achieve a similar distribution of animals with different body weights in two groups: a control group of four animals (pig #1-4) and a group of twelve animals that was later vaccinated and challenge-infected (pig #5-16, V+I). Animals were housed in two separate rooms on straw bedding for the first six weeks. One week before the infection, all animals were moved into a biosafety level (BSL) 2 facility at the University of Veterinary Medicine Vienna with the control group and the V+I group accommodated in separate compartments of the facility. The *Salmonella*-free status of the piglets was validated by serological testing for *Salmonella*-specific antibodies by the IDEXX Swine *Salmonella* Ab Test (IDEXX Europe) (**Figure 1**). Moreover, fecal samples were collected 9, 7 and 6 days prior to the first vaccination and tested for *Salmonella*. The methodology for this microbiological testing is described below in chapter 2.4. All piglets tested negative for *Salmonella* in the feces at the indicated time points.

Starting at five weeks of age, the V+I group was orally vaccinated twice in a 19-day interval [study days (SD) 0 and 19] with 1 ml of the live attenuated histidine-adenine

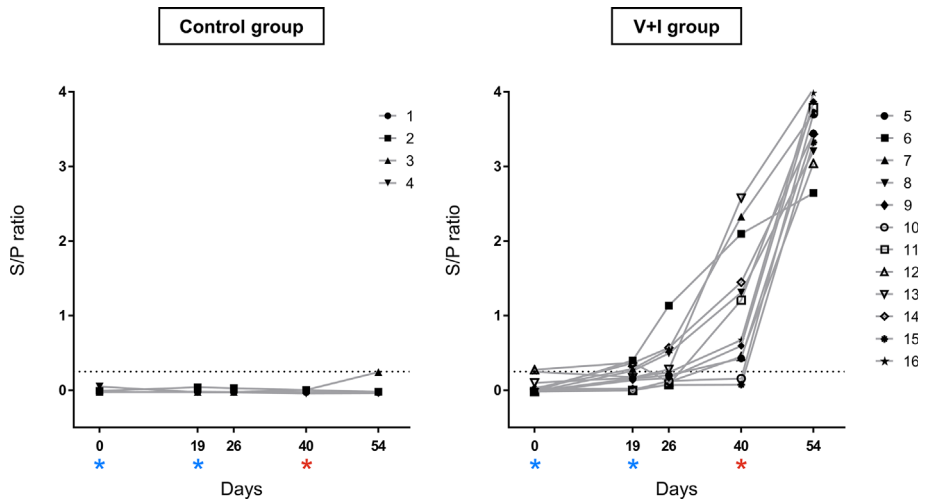

FIGURE 1 | *Salmonella*-specific antibodies in serum over the course of the experiment. *Salmonella*-specific antibodies were measured by ELISA in serum of all animals. Sample to positive (S/P) ratios were calculated by dividing optical densities (OD) of samples by OD of the positive reference control. The dotted line represents the cut-off at a S/P ratio of 0.25. Results < 0.25 were defined as negative, samples ≥ 0.25 as positive. The left graph shows ratios for control animals, the right graph for vaccinated and infected animals. Dates of vaccinations (SD0, SD19) are indicated by blue asterisks, date of infection (SD40) by a red asterisk. The combination of different symbols and numbers represent values of individual animals.

auxotrophic STM vaccine (Salmoporc, Ceva Santé Animale, Dessau-Roßlau, Germany) containing a dose of 1.33 x 10^9 colony forming units (cfu). The control group received 1 ml of tap water orally. The vaccine was applied using a button cannula attached to a 5 ml syringe. Three weeks after the second immunization (SD40), animals of the V+I group were orally challenged with 5 ml per animal containing 1 x 10^9 cfu/ml of a virulent monophasic STM strain (DT193, no. RKI 06-1900, described by (33) and provided by Ceva Innovation Center GmbH). For the application *via* an oral drencher kit (Ceva Santé Animale) the challenge strain was mixed with sugar beet solution to enhance acceptance by the animals. The control group received 5 ml of sugar beet syrup diluted in water. Two weeks after infection, necropsy was performed over five consecutive days (SDs 52–56). All animals were anaesthetized by intramuscular injection of Ketaminhydrochlorid (Narketan®, 10 mg/kg body weight, Vétoquinol, Lure Cedex, France) and Azaperon (Stresnil®, 1.3 mg/kg body weight, Elanco, Greenfield, USA) followed by euthanasia *via* intracardial injection of T61® (tetracaine hydrochloride, mebezonium iodide and embutramide, 1 ml/10 kg body weight, MSD Animal Health). The animal experiment was approved by the institutional ethics committee, the Advisory Committee for Animal Experiments (§12 of Law for Animal experiments, Tierversuchsgesetz - TVG) and the Federal Ministry for Science, Research and Economy (BMWF-68.205/0241-WF/V/3b/2016).

Clinical Examination, Necropsy and Sample Collection

Clinical monitoring of the animals was performed daily and observations were recorded and evaluated by a scoring system, considering rectal temperature, diarrhea, vomiting and changes in behavior. In addition, body weight of all animals was recorded on a weekly basis. After euthanasia inner organs of all animals were evaluated for pathological changes by visual examination. Fecal samples were taken from all animals at the beginning of the study (SDs −9, −7, −6) as well as in two-week intervals after the first vaccination (SDs 12, 26) for bacteriological examination. Blood samples from the jugular vein (*Vena jugularis*) were taken from all animals prior to vaccination (SD0) and prior to second immunization and challenge infection (SDs 19, 26, 40). On necropsy days (SDs 52–56) blood was drawn by cardiac puncture from anaesthetized animals prior to euthanasia. On these days, samples were also collected from liver, spleen, tonsils, jejunal lymph nodes, ileocolic lymph nodes, jejunum, ileum and cecum. Tissue samples from liver, jejunum, ileum and cecum were always taken from the same area of the organ. The liver sample was taken from the dorsal portion of the left lobe. The jejunum was fully unrolled and the middle determined. From this point, 15 cm of tissue was dissected in the oral and aboral direction (i.e. total length of 30 cm). For the sample of the ileum, a 30 cm section orally of the *Ostium ileale* was collected. A ligation 5 cm from the cecal tip provided the sample of the cecum.

Detection of *Salmonella*-Specific Antibodies in Serum

Serum was obtained after centrifugation of blood samples for 10 min and 1,900 × *g* at room temperature. For the detection of *Salmonella*-specific antibodies, serum samples were tested using a commercially available ELISA kit (IDEXX Swine *Salmonella* Ab Test, IDEXX Europe, Hoofddorp, The Netherlands) according to the manufacturer's instructions. Results were recorded as S/P ratios determined by the ratio between mean

optical density (OD) of each sample and mean OD of the positive control. According to the manufacturer's recommendations, S/P ratios < 0.25 were defined as negative and samples ≥ 0.25 as positive.

Microbiological Investigation

Liver, spleen, tonsils, jejunal lymph nodes, ileocolic lymph nodes, jejunum, ileum and cecum from euthanized animals as well as fecal samples taken before and after both immunizations of the animals were investigated for the presence of *Salmonella enterica*. Samples were streaked onto Xylose-Lysine-Deoxycholate (XLD) agar plates (BBL™, Becton Dickinson (BD), Heidelberg, Germany) and incubated in ambient air at 37°C for 48 h. In addition, all samples were pre-enriched in buffered peptone water (BPW, Millipore™, Merck KGaA, Darmstadt, Germany) for 24 h at 37°C. After incubation 0.1 ml of each culture was transferred to Rappaport-Vassiliadis R10 and Selenite broth (both Difco™, BD), incubated for 24 h at 42°C, and subsequently sub-cultured onto XLD agar plates (BBL™, BD) and incubated aerobically at 37°C for 48 h. Presumptive *Salmonella* colonies were confirmed by MALDI TOF mass spectrometry.

Isolation of Lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood sampled in monovettes (Kabe Labortechnik, Nümbrecht-Elsenroth, Germany) by density gradient centrifugation (Pancoll human, density: 1.077 g/ml, PAN Biotech, Aidenbach, Germany; 30 min at 920 x *g*). Lymphocytes from spleen, tonsil and mesenteric lymph nodes were isolated as described previously (31). To collect lamina propria lymphocytes (LPL) from jejunum and ileum, opened intestines were rinsed in sterile phosphate-buffered saline (PBS, PAN Biotech) and dissected into small pieces (approx. 2x2x2 mm). Subsequently, the tissue was incubated for 60 min at 37°C on a shaker in HBSS supplemented with 2 mM DTT (Carl Roth GmbH+Co.KG, Karlsruhe, Germany), 0.1 mM EDTA (Carl Roth) and 25 U/ml DNase Type 1 (ThermoFisher, Waltham, MA, USA) for the release of intra-epithelial lymphocytes. Supernatants from this incubation were discarded and the remaining tissue was placed in cell culture medium (RPMI 1640 supplemented with 100 IU/ml penicillin and 0.1 mg/ml streptomycin (all PAN Biotech)) to remove traces of DTT and EDTA for 15 min at 37°C. Thereafter, the tissue was transferred to cell culture medium supplemented with 25 U/ml DNase Type 1 and 300 U/ml Collagenase 1 (ThermoFisher) for enzymatic degradation. After incubation for 60 min on a shaker at 37°C, supernatants were collected, centrifuged at 4°C and 600 x *g* for 10 min and the cell pellet was re-suspended and filtered through a cell strainer. Cells were re-suspended in 40% Percoll® (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and under-layered with 70% Percoll before being centrifuged at room temperature for 30 min at 920 x *g*. Cells from the interphase were collected, washed twice with PBS and once with cell culture medium (as above but with 5% fetal calf serum (FCS, Merck, Darmstadt, Germany). Afterwards, cells were re-suspended in cell culture medium supplemented with gentamicin and 10% FCS. All cell preparations were counted in a Sysmex XP 300 hematology analyzer (Sysmex Europe GmbH, Norderstedt, Germany).

Preparation of *Salmonella* Antigen for *In Vitro* Stimulation

Antigens for *in vitro* stimulation of lymphocytes were prepared as follows. The vaccine strain (STM no. 421/125) and the challenge strain (STM no. RKI 06-1900) were cultured *via* two precultures in STM 6/83 medium (in-house) at 37°C and 150 rpm on a shaker. The culture was then centrifuged at 7000 x *g* for 10 min and the pellet re-suspended in PBS. After determination of colony forming units, the concentrate was heat-inactivated in a water bath at 60°C for 90 min. Subsequently, the antigen was aliquoted and stored at −80°C until use.

In Vitro Stimulation and ICS

For intracellular staining of IFN-γ, TNF-α and IL-17A, 5 x 10^5 freshly isolated cells were cultivated in 200 µl/well of round-bottomed 96-well microtiter plates (Greiner Bio One, Frickenhausen, Germany) For stimulation, wells received either heat-inactivated 1.9 x 10^8 cfu/ml STM vaccine strain or heat-inactivated 2.7 x 10^8 cfu/ml STM challenge strain. In pilot experiments, we had established that frequencies of cytokine-producing $CD4^+$ T cells plateaued at STM doses >10^8 cfu/ml (data not shown). The plates were then cultured for approximately 19 h at 37°C. Cells incubated in cell culture medium only served as negative controls. During the last 4 h of culture, Brefeldin A (BD GolgiPlug™, BD Biosciences, San Jose, CA, USA) was present in microcultures at a final concentration of 1 µg/ml. Cultivated cells were harvested and re-suspended in buffer containing PBS with 3% FCS. To analyze the phenotype of lymphocyte subsets by flow cytometry (FCM), cells were surface-stained with primary monoclonal antibodies directed to CD4 (mIgG2b, clone: 74-12-4), CD8α (mIgG2a, clone: 11/295/33, biotinylated) and CD27 (mIgG1, clone: b30c7), all produced and prepared in-house. Binding of primary antibodies was detected by the following secondary reagents: goat anti-mouse IgG2b-A488, rat anti-mouse IgG1-PE-Cy7 (both ThermoFisher) and Streptavidin-BV421 (BioLegend, San Diego, CA, USA). For exclusion of dead cells, Fixable Viability Dye eFlour780 (ThermoFisher) was used according to manufacturer's protocol with 0.025 µl reactive dye per sample. Free binding sites of secondary antibodies were blocked with whole mouse IgG molecules (2 µg per sample; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Thereafter, samples were fixed and permeabilized with BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences) according to manufacturer's instructions. This was followed by intracellular staining with IFN-γ-PE (mIgG1, clone: P2G10, BD Biosciences), TNF-α-BV605 (mIgG1, clone: Mab11, BioLegend) and IL-17A-A647 (mIgG1, clone: SCPL1362, BD Biosciences). All incubation steps were performed in 96-well round-bottom plates for 20 min at 4°C with the exception of the intracellular staining step that was carried out for 30 min. FCM analyses were performed on a FACSCanto™ II (BD Biosciences). Data of at least 1 x 10^6 lymphocytes per sample for PBMC,

spleen, tonsils and lymph nodes were recorded. For cells isolated from jejunum and ileum at least 3 x 10^5 lymphocytes were recorded. Data were analyzed with FlowJo™ Software for Windows (Version 10.4.1; FlowJo, Ashland, OR, USA).

Statistical Analysis

Data for the graphs in **Figures 1**, **7**, and **S2** including calculation of mean, median and standard deviations were analyzed with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed in R version 3.6.2 (R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/). We fitted univariate linear mixed models using function *lmer* in R package *lme4* v1.1-21 (34) with log10 transformed frequencies of cytokine-producing $CD4^+$ T cells as "response". Main effects of animal treatment and *in vitro* stimulation and the interaction between them were modeled as fixed categorical effects with two levels (control versus infected) and three levels (medium, vaccine strain and challenge strain), respectively. A random intercept pig effect was included in the model to account for the covariance structure (multiple observations per pig) in our data. For the hypothesis testing, we used maximum likelihood estimation by setting option REML to false. All assumptions for linear mixed models were met. Residuals and random intercepts were normally distributed and residuals homoscedastic. We verified the absence of collinearity *via* generalized variance inflation factors (35) using function *vif* in package *car* v3.0-8 (36). We calculated contrasts between least square means of animal treatment and *in vitro* stimulation levels respectively with package *emmeans* v1.4.7 (37). Significance was declared at a multiple testing corrected 10% false discovery rate (38). We further performed Principal Component Analysis with package *factoextra* v1.0.7 (39). We produced biplots using function *fviz_pca_biplot*, which display PCA scores of samples (shown as dots) and loadings of each variable (shown as vectors) in the same graph. Dots that are close to each other represent samples with similar values. The longer a vector of a variable the bigger the influence of this variable on that principal component. Vectors pointing in a similar direction, forming small angles between them can be interpreted as positively correlated, vectors forming an angle of 90° as uncorrelated and vectors pointing in opposing directions as negatively correlated. We also produced heatmaps with function *heatmap.2* in package *gplots* v3.0.3. (40). Hierarchical clustering using option *method= "ward.D2"*, based on Euclidian distances for rows and columns are shown as dendrograms. For these multivariate descriptive plots, centered and scaled frequencies of cytokine-producing $CD4^+$ T cells corrected for individual pig effects were used. We calculated these residuals by subtracting restricted maximum likelihood BLUP animal effects, (option REML set to true), estimated from the same linear mixed model as used for hypothesis testing, from the log10 transformed raw frequencies of cytokine-producing $CD4^+$ T cells. During statistical analysis, one animal from the control group (pig #4) was found to be an outlier in the majority of analyzed tissues and was therefore excluded from the ICS data set and subsequent multivariate plots as well as hypothesis testing of cytokine-producing phenotypes for all tissues.

RESULTS

Clinical Signs, Serology, and Microbiological Investigation

Due to a lack of knowledge on the T-cell response of pigs against STM, the animal experiment for this study was designed to achieve a strong stimulation of the immune system of pigs in the V+I group by a two-time vaccination and subsequent challenge infection, based on previous observations in demonstrating the efficacy of the Salmoporc vaccine (11). With this study design, no adverse effects on the health of the pigs following infection were expected. Indeed, average daily gain of weight developed homogeneously for both groups for the duration of the study. After moving to the BSL2 animal facility, both groups showed an increase in rectal temperature for one day with temperatures ranging between 39.3°C and 40.4°C and an average increase of 0.35°C in the control group and 0.2°C in the V+I group. This rise in temperature can most likely be attributed to the stress of being moved and adapting to a new environment. After that, all animals showed rectal temperatures within a physiologic range until the end of the study. In addition, mild signs of diarrhea (e.g. pasty feces) were observed in a small number of V+I animals during a three-day interval after challenge infection (data not shown).

To confirm a successful STM infection of the animals, production of serum antibodies as well as STM presence in organs were investigated. The humoral response against STM was evaluated by use of the commercially available IDEXX Swine *Salmonella* Ab Test (**Figure 1**). Results of the *Salmonella* antibody ELISA confirmed the *Salmonella*-free status of the animals at the beginning of the study (SD0). All pigs from the control group remained serologically negative for *Salmonella* sp. throughout the whole course of the experiment. S/P ratios of animals from the V+I group rose moderately after the second immunization and a further substantial increase was observed after challenge infection on SD40.

Samples from spleen, liver, tonsils, jejunal lymph nodes, ileocolic lymph nodes, jejunum, ileum and cecum taken at necropsy for each animal were analyzed for the presence of *Salmonella enterica*. No *Salmonella* spp. could be detected in the control group in any of the sampled organs (**Figure 2**). Regarding the V+I group, *Salmonella* spp. could be isolated from all three selected parts of the gut, the jejunal lymph nodes and the ileocolic lymph nodes from at least two animals. Additionally, *Salmonella* spp. were also identified in the tonsils of all V+I animals. Spleen and liver were negative for both control group and V+I group. As the pigs were kept under isolated conditions and the vaccine strain has a limited persistence, it can be assumed that the detection of *Salmonella* spp. is the STM challenge strain. Fecal samples collected prior to (SDs -9, -7, -6) and after both vaccinations (SDs 12, 26) of all animals (control

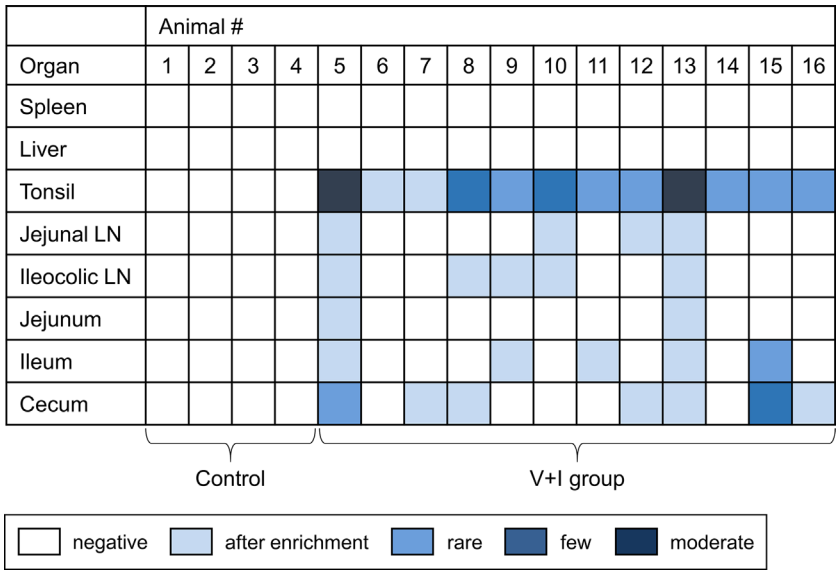

FIGURE 2 | Detection of *Salmonella* Typhimurium on the day of necropsy. Microbiological analysis was performed for spleen, liver, tonsil, jejunal lymph node, ileocolic lymph node, jejunum, ileum and cecum. Results are displayed as a heat map: dark blue/blue boxes indicate detection of *Salmonella* Typhimurium by agar isolation. Light blue boxes indicate isolation after enrichment. White boxes display negative findings for *Salmonella* Typhimurium.

and V+I group) tested negative for *Salmonella* spp. as well (data not shown).

Production of IFN-γ, TNF-α, and/or IL-17A by CD4+ T Cells After STM Stimulation

Main focus of the study was the analysis of a *Salmonella*-specific $CD4^+$ T-cell response in regard to IFN-γ, TNF-α, and IL-17A production. To that end, PBMC as well as lymphocytes isolated from spleen, tonsils, jejunal lymph nodes, ileocolic lymph nodes, jejunum and ileum collected on final SDs were stimulated *in vitro* with either the vaccine strain or the challenge infection strain. Cells cultivated in medium-only served as negative controls. For analysis of cytokine-producing cells and their phenotype, multicolor FCM was performed. To identify *Salmonella*-specific $CD4^+$ T cells, $CD4^+$ cells were gated within live lymphocytes and further analyzed for IFN-γ, TNF-α and IL-17A production (**Figure S1A**). Subsequently, Boolean gating was applied, resulting in seven possible cytokine-producing phenotypes: IFN-γ single-producing, TNF-α single-producing, IL-17A single-producing, IFN-γ/TNF-α co-producing, IFN-γ/IL-17A co-producing, TNF-α/IL-17A co-producing and IFN-γ/TNF-α/IL-17A triple-producing cells. Raw frequencies of these cytokine-producing phenotypes for all organs and *in vitro* stimulations are listed in **Table S1**.

The frequencies of cytokine-producing $CD4^+$ T cells varied between different animals and organs following stimulation with STM (**Figures S1B–D**). Comparing frequencies of cytokine-producing $CD4^+$ T cells across organs, highest frequencies of *Salmonella*-specific $CD4^+$ T cells for all cytokine-related phenotypes could be observed in LPL preparations of jejunum and ileum (**Figure S2**, N.B. the scaling of the y-axes in this figure varies between jejunum/ileum, lymphoid organs and blood/spleen).

To work out the significance of noted differences between control group and V+I animals, we chose to model the proportions of cytokine-producing $CD4^+$ T cells for all phenotypes, animals and organs in a generalized linear mixed model. All samples from control animals as well as non-stimulated samples from V+I animals that were cultivated in medium-only served as reference values. The effect of each pig as an individual strongly influenced the data and therefore residuals were calculated for each sample to minimize the effect of the factor 'animal'. Principal component analysis (PCA) and heat map analysis were carried out on residuals of cytokine-producing $CD4^+$ T cells. With the exception of IFN-γ single-producing $CD4^+$ T cells in blood, no significant differences in frequency of cytokine-producing $CD4^+$ T cells were found between stimulation with the vaccine strain and the challenge infection strain (**Table S2**). Furthermore, no separate clustering could be observed between both stimulation variants in PCA and heat map analysis (data not shown). For these reasons, data from both stimulation variants were combined for all subsequent analyses.

Cluster Analysis for STM-Stimulated IFN-γ/TNF-α/IL-17A Producing CD4+ T Cells

STM primarily infects the gastrointestinal tract and does so by breaching the gut epithelial barrier. To examine the local T-cell immune response at this site of invasion, cluster analysis was first conducted for the two intestinal sections (jejunum, ileum, **Figure 3**). Dimension 1 of the PCA revealed a clear separation of the gut tissue-derived data points into two fractions: Samples from V+I animals stimulated with STM clustered together (orange dots) on one side. On the opposite side, all samples from control animals (blue dots if stimulated with STM antigen, green dots if cultivated in medium) as well as V+I samples cultivated in medium-only

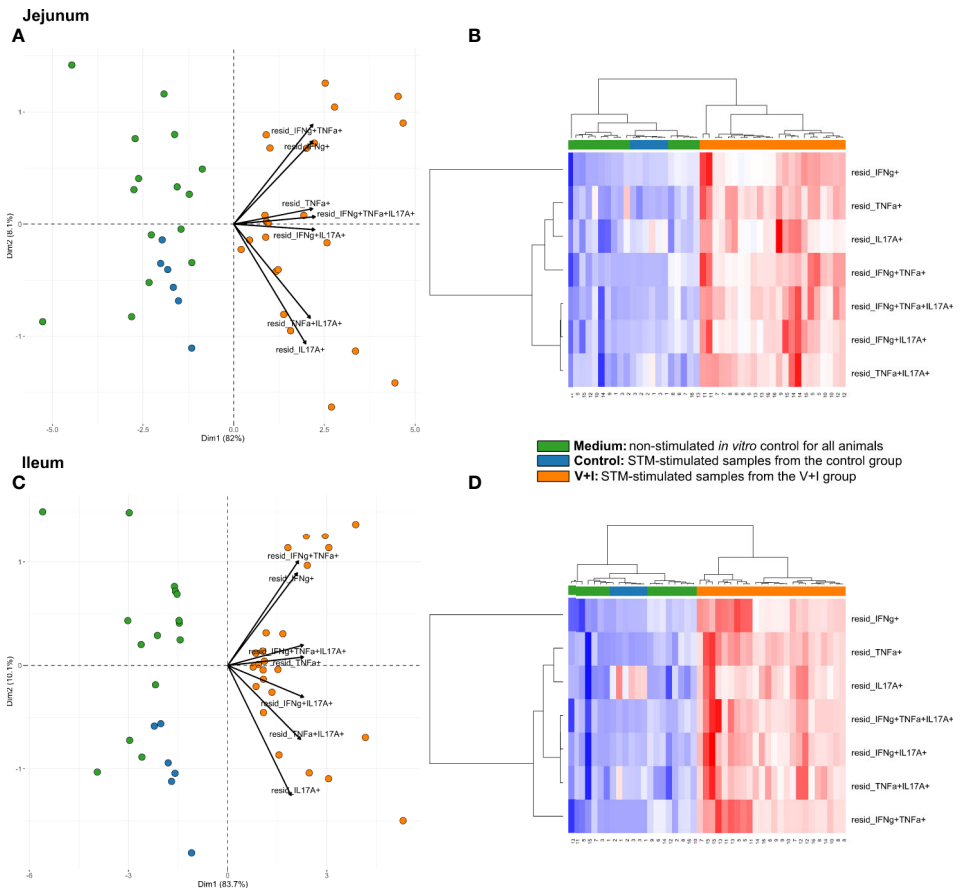

FIGURE 3 | Unsupervised cluster analysis for *Salmonella* Typhimurium (STM)-stimulated interferon-γ (IFN-γ)/tumor necrosis factor-α (TNF-α)/interleukin-17A (IL-17A) producing CD4+ T cells isolated from jejunum and ileum. **(A)** Principal component analysis (PCA) based on residuals calculated from cytokine-producing CD4+ T cells derived from jejunum and stimulated *in vitro* with STM or medium-only. Each colored circle represents one of three samples derived from one animal. Samples stimulated with STM (vaccine or challenge strain) are depicted in orange for V+I animals and blue for control animals; samples from both groups cultivated in medium-only are shown in green. Arrows depict cytokine-producing phenotypes. **(B)** Heat map of residuals calculated from cytokine-producing CD4+ T cells derived from jejunum and stimulated *in vitro* with STM or medium-only. Heat maps, generated from data converted to Z-scores, show increase (red) or decrease (blue) of relative abundance of cytokine-producing CD4+ T cells. Each row represents a cytokine-producing phenotype and each column a sample. Numbers below columns indicate numbers of the individual animals. Samples stimulated with STM (vaccine or challenge strain) are indicated by orange boxes for V+I animals and blue boxes for control animals; samples from both groups cultivated in medium-only are indicated by green boxes. **(C, D)** Same as **(A)**, **(B)** but for cytokine-producing CD4+ T cells derived from the ileum.

(remaining green dots) formed a cluster (**Figures 3A, C**). Hence, Dimension 1 resulted out of the animal treatment (control vs. vaccination and infection) as well as stimulation of the individual samples (medium vs. STM antigen). For both intestinal sections, Dimension 1 accounted for more than 80% of the variation. Dimension 2, which accounted for 8.1% and 10.1% of variation in jejunum and ileum, respectively, is associated with the type of cytokine production. Predominantly IFN-γ single- and IFN-γ/TNF-α co-producing cells clustered together in the upper quadrant while IL-17A containing phenotypes were grouped toward the other direction. Findings of the PCA were corroborated by results of the heat map analysis (**Figures 3B, D**). Heat maps show increases (red) and decreases (blue) of relative abundance of cytokine-producing CD4+ T cells. Relative abundances of phenotypes were markedly increased in samples from the V+I group that were stimulated with STM when comparing it to samples from control animals and medium-only samples. This resulted in two distinct clusters where STM-stimulated V+I samples are clearly separated from samples of control animals and samples from all animals that were cultivated in medium. Again similar to PCA results, hierarchical clustering of cytokine phenotypes in dendrograms showed that IL-17A+ phenotypes such as IFN-γ/TNF-α/IL-17A triple-producing, IFN-γ/IL-17A co-producing and TNF-α/IL-17A co-producing CD4+ T cells are closely related phenotypes while IFN-γ/TNF-α co-producing and IFN-γ single-producing CD4+ T cells clustered apart from them. To determine whether the observed differences between the control and the V+I group were significant, *p*-values were calculated for all phenotypes and organs and corrected for multiple testing where necessary (*p*-values below 0.1 were considered significant). Significant differences between control group and V+I group were found for

5 out of 7 phenotypes in both intestinal sections (**Table 1**). The phenotypes not reaching significant difference between groups were IL-17A single-producing $CD4^+$ T cells in both intestinal tissues as well as IFN-γ single-producing $CD4^+$ T cells in the jejunum and TNF-α/IL-17A co-producing $CD4^+$ T cells in the ileum.

Looking at the set of lymphatic organs, clustering in both intestinal lymph nodes was similar to that observed in jejunum and ileum, for both PCA and heat maps (**Figures 4A–D**). In both mesenteric lymph nodes, STM-stimulated V+I samples clustered away from control and medium-only samples with Dimension 1 accounting for approximately 70% of the variation (**Figures 4A, C**). Of note, IFN-γ single-producing $CD4^+$ T cells clustered separately from all other phenotypes in the PCA. This is most probably due to an increase in abundance of cytokine-producing cells in some control animals for this phenotype as displayed in the corresponding heat maps (**Figures 4B, D**). Accordingly, differences in relative abundance of IFN-γ single-producers between groups did not reach significance for either lymph node (**Table 2**). Comparable to observations in the intestine, phenotypes that included IL-17A also formed closely related clusters in both lymph nodes as illustrated by heat map dendrograms. *P*-values for both lymph nodes revealed significant differences between control conditions and STM-stimulated samples from V+I animals for TNF-α single-producing, IFN-γ/TNF-α co-producing and TNF-α/IL-17A co-producing $CD4^+$ T cells. Additional significant differences were reached for IFN-γ/IL-17A co-producing and IFN-γ/TNF-α/IL-17A triple-producing $CD4^+$ T cells in ileocolic lymph nodes. In the tonsil, however, separation of STM-stimulated V+I samples from control animals and medium-only samples was less distinct (**Figures 4E, F**). Correspondingly, no significant differences in abundance of cytokine-producing phenotypes could be detected between groups in the tonsil (**Table 2**).

Cluster analysis for STM-stimulated cytokine-producing $CD4^+$ T cells was also performed for blood and spleen to gain more insight into the systemic $CD4^+$ T-cell immune response against STM (**Figure 5**). With a few exceptions, control and medium-only samples largely clustered away from STM-stimulated V+I samples and Dimension 1 accounted for around 50% of the variation in blood and spleen (**Figures 5A, C**, respectively). In contrast to analyzed gut tissue and lymph nodes, relative abundances of cytokine-producing $CD4^+$ T cells scattered across animal groups and the type of *in vitro* stimulation (medium versus STM antigen) (**Figures 5B, D**). Nonetheless, significant differences between control and V+I group could be detected for IFN-γ/TNF-α co-producing $CD4^+$ T cells in blood and spleen, with additional significant differences for IFN-γ/IL-17A and IFN-γ/TNF-α/IL-17A co-producing $CD4^+$ T cells in the blood (**Table 3**). Of note, IFN-γ/TNF-α co-producing $CD4^+$ T cells constituted the only phenotype which was significantly different between control and V+I animals in all organs, with the exception of the tonsil.

Cluster Analysis of *Salmonella*-Specific Cytokine-Producing $CD4^+$ T Cells Across Organs and Phenotypes

Due to marked differences between organs in terms of both frequencies of cytokine-producing $CD4^+$ T cells and the type of cytokine production, we carried out a cluster analysis for STM-stimulated samples from V+I animals that encompassed all seven organs. Here, the formation of three clusters by PCA could be observed (**Figure 6**). Blood and spleen as the two systemic organs formed one cluster with tonsil and lymph nodes forming another tight cluster of lymphatic origin on the same side, while all samples from the gut tissue clustered separately. As can be seen from the raw data (**Figure S2**), the abundances of *Salmonella*-specific cytokine-producing $CD4^+$ T cells in jejunum and ileum highly surmounted those detected in the other organs as indicated by the arrows for all cytokine-producing phenotypes pointing toward the cluster of gut tissue samples (**Figure 6**). Accordingly, Dimension 1 accounted for 88.7% variability in the data set. Of note, Dimension 2 accounted only for 5.2% of the

TABLE 1 | Calculation of contrasts of *Salmonella*-specific $CD4^+$ T-cell cytokine responses between control and V+I pigs in jejunum and ileum.

Tissue	Phenotype	Estimate[a]	SE[b]	t-ratio[c]	*p*-value[d]	fdr.p[e]
Jejunum	IFN-γ^+	−1.942235	0.946641	−2.051713	0.052421	0.102649
	TNF-α^+	−0.349227	0.109627	−3.185597	0.004483	0.048073
	IL-17A$^+$	−0.194306	0.162531	−1.195501	0.244622	0.342471
	IFN-γ^+TNF-α^+	−0.696878	0.262535	−2.654421	0.014868	0.051243
	IFN-γ^+IL-17A$^+$	−0.107349	0.045450	−2.361912	0.027440	0.074697
	TNF-α^+IL-17A$^+$	−0.087403	0.040816	−2.141406	0.043547	0.096991
	IFN-γ^+TNF-α^+IL-17A$^+$	−0.116906	0.039699	−2.944789	0.007783	0.048073
Ileum	IFN-γ^+	−1.722149	0.661531	−2.603277	0.016732	0.051243
	TNF-α^+	−0.318299	0.105505	−3.016924	0.006867	0.048073
	IL-17A$^+$	−0.030348	0.090255	−0.336242	0.739993	0.771482
	IFN-γ^+TNF-α^+	−0.608607	0.231357	−2.630603	0.016001	0.051243
	IFN-γ^+IL-17A$^+$	−0.132379	0.045710	−2.896093	0.008366	0.048073
	TNF-α^+IL-17A$^+$	−0.079415	0.040456	−1.963030	0.062708	0.113803
	IFN-γ^+TNF-α^+IL-17A$^+$	−0.144465	0.047416	−3.046742	0.005979	0.048073

[a]*estimate: contrasts between least square means of control versus V+I pigs.*
[b]*SE: standard errors of the estimated contrasts.*
[c]*t-ratio: t test statistics.*
[d]*p-value: nominal p-values.*
[e]*fdr.p: p-values were corrected for multiple testing via false discovery rate. p-values < 0.1 were considered significant and are highlighted in gray.*

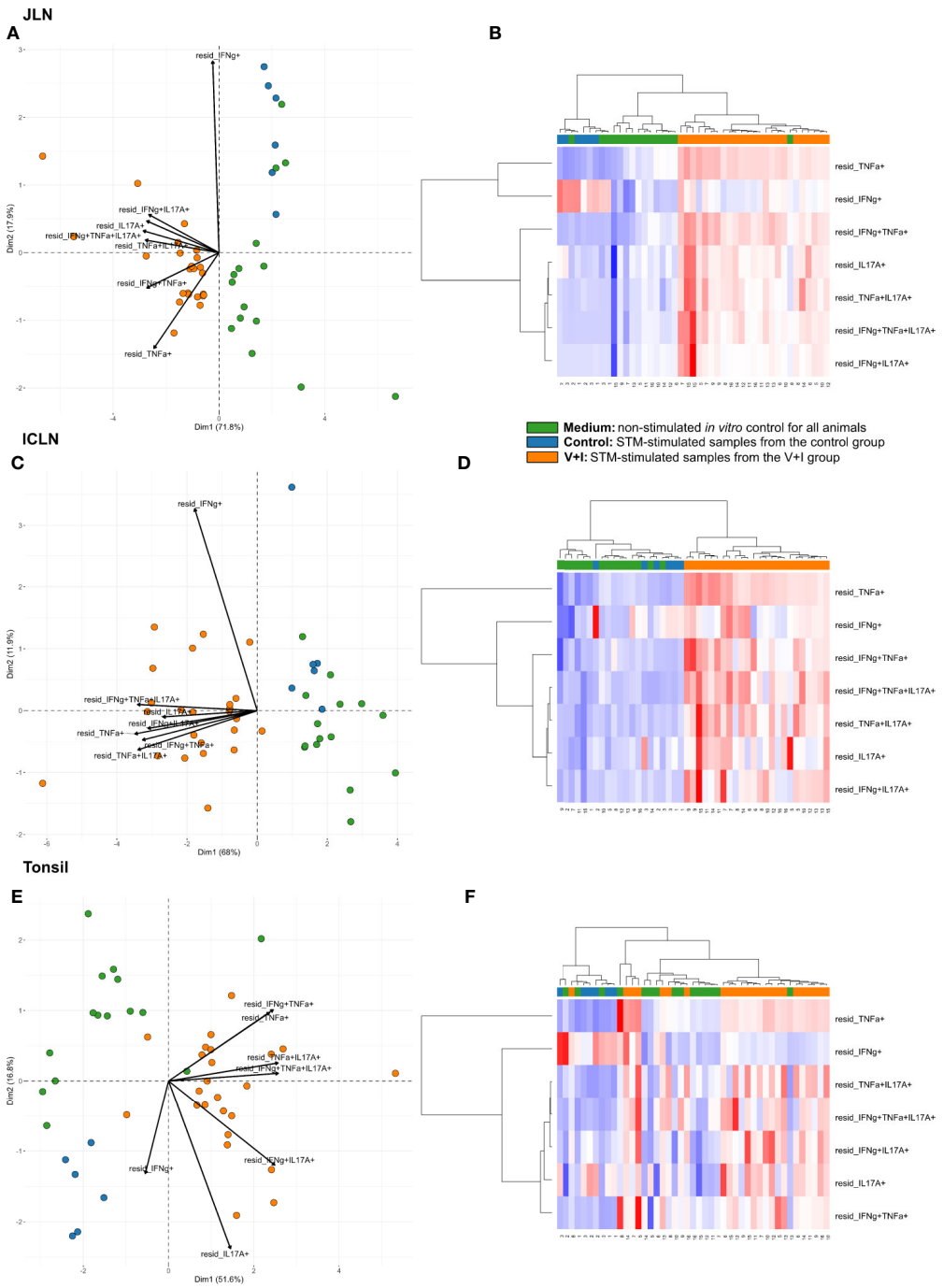

FIGURE 4 | Unsupervised cluster analysis for *Salmonella* Typhimurium (STM)-stimulated interferon-γ (IFN-γ)/tumor necrosis factor-α (TNF-α)/interleukin-17A (IL-17A) producing CD4+ T cells isolated from jejunal lymph node (JLN), ileocolic lymph node (ICLN) and tonsil. **(A)** Principal component analysis (PCA) based on residuals calculated from cytokine-producing CD4+ T cells derived from jejunal lymph node and stimulated *in vitro* with STM or medium-only. Each colored circle represents one of three samples derived from one animal. Samples stimulated with STM (vaccine or challenge strain) are depicted in orange for V+I animals and blue for control animals; samples from both groups cultivated in medium-only are shown in green. Arrows depict cytokine-producing phenotypes. **(B)** Heat map of residuals calculated from cytokine-producing CD4+ T cells derived from jejunal lymph node and stimulated *in vitro* with STM or medium-only. Heat maps, generated from data converted to Z-scores, show increase (red) or decrease (blue) of relative abundance of cytokine-producing CD4+ T cells. Each row represents a cytokine-producing phenotype and each column a sample. Numbers below columns indicate numbers of the individual animals. Samples stimulated with STM (vaccine or challenge strain) are indicated by orange boxes for V+I animals and blue boxes for control animals; samples from both groups cultivated in medium-only are indicated by green boxes. **(C–F)** Same as **(A)**, **(B)** but for CD4+ T cells derived from ileocolic lymph node **(C, D)** and tonsil **(E, F)**.

TABLE 2 | Calculation of contrasts of *Salmonella*-specific CD4+ T-cell cytokine responses between control and V+I pigs in jejunal lymph node (JLN), ileocolic lymph node (ICLN) and tonsil.

Tissue	Phenotype	Estimate[1]	SE[2]	t-ratio[3]	*p*-value[4]	fdr.p[5]
JLN	IFN-γ+	0.067079	0.072485	0.925421	0.367107	0.473375
	TNF-α+	−0.127268	0.047231	−2.694616	0.014723	0.051243
	IL-17A+	−0.008054	0.010468	−0.769364	0.451359	0.552915
	IFN-γ+TNF-α +	−0.048463	0.019869	−2.439113	0.024844	0.071611
	IFN-γ+IL-17A+	−0.004625	0.003549	−1.303352	0.205662	0.305376
	TNF-α+IL-17A+	−0.012488	0.005579	−2.238111	0.035507	0.084510
	IFN-γ+TNF-α+IL-17A+	−0.016620	0.008079	−2.057324	0.051583	0.102649
ICLN	IFN-γ+	0.005701	0.015205	0.374958	0.711656	0.758068
	TNF-α+	−0.057656	0.020898	−2.758968	0.012239	0.051243
	IL-17A+	−0.001802	0.001700	−1.060605	0.298201	0.394914
	IFN-γ+TNF-α +	−0.013547	0.004851	−2.792814	0.010159	0.049781
	IFN-γ+IL-17A+	−0.001434	0.000554	−2.585914	0.015449	0.051243
	TNF-α+IL-17A+	−0.003867	0.001238	−3.124297	0.004408	0.048073
	IFN-γ+TNF-α+IL-17A+	−0.004843	0.001243	−3.897945	0.000705	0.034531
Tonsil	IFN-γ+	0.034242	0.020767	1.648844	0.113169	0.178880
	TNF-α+	−0.102940	0.050402	−2.042367	0.054467	0.102649
	IL-17A+	0.001278	0.005386	0.237207	0.814933	0.831910
	IFN-γ+TNF-α +	−0.014022	0.007906	−1.773586	0.089954	0.157420
	IFN-γ+IL-17A+	−0.003559	0.002034	−1.749303	0.094375	0.159461
	TNF-α+IL-17A+	−0.004241	0.003743	−1.133177	0.272138	0.370410
	IFN-γ+TNF-α+IL-17A+	−0.004049	0.002521	−1.606028	0.124307	0.190346

[1]*estimate: contrasts between least square means of control versus V+I pigs.*
[2]*SE: standard errors of the estimated contrasts.*
[3]*t-ratio: t test statistics.*
[4]*p-value: nominal p-values.*
[5]*fdr.p: p-values were corrected for multiple testing via false discovery rate. p-values < 0.1 were considered significant and are highlighted in gray.*

variability, but still gave a clear separation of blood and spleen versus the lymphatic organs. Combining this with the cytokine production phenotypes, where IFN-γ-single producers and IFN-γ/TNF-α co-producing cells separated from the other phenotypes, this indicates that IL-17A-producing phenotypes are less prominent in the systemic immune response found in blood and spleen.

Clustering among cytokine-producing phenotypes as seen in heat map dendrograms (**Figures 3**, **4**, and **5B, C**) also featured such a grouping. It could be observed across organs that IL-17A-containing phenotypes formed closely related clusters whereas IFN-γ single-, TNF-α single- and IFN-γ/TNF-α co-producing CD4+ T cells largely clustered separately. To study this from an alternate point of view, heat maps were calculated individually for all cytokine-producing phenotypes (**Figure S3**). For the majority of investigated phenotypes, distinct clusters emerged for STM-stimulated V+I samples on the one hand and control and medium-only samples on the other hand. Matching with observations from **Figure 6**, the dendrograms showed that organs predominantly form clusters according to their respective origins from systemic, lymphatic or gut tissue.

Expression of CD8α and CD27 on *Salmonella*-Specific Cytokine-Producing CD4+ T Cells

It has been shown previously that porcine CD4+ T cells can be differentiated into naïve CD4+ T cells with a CD8α-CD27+ phenotype, whereas CD8α+CD27+ and CD8α+CD27- CD4+ T cells constitute central memory (Tcm) and effector memory (Tem) subsets, respectively (32). We therefore analyzed CD8α alongside CD27 expression for all seven cytokine-producing phenotypes in all organs (**Figure 7**). The gating strategy for the identification of these phenotypes is shown in **Figure S4**. Representative raw data for the CD8α/CD27 expression of cytokine-producing CD4+ T cells following STM-stimulation is shown in **Figure S5**. As illustrated in **Figure 7**, hardly any *Salmonella*-specific cytokine-producing CD4+ T cells with the hitherto uncharacterized CD8α-CD27- phenotype were found. Also, CD8α-CD27+ naïve T cells showed only a low capacity for cytokine production with some exceptions such as for single TNF-α and IL-17A-producers in the blood. Instead, *Salmonella*-specific cytokine-producing CD4+ T cells predominantly had a CD8α+CD27- effector memory phenotype, especially in the gut tissue. While a CD8α+CD27+ central memory population was present primarily for IFN-γ/TNF-α double- and IFN-γ/TNF-α/IL-17A triple-producing CD4+ T cells within analyzed non-gut locations, cytokine phenotypes present in jejunum and ileum were almost exclusively effector memory T cells.

DISCUSSION

Information on the porcine T-cell response against STM is still scarce. For the most part, previous studies have focused on detecting local and systemic immune responses on mRNA level and discovered that STM infection in the pig causes upregulation of mRNA expression of cytokines like IFN-γ, TNF-α, and IL-1β (16–20). However, the phenotype of the cells producing these mRNA transcripts was not investigated. Only one study also measured the expression of transcription factors T-bet, Gata-3

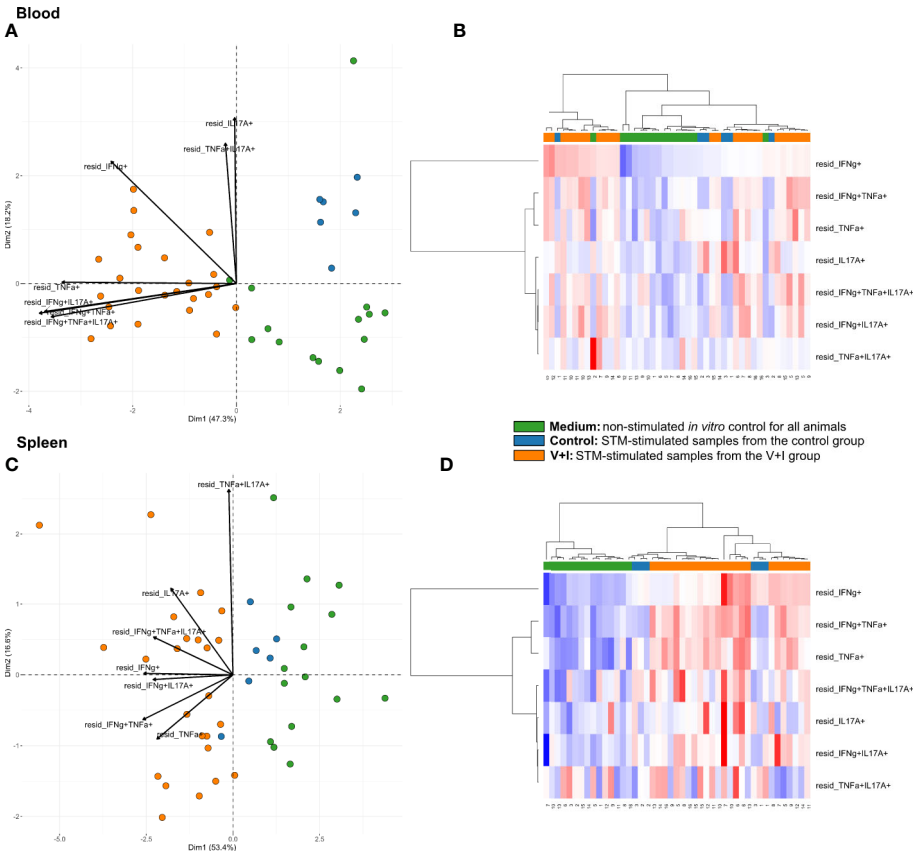

FIGURE 5 | Unsupervised cluster analysis for *Salmonella* Typhimurium (STM)-stimulated interferon-γ (IFN-γ)/tumor necrosis factor-α (TNF-α)/interleukin-17A (IL-17A) producing CD4+ T cells isolated from blood and spleen. **(A)** Principal component analysis (PCA) based on residuals calculated from cytokine-producing CD4+ T cells derived from blood and stimulated *in vitro* with STM or medium-only. Each colored circle represents one of three samples derived from one animal. Samples stimulated with STM (vaccine or challenge strain) are depicted in orange for V+I animals and blue for control animals; samples from both groups cultivated in medium-only are shown in green. Arrows depict cytokine-producing phenotypes. **(B)** Heat map of residuals calculated from cytokine-producing CD4+ T cells derived from blood and stimulated *in vitro* with STM or medium-only. Heat maps, generated from data converted to Z-scores, show increase (red) or decrease (blue) of relative abundance of cytokine-producing CD4+ T cells. Each row represents a cytokine-producing phenotype and each column a sample. Numbers below columns indicate numbers of the individual animals. Samples stimulated with STM (vaccine or challenge strain) are indicated by orange boxes for V+I animals and blue boxes for control animals; samples from both groups cultivated in medium-only are indicated by green boxes. **(C, D)** Same as **(A)**, **(B)** but for cytokine-producing CD4+ T cells derived from the spleen.

and Foxp3 in CD4+ T cells following STM infection (41) but the results were not fully conclusive. Differently, in the murine model CD4+ T cells are widely recognized as the main immune cell subset responsible for protection against STM (21, 22). Hence, we decided to lay the focus of our study on CD4+ T cells.

Considering also the lack of knowledge on the magnitude of the T-cell response in swine to STM, we decided to stimulate the immune system by a double vaccination with Salmoporc and subsequent challenge infection with a virulent STM strain. We chose this regimen because it was also used in the past to demonstrate the efficacy of the vaccine. These studies showed that, in comparison to two vaccinations or single infection, STM-specific IgG titers in blood were highest after this combination of two vaccinations and subsequent challenge infection (11). Hence, we assumed that this might also indicate an activation of CD4+ T cells at the systemic level. We further reasoned that this would probably coincide with activation of T cells in the gut mucosa, since the vaccine and the challenge infection were applied orally.

Of the seven organs analyzed in this study, the highest colonization with STM was found in the tonsils. This is not surprising as the tonsil is known to be invaded by various bacteria, with STM frequently establishing a persistent infection in this location (42, 43). Of note, the tonsil was the tissue with the lowest frequencies of cytokine-producing CD4+ T cells and no significant differences for any cytokine phenotype were found between V+I animals and the control conditions. One possible explanation might be the location of the pathogen within the organ. Unlike jejunum and ileum where STM invades the epithelial cells, it mainly resides extracellularly in porcine tonsils (44), thus making it more difficult for the immune system to attack the pathogen. Studies in recent years also present evidence that bacterial gene expression related to survival and persistence of *Salmonella* in the tonsil is vastly different from

TABLE 3 | Calculation of contrasts of *Salmonella*-specific CD4+ T-cell cytokine responses between control and V+I pigs in blood and spleen.

Tissue	Phenotype	Estimate[1]	SE[2]	t-ratio[3]	*p*-value[4]	fdr.p[5]
Blood	IFN-γ+	−0.140874	0.310025	−0.454395	0.654487	0.712664
	TNF-α+	−0.018921	0.015141	−1.249649	0.225714	0.325293
	IL-17A+	0.014726	0.007054	2.087703	0.048858	0.102649
	IFN-γ+TNF-α +	−0.090341	0.038238	−2.362602	0.029046	0.074908
	IFN-γ+IL-17A+	−0.004515	0.001471	−3.069605	0.005911	0.048073
	TNF-α+IL-17A+	−0.000276	0.000590	−0.467837	0.643481	0.712664
	IFN-γ+TNF-α+IL-17A+	−0.003946	0.001757	−2.246654	0.036219	0.084510
Spleen	IFN-γ+	−0.156988	0.264683	−0.593117	0.559227	0.651342
	TNF-α+	−0.032464	0.019558	−1.659894	0.111782	0.178880
	IL-17A+	−0.002837	0.004332	−0.654929	0.519200	0.620507
	IFN-γ+TNF-α +	−0.129475	0.044655	−2.899429	0.008830	0.048073
	IFN-γ+IL-17A+	−0.002962	0.003282	−0.902414	0.376942	0.473594
	TNF-α+IL-17A+	0.000047	0.000795	0.059229	0.953292	0.953292
	IFN-γ+TNF-α+IL-17A+	−0.002789	0.004846	−0.575497	0.571586	0.651342

[1]*estimate: contrasts between least square means of control versus V+I pigs.*
[2]*SE: standard errors of the estimated contrasts.*
[3]*t-ratio: t test statistics.*
[4]*p-value: nominal p-values.*
[5]*fdr.p: p-values were corrected for multiple testing via false discovery rate. p-values < 0.1 were considered significant and are highlighted in gray.*

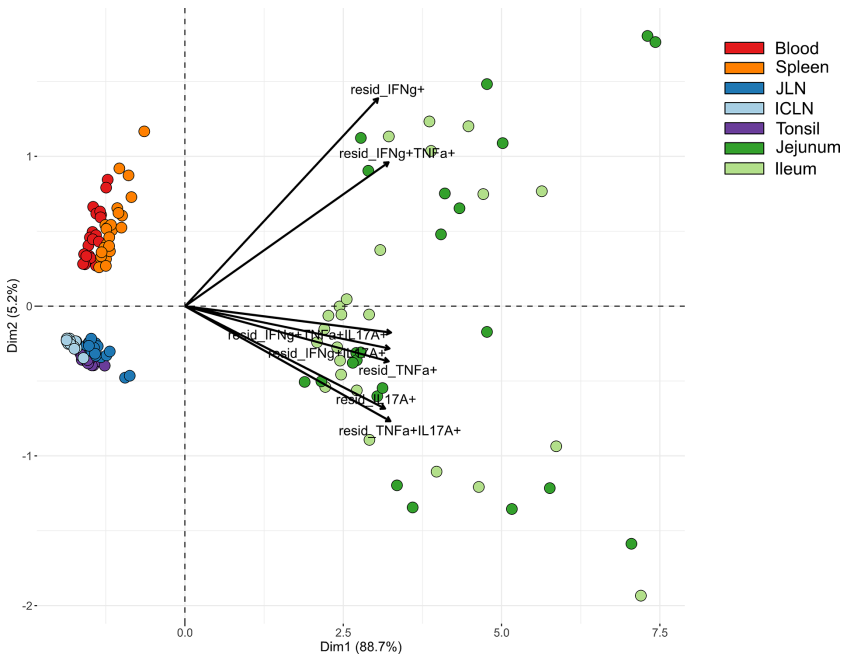

FIGURE 6 | Principal component analysis (PCA) for STM-stimulated interferon-γ (IFN-γ)/tumor necrosis factor-α (TNF-α)/interleukin-17A (IL-17A) producing CD4+ T cells isolated from various organs. PCA based on residuals calculated from cytokine-producing CD4+ T cells derived from jejunum, ileum, jejunal lymph node (JLN), ileocolic lymph node (ICLN), tonsil, blood and spleen after *in vitro* stimulation with STM. Each colored circle represents one of two samples from one animal (stimulation with either vaccine or challenge strain). Samples are colored according to the organ of origin (blood: red, spleen: orange, JLN: blue, ICLN: light blue, tonsil: purple, jejunum: green, ileum: light green). Arrows depict cytokine-producing phenotypes.

gene expression related to these mechanisms in the gut and gut-associated lymph nodes (45–47). Another factor may be the anti-inflammatory nature of the tonsil. In humans, tonsil-derived dendritic cells only induce a weak T-cell response to mucosally encountered pathogens but rather maintain immunotolerance (48). This observation fits with studies in the pig where the presence of *Actinobacillus pleuropneumoniae* in the tonsil induced an increase in IL-10 expression (49). Although IL-10 production was not measured in our study, a similar scenario is conceivable which could explain the poor induction of Th1 and Th17 responses in this organ.

Surpassing all other organs, we found the highest frequencies of cytokine-producing *Salmonella*-specific CD4+ T cells within LPLs isolated from jejunum and ileum. Of note, STM infection in

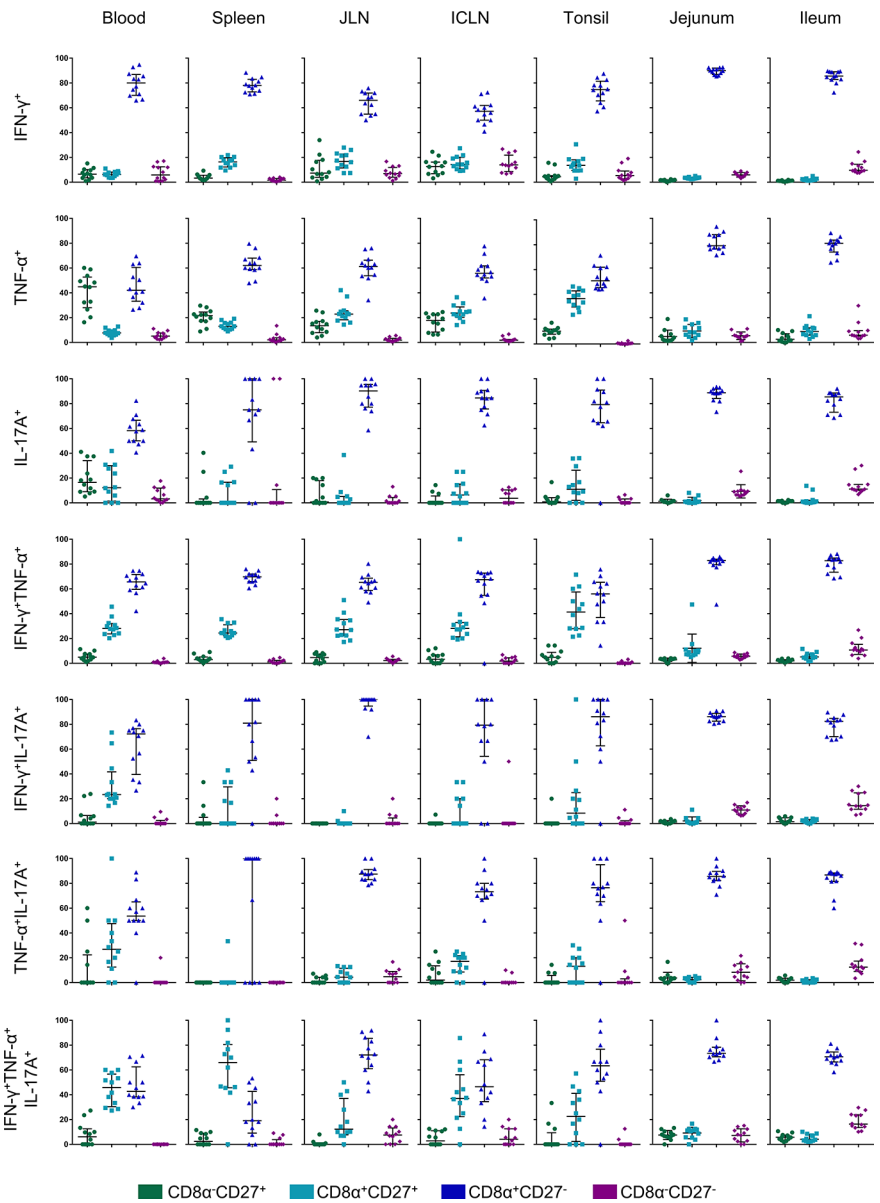

FIGURE 7 | CD8α and CD27 expression of cytokine-producing CD4+ T cells in analyzed organs. Intracellular cytokine staining was performed on lymphocytes isolated from organ tissue following overnight *in vitro* stimulation with the challenge strain. CD4+ T cells were analyzed for expression of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-17A (IL-17A) and grouped into seven cytokine-producing phenotypes. Cytokine-producing CD4+ T cells were further subgated for CD8α and CD27 to identify four CD8a/CD27-defined cell populations: CD8α-CD27+ (green), CD8α+CD27+ (light blue), CD8α+CD27- (dark blue), CD8α-CD27- (violet). Individual graphs indicate percentages of CD8a/CD27-defined cell populations for each cytokine-producing phenotype from individual animals of the V+I group. Black bars indicate the median and whiskers show the interquartile range. Data was obtained from day of necropsy of the respective animals.

the pig is mostly limited to the intestine, whereas a potential systemic infection is not well studied (50). Mouse models have shown that dissemination to mesenteric lymph nodes, blood and systemic organs can take place (51). We did not find *Salmonella* in spleen and liver of any of the animals two weeks post infection. As the immune system of V+I animals was already primed by vaccination prior to infection, it is possible that the infection was either already cleared or did not even spread to these systemic locations. In accordance to this, only few phenotypes of cytokine-producing CD4+ T cells showed significant rises in blood and spleen of V+I animals (**Table 3**). More phenotypes significantly different between V+I and control pigs were found for the investigated gut-draining lymph nodes (**Table 2**), whereas in the gut sections nearly all of the seven possible cytokine-producing phenotypes were induced (**Table 1**). This complies with studies in mice and humans, where T cells residing in the lamina propria have already been recognized as an important element of the response against STM and *Salmonella* Typhi

(52–54). The presence of a very high abundance of *Salmonella*-specific cytokine-producing CD4$^+$ T cells in the intestinal lamina propria of V+I animals in our study suggests that they also play a vital role in the mucosal immune response against STM infection in the pig.

Looking more closely at the seven possible cytokine-producing phenotypes in the different organs, it became apparent that IL-17A-containing phenotypes formed closely related clusters, often separating from IFN-γ and IFN-γ/TNF-α co-producing CD4$^+$ T cells. This separation is especially striking in jejunum and ileum. Th17 cells and their signature cytokine IL-17A are well known for contributing to mucosal immunity and protection against intracellular enteric pathogens (55–57). Infection with STM has previously been shown to induce the expression of Th17 cytokines in the intestinal mucosa of other species such as mice, calves and rhesus macaques (27, 58). A depletion of Th17 cells in the intestinal mucosa of rhesus macaques by infection with simian immunodeficiency virus was correlated with increased STM dissemination to mesenteric lymph nodes (27) suggesting that Th17 cells are important in disease containment. On the other hand, it has been discussed that IL-17A-induced recruitment of neutrophils and the resulting inflammation is exploited by pathogens like STM and ultimately promotes bacterial colonization (59). Since all V+I animals were vaccinated before infection and we did not detect any signs of inflammation in their intestines, a negative impact of the *Salmonella*-specific Th17 phenotypes observed in this study seems unlikely. Instead, we provide evidence that Th17 cells also seem to play a protective role in host defense against STM in swine.

In contrast to other IL-17A$^+$ populations, however, IL-17A single-producing CD4$^+$ T cells were the only cytokine phenotype that did not reach significant difference between control and V+I pigs in any of the organs. Indeed, we observed across organs that CD4$^+$ T-cell phenotypes consisting of more than one cytokine, such as IFN-γ/TNF-α, IFN-γ/IL-17A, TNF-α/IL-17A and IFN-γ/TNF-α/IL-17A, overall reached significant differences over the control group more frequently than single-cytokine producing CD4$^+$ T cells. T cells producing several cytokines, also called multifunctional (MF) T cells, have been associated with protection in several bacterial and viral infections in humans and mice (60–63) and were found to be functionally superior to their single-producing counterparts (64). Studies in swine have also demonstrated the involvement of antigen-specific MF CD4$^+$ T cells in response to various pathogens (65–68). While co-production of IFN-γ, TNF-α and IL-2 by T cells is frequently investigated, reports on these cytokines in combination with IL-17A in the context of infectious diseases are rather scarce. Concerning *Salmonella*, MF IL-17A T-cell responses have been reported in the blood and terminal ileum of humans. Vaccination or infection with *Salmonella* Typhi led to the induction of MF CD8$^+$ and CD4$^+$ T-cell responses, that, in case of MF CD8$^+$ T cells in PBMC, were demonstrated to correlate with disease outcome (54, 69, 70). To our knowledge, this is the first description of simultaneous production of IL-17A with IFN-γ and TNF-α by antigen-specific CD4$^+$ T cells in the pig and it can be speculated that MF CD4$^+$ T cells may serve as a correlate of protection for STM infection in swine. Especially IFN-γ/TNF-α co-producing CD4$^+$ T cells appear as a promising candidate as significant differences between groups were reached for six out of the seven analyzed organs for this phenotype, including the blood. Moreover, based on observations in the mouse model, it is likely that these cells contribute to STM clearance by a potent stimulation of macrophages that have engulfed the pathogen (23, 26).

Interestingly, it has recently been shown that non-cognate stimulation of Th1 cells contributes to resolution of *Salmonella* infection in mice (71, 72). This describes a mechanism where T cells are stimulated by T-cell receptor (TCR)-independent stimuli such as inflammatory cytokines without TCR recognition of cognate antigen presented by antigen presenting cells. Since we analyzed total CD4$^+$ T cells in our study and used whole bacterial antigen for *in vitro* re-stimulation, it is possible that a fraction of the measured cytokine production by CD4$^+$ T cells may be derived from non-cognate stimulation and not *via* direct stimulation of the TCR. So far, these bystander responses have mostly been described in effector or memory CD4$^+$ T cells (73, 74). Considering the strong stimulation applied in our study with two vaccinations and a challenge infection, it is conceivable that some of the T cells in our analyses had already reached a differentiation status that may have enabled them to respond in a non-cognate manner. Although that could lead to slightly overestimated numbers of STM-specific CD4$^+$ T cells in V+I animals, it might more accurately reflect the situation occurring *in vivo*.

For investigation of further functional differentiation, we looked at the expression of CD8α and CD27, which have been proposed for the distinction between central (Tcm) and effector memory (Tem) CD4$^+$ T cells in the pig (32). *Salmonella*-specific cytokine-producing CD4$^+$ T cells in all organs predominantly expressed CD8α while lacking CD27, corresponding to a Tem phenotype in the pig. Conversely, previous studies on viral infections in swine have shown that the CD4$^+$ Tcm subset was capable of IFN-γ, TNF-α and/or IL-2 production (66, 75). However, we did detect two phenotypes, namely IFN-γ/TNF-α co-producing along with IFN-γ/TNF-α/IL-17A triple-producing CD4$^+$ T cells, with a sizable cell population co-expressing CD8α and CD27 in non-gut tissues, indicating a Tcm subset. As IFN-γ/TNF-α co-producing CD4$^+$ T cells were also present in significantly higher amounts in V+I compared to control animals in the blood, they might be exploited as a phenotype for STM T-cell immunity in the pig.

In jejunum and ileum, almost the entirety of all cells regardless of the cytokine phenotype displayed features of Tem cells. Indeed, it is mainly Tem cells that reach non-lymphoid tissues like the intestine (76, 77). The mucosal memory pool contains recirculating as well as resident memory T cells (Trm) that stay in the tissue long term to initiate an immediate immune response against enteric pathogens (77, 78). In humans, *Salmonella* Typhi-specific CD4$^+$ Trm cells were induced in the ileal mucosa after Ty21a immunization (54). Moreover, resident memory Th1 cells have been shown to be indispensable for protection against STM infection in mice (30). Unfortunately, due to a lack of available antibodies for markers of

tissue-residency, Trm cells cannot yet be identified in the pig. Nevertheless, we can speculate that a part of the population now identified as Tem cells in the gut may represent Trm cells. These cells may have been already induced by the vaccination, settled down permanently in the intestine and were re-activated upon challenge infection.

In conclusion, by vaccinating piglets with a live attenuated STM vaccine and challenging them with a virulent STM strain, we could show the induction of STM-specific multifunctional $CD4^+$ T cells across organs with a strong enrichment in the intestinal mucosa. These cells predominantly possessed an effector memory phenotype. Their multifunctional cytokine profile suggests an involvement in protective immunity against STM infection and IFN-γ/TNF-α co-producing $CD4^+$ T cells in the blood might be investigated further as a marker for long-term protective immunity against STM infections. Finally, we think that our study forms an important foundation for more in-depth studies on the T-cell response in pigs against STM following only vaccination or infection. In this way, the postulated correlates of protection will be further corroborated.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Advisory Committee for Animal Experiments, University of Veterinary Medicine, Vienna and the Federal Ministry for Science, Research and Economy (BMWF-68.205/0241-WF/V/3b/2016).

AUTHOR CONTRIBUTIONS

SSp, TT, VF, AS, and WG conceived the idea and designed the project. ES, CK, and AL organized the animal experiment and necropsy with sample collection. EV, AP, JL, KM, and MS performed lymphocyte isolation and *in vitro* stimulation. SSc performed flow cytometry experiments. JS conducted bacteriological analysis. MD performed statistical analysis. SSc and WG analyzed the experiments, interpreted the data, and wrote the manuscript. SSp, TT, VF, AL, and AS assisted with interpretation of the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.603089/full#supplementary-material

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Conflict of Interest: SSp, TT, and VF are employed by Ceva Innovation Center GmbH.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Figure S1

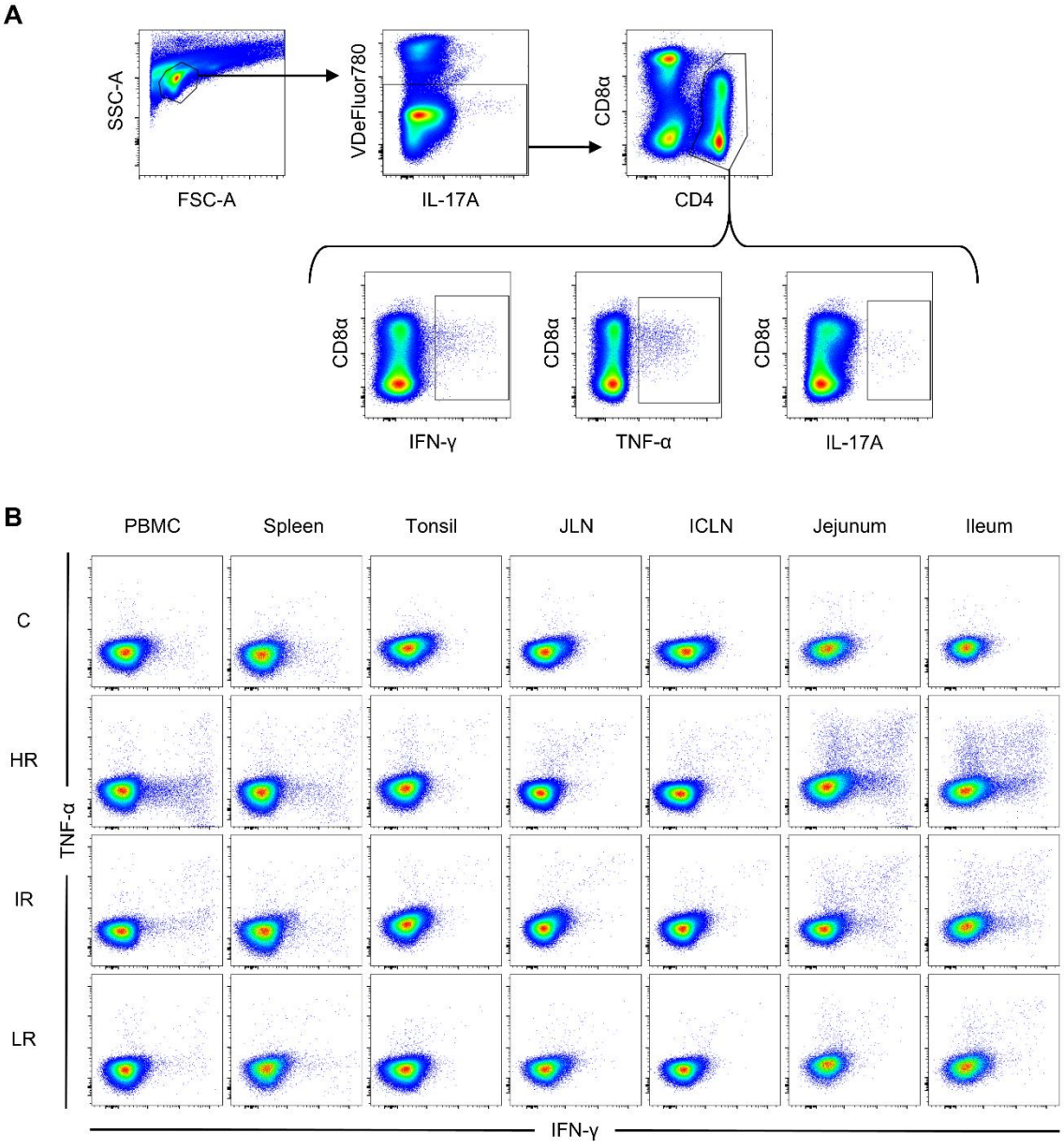

Figure S1 continued

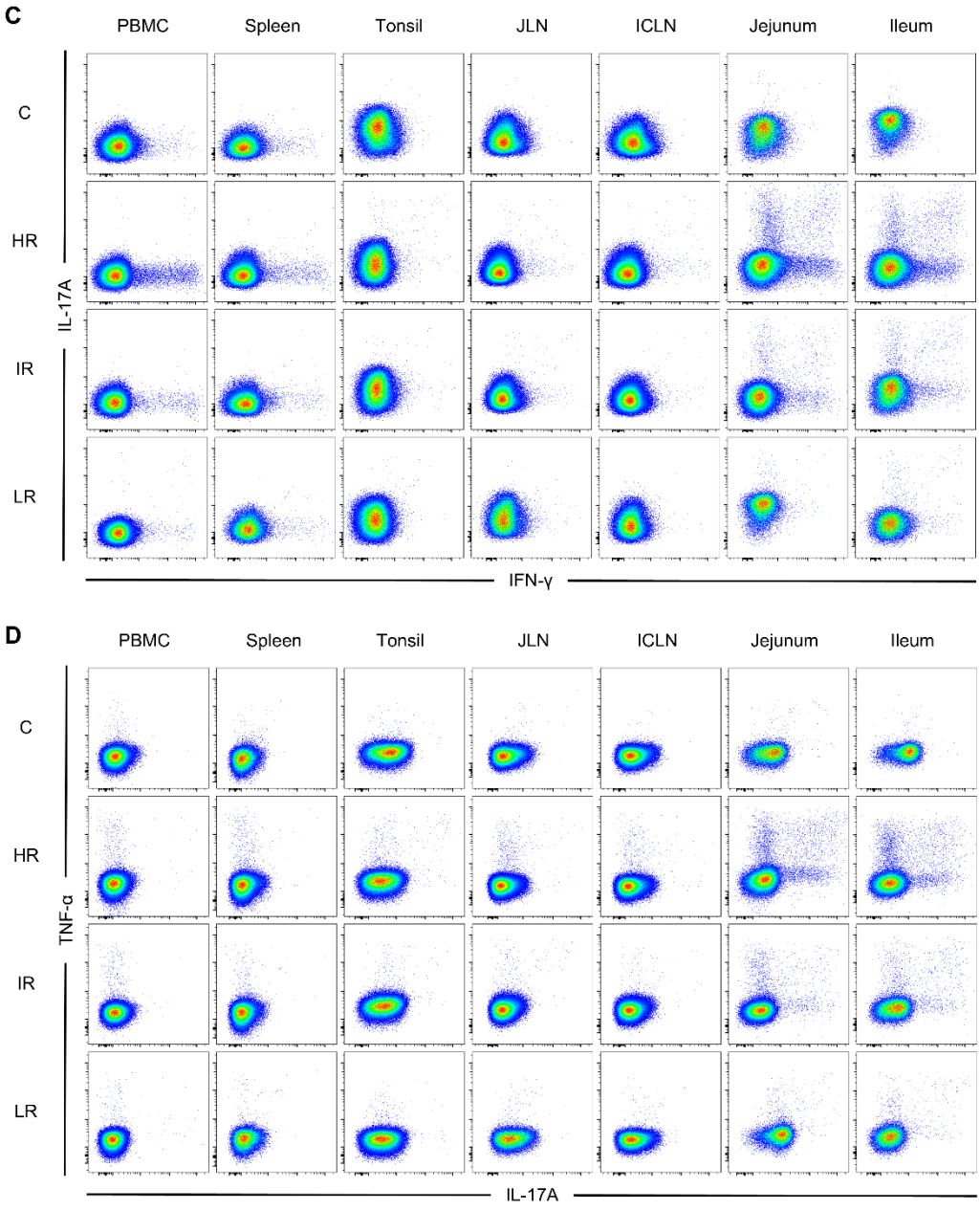

Figure S1. Gating strategy and representative raw data for FCM analysis of $CD4^+$ T cells. Intracellular cytokine staining was performed on lymphocytes isolated from various locations following overnight *in vitro* stimulation with STM antigen. (A) Representative gating strategy for the intracellular cytokine staining in $CD4^+$ T cells is shown (Sw #9, jejunal lymph node, stimulation with the vaccine strain). Cells were gated according to their light scatter properties and further subgated for live cells (VDeFlour780 negative). Subsequently, a gate was set on $CD4^+$ cells and a Boolean gating for IFN-γ, TNF-α and IL-17A was performed. (B-D) Co-production of IFN-γ/TNF-α (B), IFN-γ/L-17A (C) and TNF-α/IL-17A (D) in $CD4^+$ T cells in analyzed organs. Representative data from one high responder (HR), one intermediate responder (IR) and one low responder (LR) is shown. Additionally, one representative control (C) animal is shown for each organ.

Figure S2

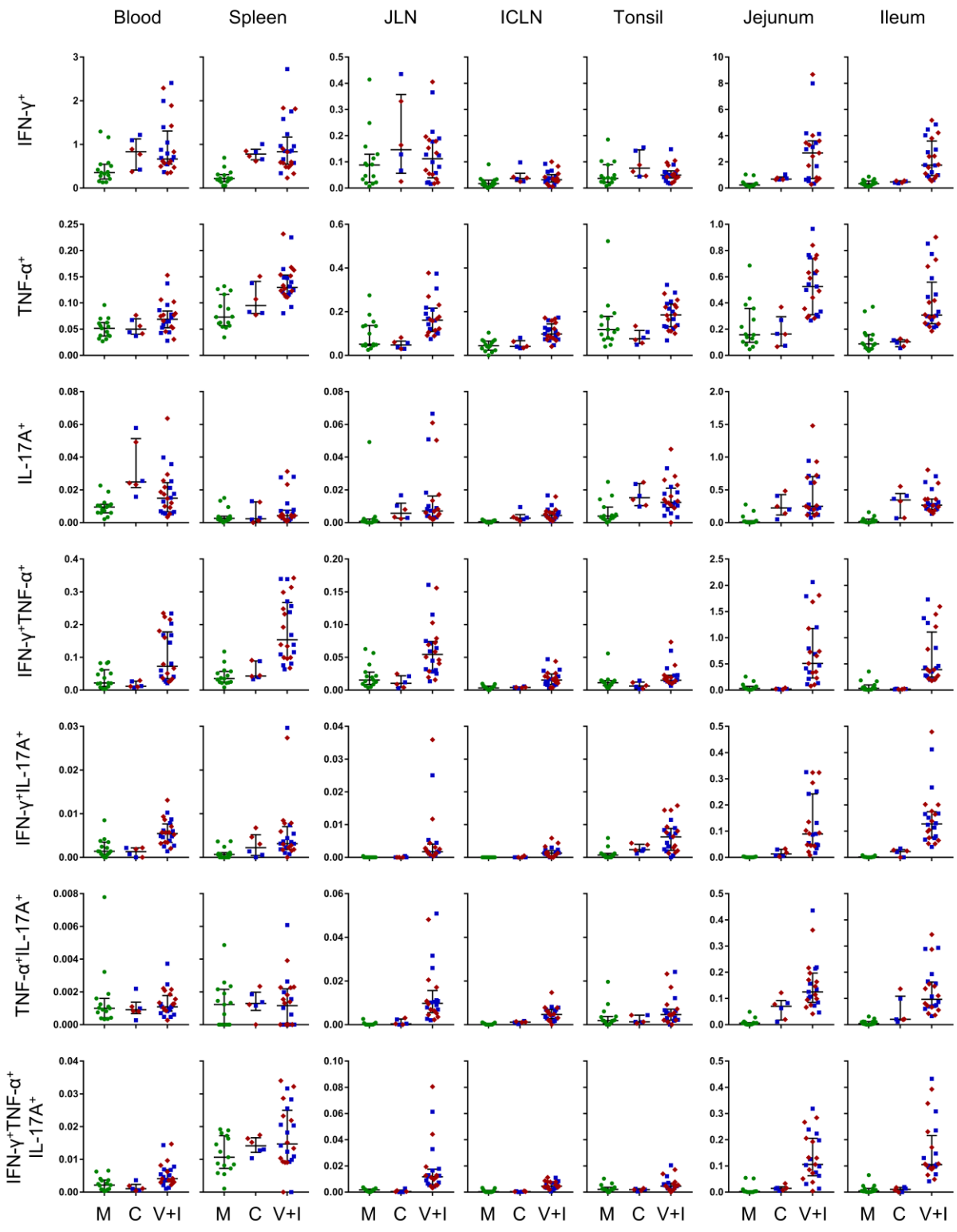
Blood
Spleen
JLN
ICLN
Tonsil
Jejunum
Ileum
IFN-γ+
TNF-α+
IL-17A+
IFN-γ+TNF-α+
IFN-γ+IL-17A+
TNF-α+IL-17A+
IFN-γ+TNF-α+ IL-17A+
M C V+I
Medium
Salmoporc (vaccine strain)
Challenge (infection strain)
M: non-stimulated *in vitro* control for all animals
C: STM-stimulated samples from the control group
V+I: STM-stimulated samples from the V+I group

Figure S2. Frequencies of cytokine-producing $CD4^+$ T cells in blood, spleen, tonsil, jejunal lymph node (JLN), ileocolic lymph node (ICLN), jejunum and ileum. $CD4^+$ T cells were gated within live lymphocytes and further analyzed for IFN-γ, TNF-α and IL-17A production by Boolean gating. Individual graphs indicate percentages of cytokine-producing $CD4^+$ T cells from individual animals of the control (C) and the V+I group (V+I) within total $CD4^+$ T cells. Cells were stimulated with the vaccine strain (Salmoporc, blue diamonds) or the challenge infection strain (Challenge, red squares) or cultivated in medium-only (Medium, green circles). Samples from both groups in medium-only (M) are displayed on the left side of each graph. Black bars indicate the median and whiskers show the interquartile range. Data was obtained from the day of necropsy of the respective animals.

Figure S3

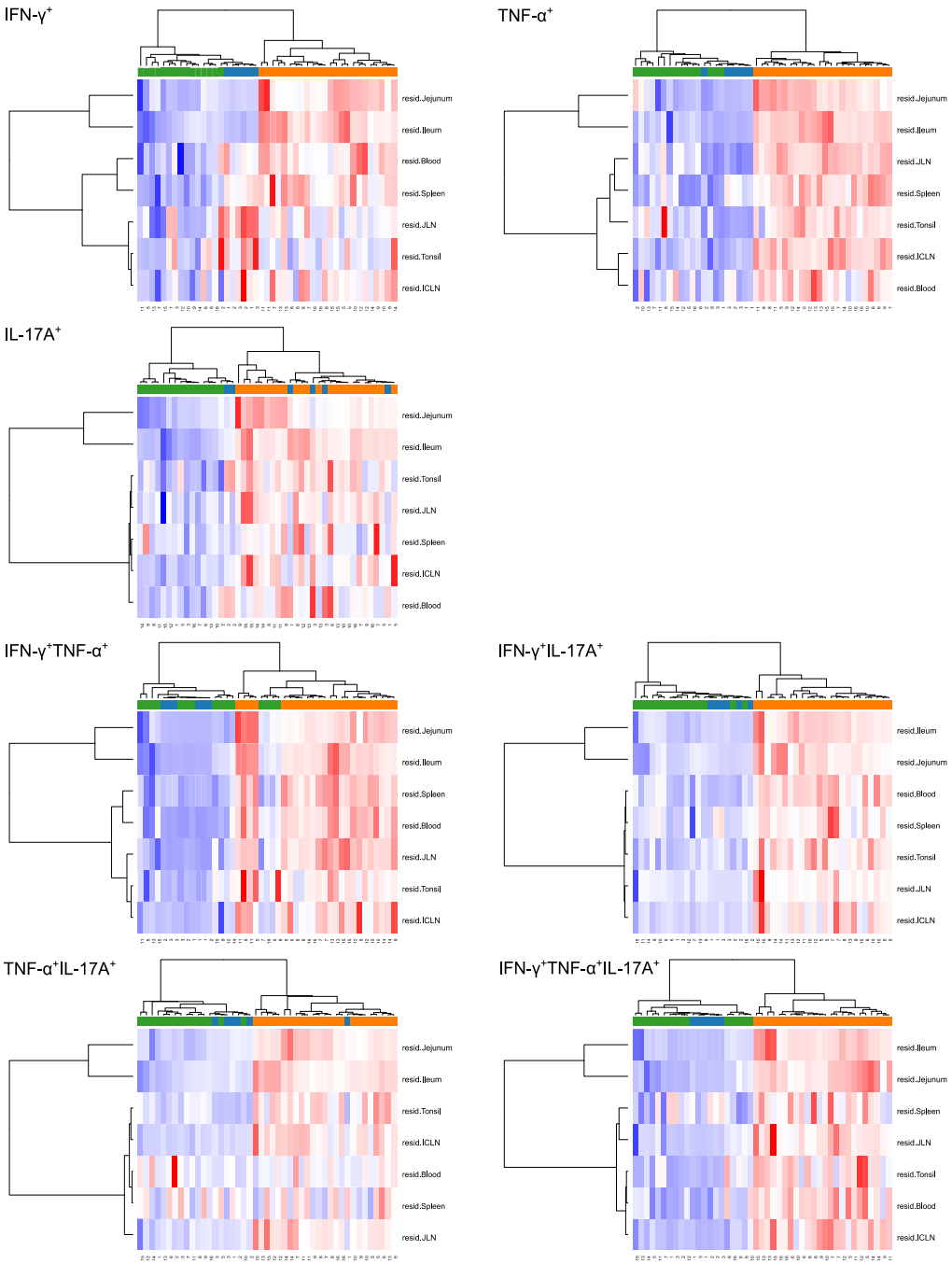

Figure S3. Heat map analysis for STM-stimulated IFN-γ/TNF-α/IL-17A producing CD4$^+$ T cells isolated from various organs. Heat map of residuals calculated from cytokine-producing CD4$^+$ T cells derived from jejunum, ileum, jejunal lymph node (JLN), ileocolic lymph node (ICLN), tonsil, blood and spleen stimulated *in vitro* with STM or medium-only. Each row represents an organ and each column a sample. Numbers under each column indicate animal numbers. Samples stimulated with STM (vaccine or challenge strain) are indicated by orange boxes for V+I animals and blue boxes for control animals; samples from both groups cultivated in medium-only are indicated by green boxes. Heat maps were generated from data converted to Z-scores. Relative increase (red) or decrease (blue) of abundance of cytokine-producing CD4$^+$ T cells is shown.

Figure S4

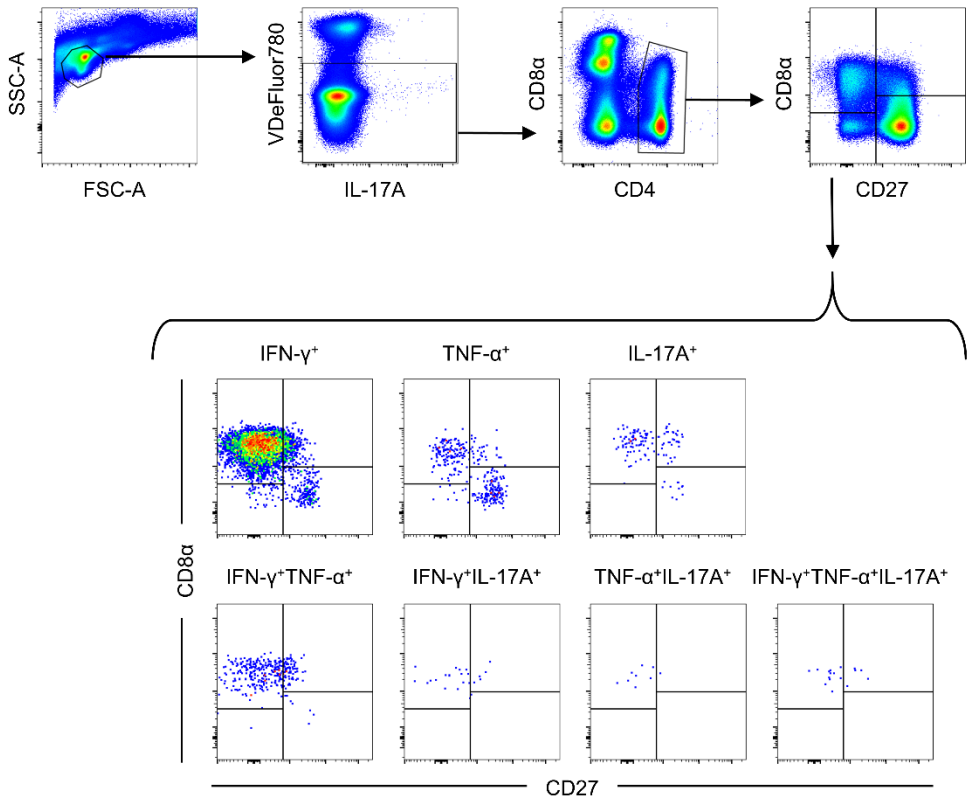

Figure S4. Gating strategy for identification of phenotypes based on CD8α/CD27 expression of cytokine-producing $CD4^+$ T cells. Blood and organ-derived T cells were analyzed for CD8α/CD27-defined phenotypes within cytokine-producing $CD4^+$ T cells. Representative data depicting gates used to identify CD8α/CD27-defined phenotypes from cytokine-producing $CD4^+$ T cells of one pig is shown in pseudo-color plots. Dots were enlarged in plots of cytokine-producing phenotypes for better visibility of rare populations.

Figure S5

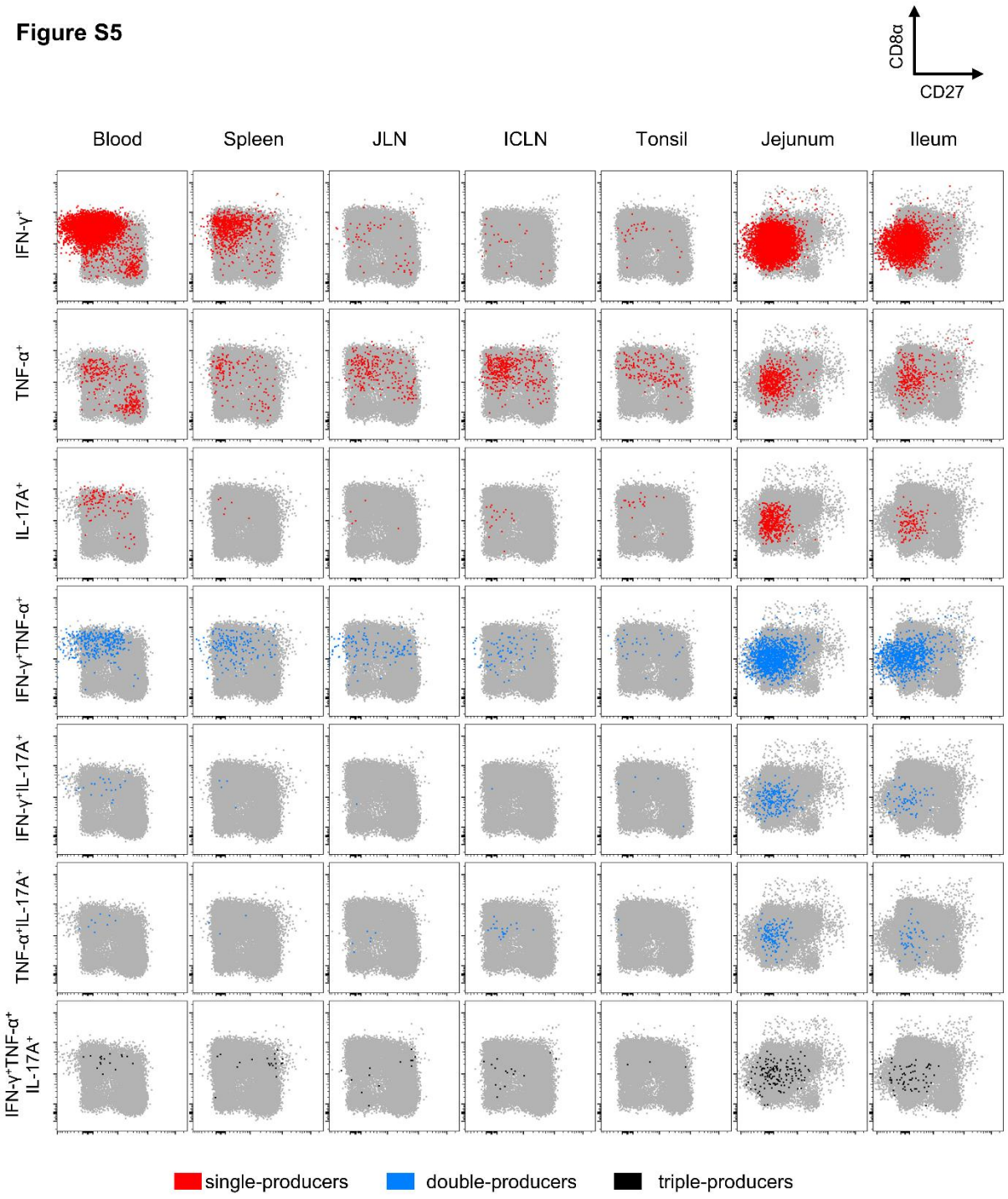

Figure S5. CD8α and CD27 expression of cytokine-producing $CD4^+$ T cells in analyzed organs. CD8α (y-axis) and CD27 expression (x-axis) was analyzed in total $CD4^+$ T cells (light grey dots, background) and $CD4^+$ T cells producing a single cytokine (red dots, top), two cytokines (blue dots, middle) or three cytokines (black dots, bottom). Raw data of one representative animal (Sw #11) is shown. Approximate numbers of $CD4^+$ T cells depicted per organ and cytokine-producing phenotype: Blood: 4.2 x 10^5, Spleen: 1.3 x 10^5, JLN: 2.6 x 10^5, ICLN: 2.8 x 10^5, Tonsil: 1.7 x 10^5, Jejunum: 7.3 x 10^4, Ileum: 7.4 x 10^4.

vaccines

MDPI

Article

T-Cell Cytokine Response in *Salmonella* Typhimurium-Vaccinated versus Infected Pigs

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Abstract: Vaccination with the live attenuated vaccine Salmoporc is an effective measure to control *Salmonella* Typhimurium (STM) in affected swine populations. However, the cellular immune response evoked by the Salmoporc vaccine including differences in vaccinated pigs versus non-vaccinated pigs upon STM infection have not been characterized yet. To investigate this, tissue-derived porcine lymphocytes from different treatment groups (vaccination-only, vaccination and infection, infection-only, untreated controls) were stimulated in vitro with heat-inactivated STM and abundances of IFN-γ, TNF-α and/or IL-17A-producing T-cell subsets were compared across organs and treatment groups. Overall, our results show the induction of a strong $CD4^+$ T-cell response after STM infection, both locally and systemically. Low-level induction of STM-specific cytokine-producing $CD4^+$ T cells, notably for the IFN-γ/TNF-α co-producing phenotype, was detected after vaccination-only. Numerous significant contrasts in cytokine-producing T-cell phenotypes were observed after infection in vaccinated and infected versus infected-only animals. These results suggest that vaccine-induced STM-specific cytokine-producing $CD4^+$ T cells contribute to local immunity in the gut and may limit the spread of STM to lymph nodes and systemic organs. Hence, our study provides insights into the underlying immune mechanisms that account for the efficacy of the Salmoporc vaccine.

Keywords: *Salmonella* Typhimurium; pig; multifunctional T cells; interferon-γ; tumor necrosis factor-α; Interleukin-17A; lamina propria lymphocytes

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1. Introduction

Salmonella Typhimurium (STM), a Gram-negative facultative intracellular bacterium within the family of the *Enterobacteriaceae*, can infect a broad range of host species. Non-typhoidal *Salmonella* (NTS) serovars such as STM are among the most frequent causes of gastrointestinal infections in humans [1,2] but invasive strains can also lead to more severe

manifestations such as septicemia and meningitis in children and immunocompromised individuals [3,4]. In industrialized countries, the consumption of contaminated food of animal origin poses the greatest risk of STM transmission to humans; this applies in particular to products originating from pigs [5–7]. Although STM infection in weaner pigs can manifest as enterocolitis with symptoms such as fever and diarrhea, pigs more often act as subclinical carriers [8,9] making it harder to recognize and eradicate the disease on the farm level. Considering the rise of resistance to antimicrobials observed in many *Salmonella* strains [10], alternative measures such as vaccination are promising strategies to control *Salmonella* in affected swine herds [11]. Indeed, the live attenuated histidine–adenine auxotrophic vaccine Salmoporc (Ceva Santé Animale, Libourne, France), which is commercially available for the use in pigs in Europe, was confirmed in its effect of reducing clinical signs, tissue colonization and shedding in numerous studies [12–18].

Furthermore, vaccination with Salmoporc was shown to induce *Salmonella*-specific IgM, IgG and IgA antibodies [15,19] as well as differentiation of T follicular helper (Tfh) cells [20] in immunized pigs. The exact role of the humoral immune response in controlling STM infections has been subject to much debate and limited data are available on this in pigs. B-cell deficient mice were able to resolve infection with attenuated STM strains, but succumbed to infection with virulent strains [21,22]. Moreover, antibodies were shown to be protective in NTS bacteremia in African children [23].

When it comes to bacterial clearance of STM infection, however, there is consensus that a $CD4^+$ T-cell response is vital [24,25]. Especially Th1 cells with their capacity for interferon-γ (IFN-γ) production, leading to an activation of antimicrobial macrophages, are essential for the resolution of infection in the mouse model [26–28]. In line with these findings, in HIV-infected humans with their reduction of $CD4^+$ T cells otherwise non-invasive nontyphoidal *Salmonella* can cause systemic infection and disease [29]. Interleukin-17A (IL-17A)-producing Th17 cells assist in bacterial clearance through recruitment of neutrophils [30]. This corresponds to previous studies in pigs where mRNA level expression of cytokines in STM-infected piglets also suggested a T helper (Th) 1-driven response [31,32].

In a previous experiment, we could demonstrate the induction of multifunctional STM-specific $CD4^+$ T cells in pigs that received a two-time Salmoporc immunization with a subsequent STM challenge [33]. Since all pigs in that study were both vaccinated and infected it remained unclear to what extent the vaccination influenced the $CD4^+$ T-cell response measured after STM challenge infection. In humans, immunization with a live attenuated typhoid vaccine was shown to elicit *Salmonella* Typhi (*S*. Typhi)-specific multifunctional $CD4^+$ T cells in the blood that predominantly produced IFN-γ and tumor necrosis factor-α (TNF-α) [34] as well as mucosal immunity at the site of infection driven by $CD4^+$ and $CD8^+$ T cells [35,36]. Although *S*. Typhi as a typhoidal serovar differs from NTS in many aspects [37], it is conceivable that the Salmoporc vaccine might have similar effects on the porcine immune system.

With the aim of addressing these knowledge gaps, we characterized the STM-specific response of $CD4^+$, $CD8^+$ and $CD4^-CD8\beta^-$ porcine T cells (the latter mainly representing γδ T cells) that were isolated from systemic organs such as blood and spleen as well as from local sites of immunity such as mesenteric lymph nodes and the gut. Frequencies of STM-specific IFN-γ, TNF-α and/or IL-17A-producing T cells measured in these tissues at 7 and 21 days post Salmoporc vaccination and/or STM infection were then compared between only vaccinated (VAC), vaccinated and infected (V+I), only infected (INF) and untreated control (CON) pigs.

2. Materials and Methods

2.1. Animals

Sera from sows (Large White × Landrace) at a university-owned pig farm in Lower Austria were tested by the IDEXX Swine *Salmonella* Ab test (IDEXX Europe, Hoofddorp, The Netherlands) prior to the study. The five sows with the lowest sample to positive control (S/P) ratios (S/P ratios: 0.295–0.473, cut-off for positivity: 1.0) were selected

and 44 female and male castrated pigs (Large White × Landrace × Pietrain) from those sows were included in the study at the age of four weeks. All piglets underwent routine vaccinations against PCV-2 (Ingelvac CircoFLEX®, Boehringer-Ingelheim, Ingelheim am Rhein, Germany) at three weeks of age and *Mycoplasma hyopneumoniae* (M^+PAC®, MSD Animal Health, Kenilworth, NJ, USA) in the first and third week of life at the farm. The *Salmonella*-free status of the piglets at the start of the study was validated by serological testing for *Salmonella*-specific antibodies by the IDEXX Swine *Salmonella* Ab test (IDEXX Europe) and by microbiological testing of fecal samples collected on three consecutive days after arrival (study days (SD) −11, −10, −9). Antibody S/P ratios were below the positivity cut-off for piglets (0.25). Additionally, no *Salmonella* were detectable in the fecal samples of the piglets at the stated time points.

2.2. Vaccination and Infection Experiment

For the duration of the study, all animals were housed in a biosafety level (BSL) 2 facility at the University of Veterinary Medicine Vienna with different treatment groups residing in separate compartments of the isolation unit. Upon arrival at four weeks of age, the animals were weighed and the data were used to achieve equal distribution of animals with different body weights into four groups (Figure 1). Starting at an age of 6 weeks, groups 1 (VAC) and 2 (V+I) were immunized twice orally in a 3-week interval (SD0 and SD21, Figure 1) with 1.0 mL of the live attenuated histidine–adenine auxotrophic STM vaccine (Salmoporc, Ceva Santé Animale, Libourne, France) containing a dose of 1.33×10^9 colony forming units (cfu). Groups 3 (INF) and 4 (CON) received 1.0 mL of tap water orally. Three weeks after the second immunization (SD42), groups 2 (V+I) and 3 (INF) were infected orally with 5 mL per animal containing 2×10^8 cfu/mL of a virulent monophasic STM strain (DT193, no. RKI 06-1900, described by [38] and provided by Ceva Innovation Center GmbH, Dessau-Roßlau, Germany). For the infection, the challenge strain was mixed with sugar beet solution to improve acceptance by the animals. The control group (CON) received 5 mL of sugar beet syrup diluted in water. Both vaccine and challenge strain solutions were applied orally using an oral drencher kit provided by Ceva Santé Animale. Necropsy was performed over two or three consecutive days at 7 and 21 days post vaccination (dpv) and/or infection (dpi): SDs 28/29 were summarized as 7 dpv, SDs 42/43 as 21 dpv, SDs 49–51 as 7 dpi and SDs 63–65 as 21 dpi (Figure 1, bottom). All animals were anaesthetized by intramuscular injection of Ketaminhydrochlorid (Narketan®, 10 mg/kg body weight, Vétoquinol, Lure Cedex, France) and Azaperon (Stresnil®, 1.3 mg/kg body weight, Elanco, Greenfield, IN, USA) followed by euthanasia via intracardial injection of T61® (tetracaine hydrochloride, mebezonium iodide and embutramide, 1.0 mL/10 kg body weight, MSD Animal Health, Kenilworth, NJ, USA). One animal (Sw#3, Group 4, CON) was unexpectedly found dead on SD7. All results from this animal were excluded from the analyses. The animal experiment was approved by the institutional ethics committee, the Advisory Committee for Animal Experiments (§12 of Law for Animal experiments, Tierversuchsgesetz-TVG) and the Federal Ministry for Science, Research and Economy (BMBWF-68.205/0095-V/3b/2018).

2.3. Clinical Examination, Necropsy, and Sample Collection

Animals were examined daily, and observations were evaluated by a scoring system, taking rectal temperature, diarrhea, vomiting, and changes in behavior into account. Additionally, body weights of all pigs were recorded weekly. After euthanasia, a general pathological examination of the inner organs of all animals was performed. Fecal samples from all animals were collected at the beginning of the study (SDs −11, −10, −9) for STM analysis. Serum samples from the jugular vein (*Vena jugularis*) were taken from all animals upon arrival (SD-11) and from all animals still alive at SD42 prior to challenge infection. At every day of necropsy (SD28/29, 42/43, 49–51, 63–65) blood was drawn by cardiac puncture from the respective animals after anesthesia prior to euthanasia. On these days, samples were collected from liver, spleen, jejunal lymph nodes (JLN), ileocolic lymph nodes

(ICLN), jejunum, ileum, and cecum. Tissue samples from liver, jejunum, ileum, and cecum were always taken from the same part of the organ as described in [33].

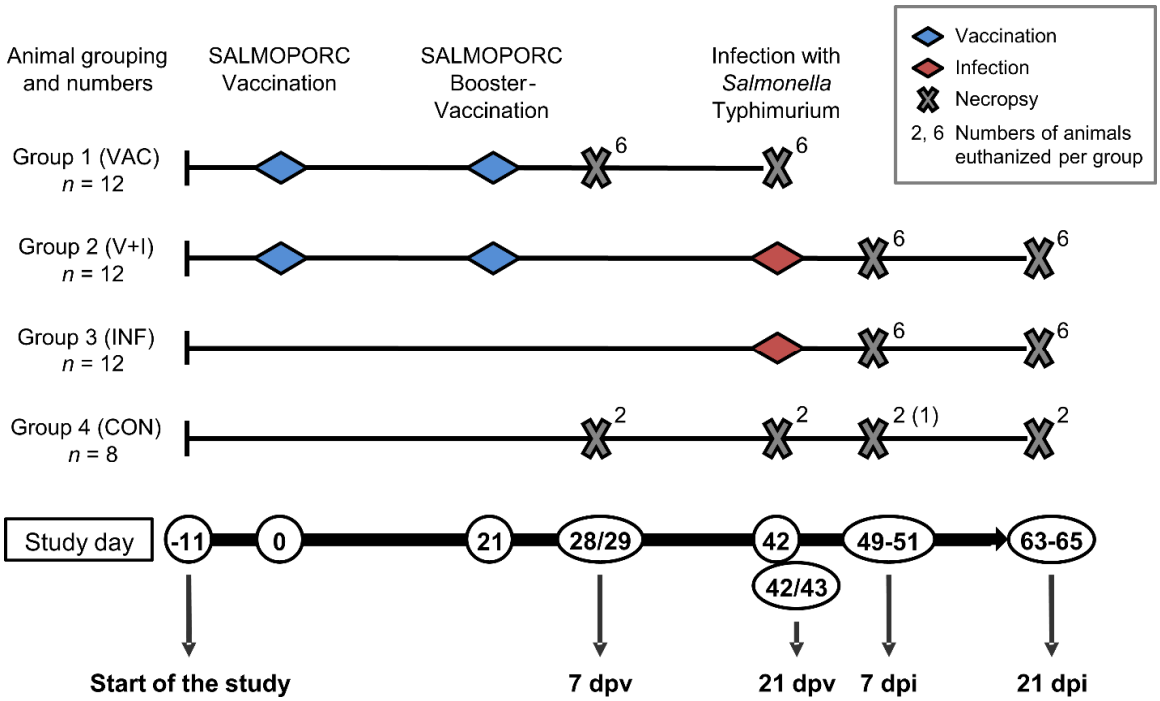

Figure 1. Animal grouping and time schedule for the study. At the beginning of the study (SD -11, study start), 44 piglets at an age of four weeks were divided into four groups: animals that were only vaccinated (group 1, VAC), animals that were vaccinated and challenged (group 2, V+I), animals that only received the challenge infection (group 3, INF) and non-vaccinated non-infected control animals (group 4, CON). First vaccination (SD0), booster vaccination (SD21) and challenge infection (SD42) are indicated by blue and red diamonds, respectively. Animals were euthanized 7 days post vaccination (7 dpv, SDs 28/29), 21 days post vaccination (21 dpv, SDs 42/43), 7 days post infection (7 dpi, SDs 49–51) and 21 days post infection (21 dpi, SDs 63–65) as marked by grey crosses. Superscript numbers above crosses indicate numbers of animals euthanized from each group. Due to the loss of animal Sw#3, only one control animal was available for the necropsy date SDs 49–51.

2.3. Clinical Examination, Necropsy, and Sample Collection

Animals were examined daily, and observations were evaluated by a scoring system, taking fecal consistency, vomiting, and changes in behavior into account. Additionally, body weights of all pigs were recorded weekly. After euthanasia, a general pathological examination of the inner organs of all animals was performed. Fecal samples from all animals were collected at the beginning of the study (SDs −11, −10, −9) for STM analysis. Serum samples from the jugular vein (*Vena jugularis*) were taken from all animals upon arrival (SD −11) and from all animals still alive at SD42 prior to challenge infection. At every day of necropsy (SD28/29, 42/43, 49–51, 63–65) blood was drawn by cardiac puncture from the respective animals after anesthesia prior to euthanasia. On these days, samples were collected from liver, spleen, jejunal lymph nodes (JLN), ileocolic lymph nodes (ICLN), jejunum, ileum, and cecum. Tissue samples from liver, jejunum, ileum, and cecum were always taken from the same part of the organ as described in [33].

2.4. Detection of *Salmonella*-Specific Antibodies in Serum

Blood samples were centrifuged for 10 min and 1900× *g* at room temperature and resulting sera were tested for *Salmonella*-specific antibodies by use of a commercially available ELISA kit (IDEXX Swine *Salmonella* Ab test, IDEXX Europe) according to the manufacturer's instructions. Ratios between mean optical density (OD) of each sample and mean OD of the positive control were calculated and reported as S/P ratios. In addition, serum samples were used to detect *Salmonella*-specific IgM, IgA, and IgG antibodies using an in-house ELISA as described in [15]. Briefly, 96-well plates (Nunc Maxisorp™, Thermo Fisher, Waltham, MA, USA) were coated with 10 µg/mL of the Salmoporc vaccine strain (provided by Ceva Innovation Center GmbH, Dessau-Roßlau, Germany) at 4 °C overnight. Plates were then washed and blocked for 2 h with 1% BSA blocking buffer. Swine sera were diluted 40-fold for IgM, 12-fold for IgA, and 200-fold for IgG detection. These swine sera, after pre-incubation with soluble *E. coli* proteins (provided by Ceva Innovation Center GmbH, Dessau-Roßlau, Germany), were incubated with plate-immobilized STM antigen for 2 h at room temperature. Detection of IgM, IgA, and IgG was achieved with Ig-class-specific secondary goat anti-swine antibodies conjugated to horseradish peroxidase (Bethyl Laboratories, Montgomery, TX, USA) and subsequent incubation with TMB/E (3,3′,5,5′-tetramethylbenzidine) substrate (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The reaction was stopped with sulfuric acid and optical densities were measured at a wavelength of 450 nm. An internal reference serum, derived from the combined sera of two serum samples were used to detect *Salmonella*-specific IgM, IgA, and IgG antibodies using an in-house ELISA as described in [15]. Briefly, 96-well plates (Nunc Maxisorp™, Thermo Fisher, Waltham, MA, USA) were coated with 10 µg/mL of the Salmoporc vaccine strain

V+I animals, euthanized 7 and 21 dpi, respectively, and two INF animals, also euthanized 7 and 21 dpi, served as a positive control on all plates. Results are given as S/P ratio obtained by the ratio between mean OD of individual samples and mean OD of the reference serum.

2.5. Microbiological Investigation

Fecal samples taken at the start of the study as well as samples of liver, spleen, JLN, ICLN, jejunum, ileum, and cecum collected at necropsy were examined for the presence of *Salmonella enterica* according to ISO 6579–1:2017 [39]. All samples were streaked onto Xylose-Lysine-Deoxycholate (XLD) agar plates (BBL™, Becton Dickinson (BD), Heidelberg, Germany) in a three-sector T-streak pattern and incubated at 37 °C for 48 h in ambient air. Samples were additionally pre-enriched in buffered peptone water (BPW, Millipore™, Merck KGaA, Darmstadt, Germany) for 24 h at 37 °C. Subsequently, 0.1 mL of each culture was transferred to Rappaport-Vassiliadis R10 and Selenite broth (both Difco™, BD, Heidelberg, Germany). Incubation was performed for 24 h at 42 °C, and sub-cultures streaked onto XLD agar plates (BBL™, BD, Heidelberg, Germany) that were incubated at 37 °C for 48 h under aerobic conditions. MALDI TOF mass spectrometry was employed for identification of putative *Salmonella* colonies. The content of *Salmonella* in a sample was estimated by counting colonies in the three-sector T-streak pattern (direct smear), i.e., if growth was only observed in the first streak, *Salmonella* colony counts were considered rare or few, depending on the number of colonies grown (rare <10 colonies; few >10 colonies); if colonies were observed in the second fraction, the quantity was considered moderate, and if *Salmonella* colonies were noticed in all three streaks, it was considered many. This semi-quantitative analysis was performed for the seven organs mentioned above of all VAC pigs (7 and 21 dpv), for liver, spleen, and JLN of V+I and INF pigs (7 and 21 dpi) as well as all organs from control animals (7 and 21 dpv/dpi).

Samples of ICLN, jejunum, ileum and cecum taken from V+I and INF animals were used to perform quantitative determinations of the challenge strain load (cfu per g tissue) by application of the Koch spread-plate method as described in [12]. In brief, samples from lymph nodes were cut into small pieces and the mucosa of gut samples was gently scraped off. Tissue material was subsequently transferred to centrifuge tubes (Greiner, Kremsmünster, Austria), weighed and frozen at −25 °C until analysis. After thawing, tissue homogenates prepared with an Ultra-Turrax (IKA®-Labortechnik, Staufen, Germany) were suspended in physiological saline solution at a 10-fold dilution series and plated on deoxycholate-citrate agar supplemented with ampicillin (10 µg/mL), streptomycine (50 µg/mL), sulfamerazine (100 µg/mL) and tetracycline (10 µg/mL). After incubation at 37 ± 1 °C for 24 h under aerobic conditions, bacterial colonies were counted on petri dishes and the bacterial load was calculated as cfu/g tissue. A verification of the cultures originating from the challenge strain was performed serologically (Enteroclon, sifin diagnostics GmbH, Berlin, Germany). In the cases of no bacterial growth, a qualitative examination was carried out according to DIN ISO 6579–1:2017. Samples being positive only after enrichment were rated with 10 cfu/g in the original tissue. A sample which yielded no *Salmonella* growth after enrichment was rated as 0 cfu/g tissue.

2.6. Isolation of Lymphocytes

Peripheral blood mononuclear cells were isolated from heparinized blood by density gradient centrifugation using lymphocyte separation medium (Pancoll human, density: 1.077 g/mL, PAN Biotech, Aidenbach, Germany). Lymphocytes from spleen and mesenteric lymph nodes were collected as previously described [40]. The procedure for the isolation of lamina propria lymphocytes (LPL) from jejunum and ileum was performed as outlined in [33]. A Sysmex XP 300 hematology analyzer (Sysmex Europe GmbH, Norderstedt, Germany) was used to count cell numbers in all tissue preparations.

2.7. Preparation of STM Antigen for In Vitro Stimulation

STM antigen used for the in vitro stimulation of lymphocytes were manufactured by culturing the vaccine strain (STM no. 421/125) and the challenge strain (STM no. RKI 06–1900) via two pre-cultures that were prepared in STM 6/83 medium (in-house) at 37 °C and 150 rpm. After a 10 min centrifugation at 7000× *g*, the pellets were re-suspended in PBS. Afterwards, colony forming units were assessed and concentrates were placed for 90 min in a water bath at 60 °C for heat-inactivation. Antigens were then aliquoted and frozen at −80 °C until further use.

2.8. In Vitro Stimulation and Intracellular Cytokine Staining (ICS)

For intracellular staining of IFN-γ, TNF-α and IL-17A, round-bottomed 96-well microtiter plates (Greiner Bio One, Frickenhausen, Germany) were seeded with 5×10^5 freshly isolated cells in a final volume of 200 µL/well in cell culture medium (RPMI 1640, PAN Biotech) supplemented with 10% fetal calf serum (FCS, Merck KGaA, Darmstadt, Germany). Heat-inactivated 2.5×10^8 cfu/mL STM vaccine strain or heat-inactivated 2.25×10^8 cfu/mL STM challenge strain were present in the wells for stimulation and plates were cultured for approximately 19 h at 37 °C. Cells incubated in cell culture medium only served as negative controls. Brefeldin A (BD GolgiPlug™, BD Biosciences, San Jose, CA, USA) was added to the microcultures at a final concentration of 1 µg/mL for the final four hours of cultivation. Cultivated cells were harvested and re-suspended in buffer containing PBS with 3% FCS (Merck KGaA). For flow cytometry (FCM)-based phenotyping of lymphocyte subsets, cells were surface-stained with primary monoclonal antibodies directed to CD3 (mIgG1, clone: PPT3, biotinylated, Southern Biotech, Birmingham, AL, USA), CD4 (mIgG2b, clone: 74–12-4, in-house), CD8α (mIgG2a, clone: 76–2-11, in-house) and CD8β-A488 (mIgG1, clone: PPT23, in-house). The secondary staining step included Streptavidin-BV605 (BioLegend, San Diego, CA, USA), goat anti-mouse IgG2b-APC-Cy7 and goat anti-mouse IgG2a-PE-Cy7 (both Southern Biotech, Birmingham, AL, USA). To discriminate dead cells, Fixable Viability Dye eFlour506 (Thermo Fisher, Waltham, MA, USA) was applied according to manufacturer's protocol at 0.05 µL reactive dye per sample. Free binding sites of secondary antibodies were blocked with whole mouse IgG molecules (2 µg per sample; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences, San Jose, CA, USA) was used following the manufacturer's instructions. Intracellular staining was performed using IFN-γ-PE (mIgG1, clone: P2G10, BD Biosciences, San Jose, CA, USA), TNF-α-PE/Dazzle 594 (mIgG1, clone: Mab11, BioLegend, San Diego, CA, USA) and IL-17A-eF450 (mIgG1, clone: eBio64DEC17, Thermo Fisher, Waltham, MA, USA). Except for the intracellular staining step that encompassed 30 min, all other incubation steps were carried out in 96-well round-bottom plates for 20 min at 4 °C. After two washing steps, samples were left in 50 µL Perm/Wash™ Buffer (BD Biosciences, San Jose, CA, USA) overnight at 4 °C and analyzed the following morning. FCM analyses were performed on a FACSAria™ (BD Biosciences, San Jose, CA, USA) and data of at least 1×10^6 lymphocytes per sample were recorded for blood, spleen, and lymph nodes. For LPL preparations from jejunum and ileum at least 3×10^5 lymphocytes were recorded. FCM data were analyzed with FlowJo™ Software for Windows (Version 10.5.3; BD Biosciences, San Jose, CA, USA).

2.9. Statistical Analysis

Data for the graphs in Figure 2 and Figures S5–S7 including calculation of median and interquartile range were prepared with GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Heatmaps in Figure 3A,B and Figure 4, Figures S9 and S10 were visualized using GraphPad Prism 7. Statistical analysis was performed in R version 3.6.2 (R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/, accessed on 7 August 2020).

pigs. The antibody profile of STM-specific IgG largely matched the results of the IDEXX Swine *Salmonella* Ab test (Figure 2C). Regarding STM-specific IgA, a slight rise of S/P ratios could be observed after vaccination followed by a strong increase in V+I pigs after challenge infection (Figure 2D). S/P ratios of IgA in INF animals experienced a delayed rise but eventually surpassed those of V+I animals 3 weeks post infection. Overall, largest differences between the V+I and INF group were detected at 7 dpi where STM-specific IgG and IgA levels were highest in the V+I group while IgM dominated in INF pigs.

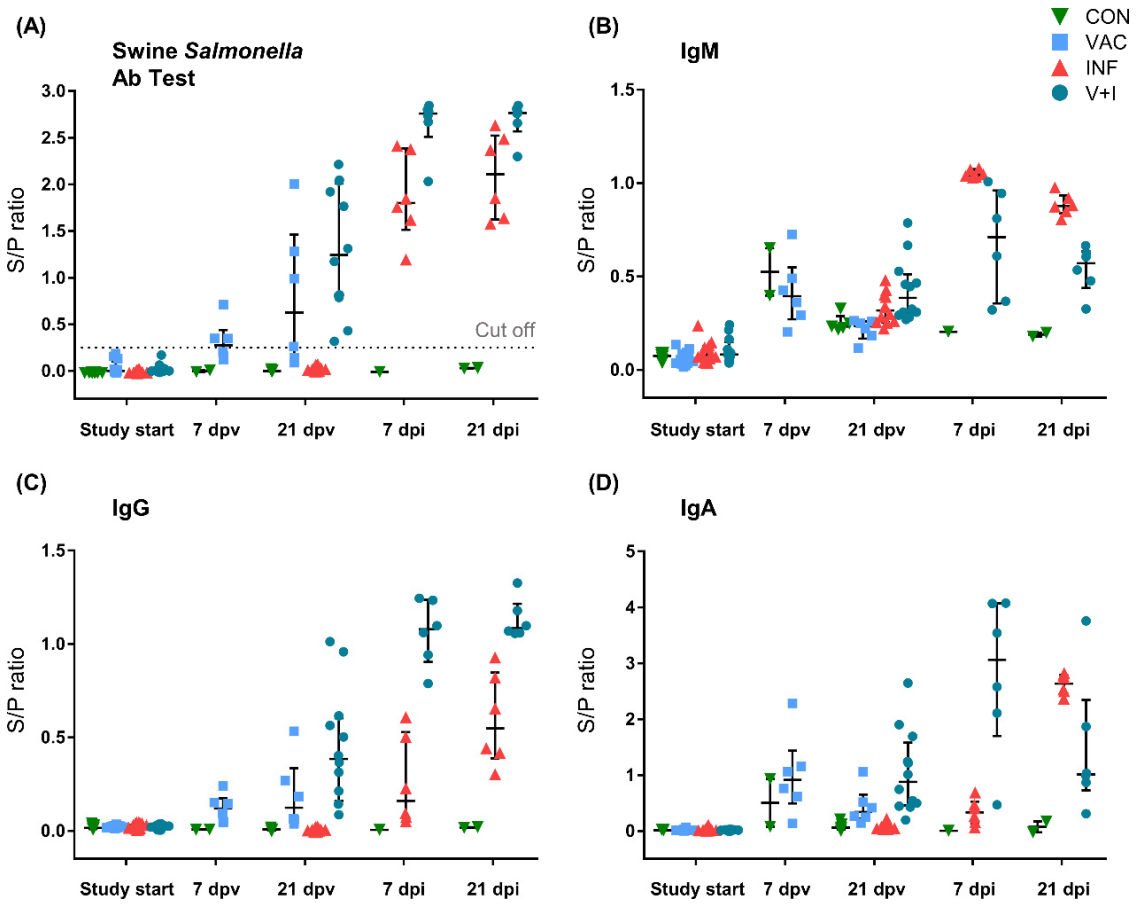

Figure 2. *Salmonella*-specific antibodies in serum. Serum samples were taken on day of arrival (SD-11, study start), 7 days post booster vaccination (7 dpv), on day of challenge infection (21 dpv) as well as 7 and 21 days after infection (7 and 21 dpi, respectively; note: number of serum samples varies on the different time points. Study start: n = 43; 7 dpv: n = 8; 21 dpv: n = 35; 7 dpi: n = 13; 21 dpi: n = 14). (**A**) *Salmonella*-specific antibodies were measured in the serum using the Swine *Salmonella* Ab Test (IDEXX). Sample to positive control (S/P) ratios were calculated by dividing optical densities (OD) of samples by OD of the positive reference control. The dotted line represents the recommended cut-off for piglets at an S/P ratio of 0.25. Results < 0.25 were defined as negative, samples ≥ 0.25 as positive. (**B**–**D**) *Salmonella*-specific IgM (**B**), IgG (**C**) and IgA (**D**) were measured in the serum by isotype-specific in-house ELISA. S/P ratios were obtained by calculating the ratio between mean OD of individual samples and mean OD of the reference serum. (**A**–**D**) Different colors and symbols represent different groups: green triangles depict control animals, light blue rectangles VAC animals, red triangles INF animals and petrol blue circles show V+I animals. The median is indicated by a central black line with whiskers showing the interquartile range.

3.3. Bacterial Load

Bacterial load analysis of the bacterial load did not detect *Salmonella* in any of the collected fecal samples before immunization (SDs –11, –10, –9) nor from any of the organs of the control group at any given time (data not shown). Similarly, spleen and liver were

2.9.1. Bacterial Load

Cfu per gram tissue, measured in tLN, jejunum, ileum, and cecum, were $\log_{10}$ transformed after adding a constant of one to each observation and used as univariate response in linear models applying function *lm* in base R fitting treatment and days after infection as fixed categorical main effects and the interaction between them. Both categorical fixed effects have two levels each; treatment: INF and V+I, and days after infection: 7 and 21 dpi, respectively. The interaction between treatment and days after infection is key in these models. We then calculated estimated marginal means (emmeans) for treatment groups for both days and tested for differences between them with package *emmeans* v1.4.7 [41]. We created bar plots to display the results of our hypothesis testing for estimated marginal means using packages *ggplot2* [42] and *ggpubr* [43] in which the fitted model is shown as black dots and the height of each bar, whiskers represent upper and lower 95% confidence intervals. *p*-values are derived from hypothesis testing of contrasts between estimated marginal means for treatment separately for both days after infection

For a more precise evaluation of STM loads in the organs most affected by STM infection, a quantitative detection of the STM challenge strain load was performed for samples of ICLN, jejunum, ileum, and cecum of V+I and INF animals 7 and 21 dpi (Figure 3C). Challenge strain numbers in ICLN as well as ileal and cecal mucosa were significantly reduced ($p < 0.1$) in V+I pigs 7 dpi compared to INF animals. The challenge strain content in the jejunal mucosa was likewise reduced in V+I animals albeit without reaching statistical significance due to large confidence intervals in both groups. The same trend could be observed at 21 dpi for all analyzed organs though without statistically significant differences between the V+I and INF group. Taken together, a lower challenge strain load was found in V+I pigs versus INF pigs for all investigated organs with differences reaching significance in ICLN, ileal, and cecal mucosa at 7 dpi.

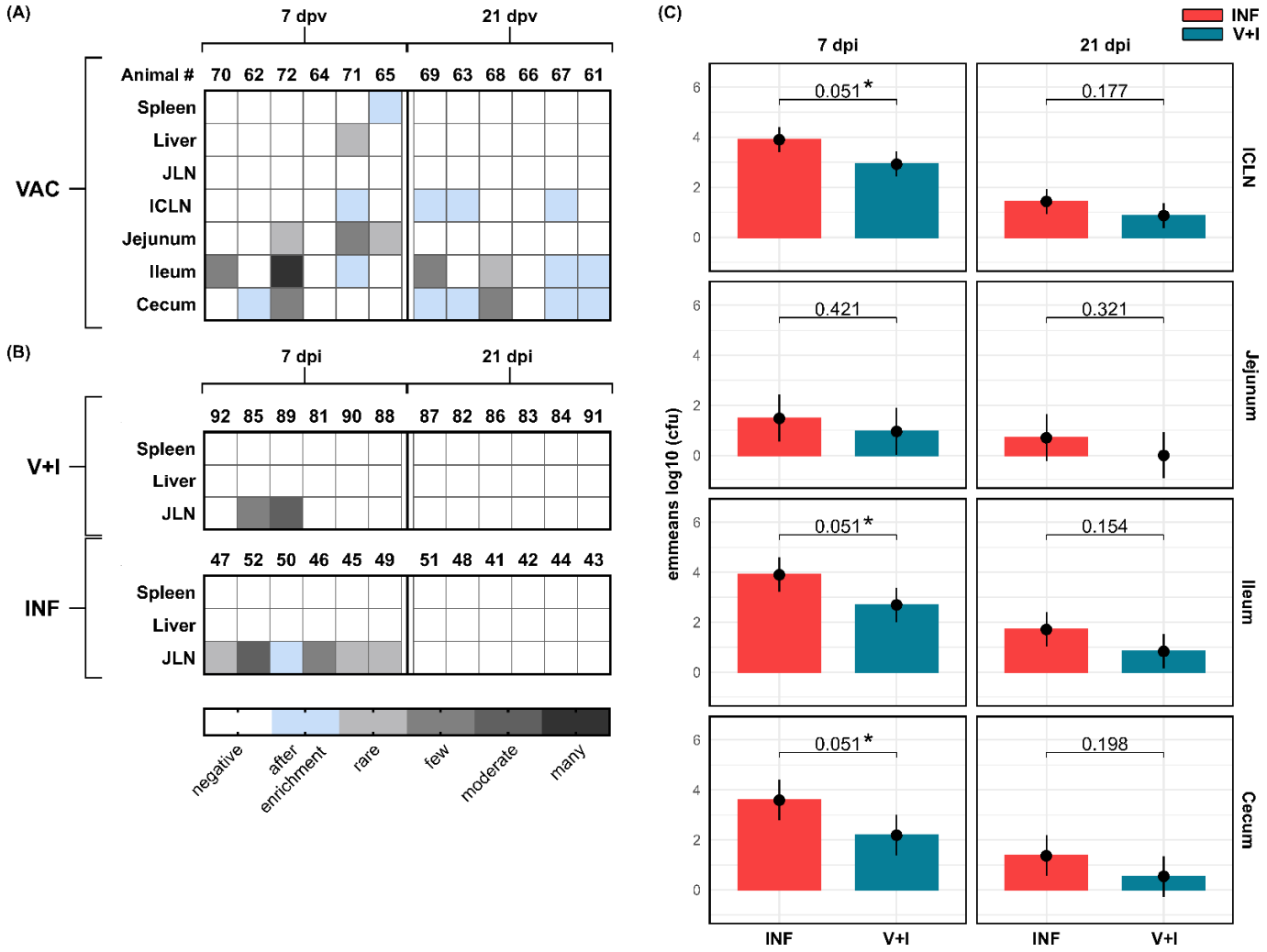

Figure 3. Detection of *Salmonella* Typhimurium in different treatment groups 7 and 21 days post vaccination (dpv)/infection (dpi). (**A**,**B**) Semi-quantitative microbiological analysis was performed for spleen, liver, JLN (all time points), ICLN, jejunum, ileum, and cecum (7/21 dpv only). Results are displayed as heat maps with a grayscale gradient depicting detection of *Salmonella* Typhimurium by agar isolation. Light blue boxes indicate isolation after enrichment. Negative findings for *Salmonella* Typhimurium are displayed in white. (**C**) Quantitative microbiological analysis was performed for ICLN, jejunum, ileum, and cecum from V+I (petrol) and INF animals (red) 7 and 21 dpi. Results are shown as bar charts. Y-axes depict estimated marginal means (emmeans) of the bacterial load (cfu/g tissue) on a $\log_{10}$ scale. Numbers above brackets show corresponding multiple testing corrected *p*-values applying a false discovery rate approach across all pairwise comparisons of contrasts between emmeans of V+I and INF animals for each day and tissue (* indicates $p < 0.1$). Black whiskers indicate the lower and upper 95% confidence intervals of emmeans.

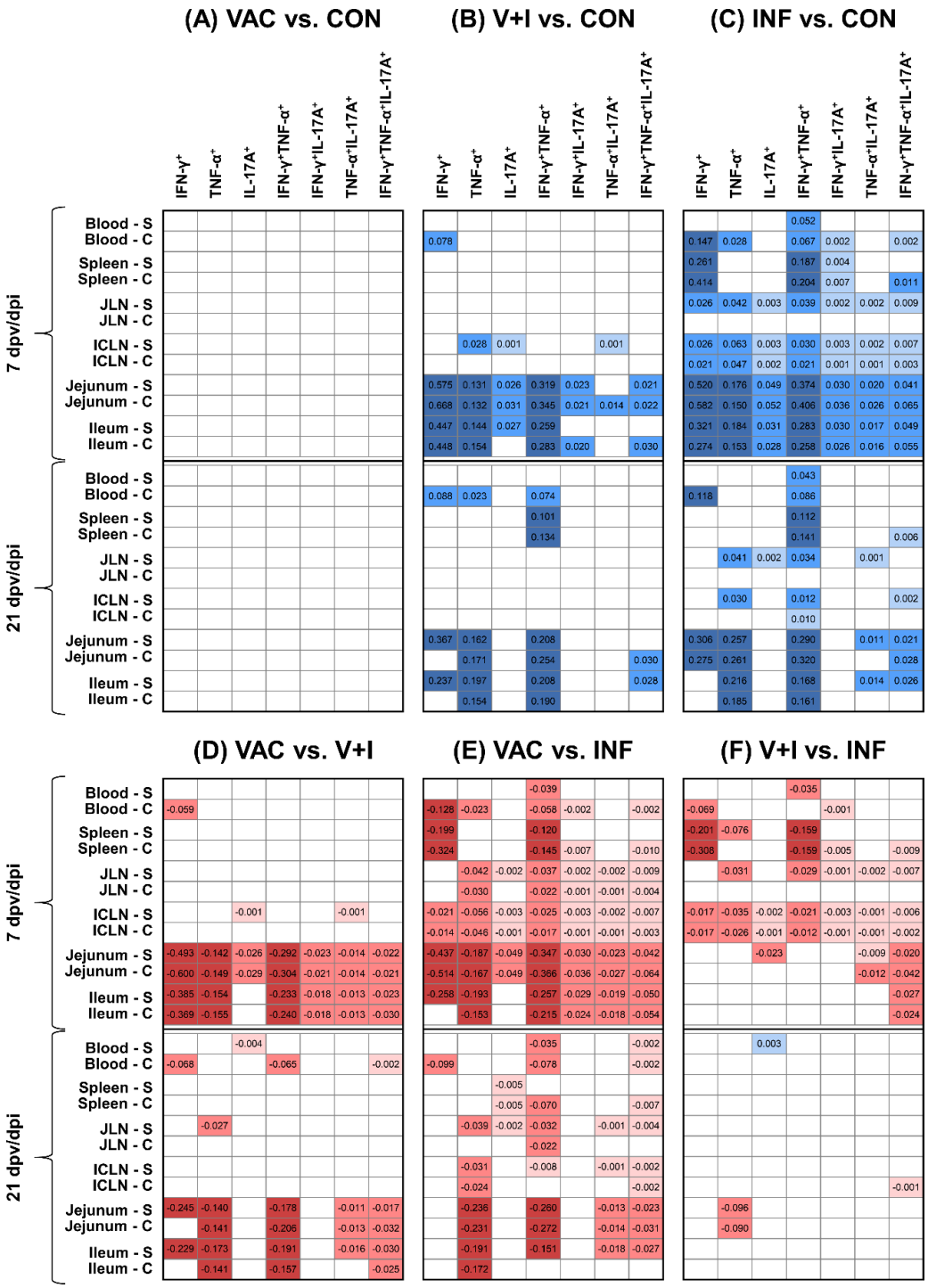

Figure 4. Contrasts of STM-stimulated cytokine-producing CD4+ T cells between treatment groups. Contrasts for (**A**) VAC versus CON, (**B**) V+I versus CON, (**C**) INF versus CON, (**D**) VAC versus V+I, (**E**) VAC versus INF, (**F**) V+I versus INF at 7 and 21 days post vaccination (dpv) or infection (dpi) in various tissues. Results are shown as effect size heatmaps. Samples were stimulated in vitro with STM antigen (S: Salmonella vaccine strain; C: challenge infection strain). White boxes represent non-significant differences between groups. Boxes with significant differences are color-coded and contain numbers with corresponding effect sizes also known as contrasts between estimated marginal means on a $\log_{10}$ level (dark blue: >0.1, blue: 0.1 to 0.01, light blue: <0.01; dark red: <−0.1, red: −0.1 to −0.01, light red: >−0.01). Boxes in blue indicate STM-stimulated cytokine-producing CD4+ T cells are more abundant in the group that is stated first in the comparison. Red boxes indicate higher abundances in the second group. Differences were considered significant at a multiple testing corrected 10% false discovery rate cut-off across all pairwise comparisons (7 and 21 dpv/dpi, four treatment groups, six tissues, and seven phenotypes).

2.9.2. T-Cell Response

We analyzed the T-cell response fitting univariate linear mixed models applying function *lmer* in R package *lme4* v1.1–21 [45]. Frequencies of cytokine-producing $CD4^+$, $CD8^+$ and $CD4^-CD8\beta^-$ T cells measured in blood, spleen, JLN, ICLN, jejunum, and ileum were $\log_{10}$ transformed after adding a constant of one to every observation. Hypothesis testing for $CD4^+$ and $CD4^-CD8\beta^-$ T cells was performed for IFN-γ single-producing, TNF-α single-producing, IL-17A single-producing, IFN-γ/TNF-α co-producing, IFN-γ/IL-17A co-producing, TNF-α/IL-17A co-producing and IFN-γ/TNF-α/IL-17A triple-producing cells. Measures of phenotypes involving IL-17A in $CD8^+$ T cells were too low, even after $\log_{10}$ transformation to be amenable for linear (mixed) models, i.e., violating the required assumptions of variance homogeneity and normal distribution of residuals and therefore excluded from hypothesis testing. To facilitate the comparison of the STM-specific T-cell response between groups after treatment, necropsy days for the different groups were aligned to 7 days post treatment (7 dpv and 7 dpi) and 21 days post treatment (21 dpv and 21 dpi). Control animals euthanized at 7 dpv/dpi and 21 dpv/dpi, respectively, served as reference values for the respective time points. Main fixed categorical effects in our model are animal treatment (four levels: VAC, V+I, INF, CON), in vitro stimulation (three levels: medium, STM vaccine strain, STM challenge strain) and days after vaccination/infection (two levels: 7 and 21 days) and a triple interaction between them, which is considered the key aspect of our model. The model also contained all pairwise lower-level interaction terms (treatment * stimulation, treatment * days after vaccination/infection, stimulation * days after vaccination/infection). We further included a fixed categorical effect of sex with two levels (male and female) as a nuisance factor. A random intercept animal effect was needed to account for the covariance structure in our data (multiple observations per pig). We used maximum likelihood estimation by setting option REML to false. All assumptions for linear mixed models for the analyzed cytokine phenotypes were met. Residuals and random intercepts were normally distributed and residuals homoscedastic. We verified the absence of collinearity via generalized variance inflation factors [46] using function *vif* in package *car* v3.0–8 [47]. We created bar plots to visualize the results of our hypothesis testing as described for the bacterial load. In short, the height of each bar and the black dots represent the respective estimated marginal means for each treatment group, stimulation level and days after vaccination/infection. Lower and upper 95% confidence intervals of estimated marginal means are shown as black whiskers. *p*-value brackets are derived from hypothesis testing of all pairwise treatment contrasts between estimated marginal means, separately for each level of stimulation and days after vaccination/infection, respectively. These are possible to estimate because of the triple interaction fitted in our models. The y-axes are scaled the same within T-cell subsets for the following three groups of tissues: blood and spleen, JLN and ICLN, jejunum and ileum, but separately for each measured phenotype, to ease interpretation of effect sizes. Multiple testing correction was applied across all tissues and phenotypes within cell type. Significance was declared at a multiple testing corrected 10% false discovery rate [44]. The following two samples were found to be extreme outliers in all T-cell subsets and consequently both removed from the raw ICS data and excluded from hypothesis testing: Animal Sw#69, spleen, stimulation with the challenge strain (due to a staining artefact); animal Sw#90, ileum, stimulation with the challenge strain (due to very low numbers of lymphocytes).

3. Results

3.1. Clinical Signs

Major aim of the study was the comparison of the T-cell immune response in VAC pigs and V+I pigs versus INF pigs. For animal welfare of INF animals, a lower infection dose was chosen compared to the one used in a previous trial where all animals underwent vaccination prior to challenge infection (1×10^9 cfu/animal compared to 5×10^9 cfu/animal, [33]). Consequently, clinical signs were very mild in all groups (data not shown). All groups showed homogeneous weight gain for most of the study (data

not shown). A reduction in average daily gain was observed in both infected groups (V+I pigs: from 1019.5 to 628.6 g/d; INF pigs: from 916.7 to 541.7 g/d) one week after infection. Additionally, rectal temperatures increased in infected animals during a ten-day period starting after the challenge infection. Rectal temperatures during this period ranged from 39.0 °C to 40.4 °C (V+I) and 39.0 °C to 40.5 °C (INF), with differences in average group temperature compared to the CON group reaching a maximum of 0.6 °C on SD50 in both infected groups. During the first three weeks of the study, animals from all groups intermittently showed slightly increased diarrhea scores, likely due to change in diet and stress caused by social group formation. After infection, V+I and INF animals showed slightly increased diarrhea scores in comparison to CON animals, but the levels were similar to what was observed in the adaption period and never exceeded an average group score of 1 (1 = pasty feces).

3.2. STM-Specific Antibodies

For the evaluation of the humoral response against STM, both the commercially available IDEXX Swine *Salmonella* Ab test (Figure 2A) and an in-house ELISA test based on whole-cell lysate of purified STM (Figure 2B,C) were used. Negative test results from the IDEXX ELISA verified the *Salmonella*-free status of all animals upon arrival (study start, Figure 2A). All pig sera from the control group remained below the cut-off value of 0.25 throughout the course of the study. S/P ratios of vaccinated animals (VAC and V+I) rose moderately after the second vaccination. A substantial increase was observed in S/P ratios of V+I and INF animals after STM infection with S/P ratios of V+I animals rising above those of INF animals. Although the IDEXX Swine *Salmonella* Ab test is directed against *Salmonella*-specific IgG, the in-house STM ELISA made it possible to distinguish between *Salmonella*-specific IgM, IgA, and IgG. No distinct group-specific impact of the vaccination on S/P ratios of STM-specific IgM was detected (Figure 2B). After infection, however, S/P ratios rose in all infected animals with the highest levels reached by INF pigs. The antibody profile of STM-specific IgG largely matched the results of the IDEXX Swine *Salmonella* Ab test (Figure 2C). Regarding STM-specific IgA, a slight rise of S/P ratios could be observed after vaccination followed by a strong increase in V+I pigs after challenge infection (Figure 2D). S/P ratios of IgA in INF animals experienced a delayed rise but eventually surpassed those of V+I animals 3 weeks post infection. Overall, largest differences between the V+I and INF group were detected at 7 dpi where STM-specific IgG and IgA levels were highest in the V+I group while IgM dominated in INF pigs.

3.3. Bacterial Load

Semi-quantitative analysis of the bacterial load did not detect *Salmonella* in any of the collected fecal samples before immunization (SDs −11, −10, −9) nor from any of the organs of the control group at any given time (data not shown). Similarly, spleen and liver were negative for all groups and time points except for two VAC animals from which the vaccine strain could be isolated at 7 dpv (Figure 3A). Although the STM vaccine strain was also found sporadically in ICLN and jejunum, it was more abundant in samples from ileum and cecum, especially 21 dpv. At 7 dpi, *Salmonella* was detected in the JLN of all INF animals (Figure 3B) but not in spleens or livers. In contrast, only in two of the V+I animals could *Salmonella* be isolated from the JLN at this time point. By day 21 post infection, JLN of both groups were negative for *Salmonella*.

For a more precise evaluation of STM loads in the organs most affected by STM infection, a quantitative detection of the STM challenge strain load was performed for samples of ICLN, jejunum, ileum, and cecum of V+I and INF animals 7 and 21 dpi (Figure 3C). Challenge strain numbers in ICLN as well as ileal and cecal mucosa were significantly reduced ($p < 0.1$) in V+I pigs 7 dpi compared to INF animals. The challenge strain content in the jejunal mucosa was likewise reduced in V+I animals albeit without reaching statistical significance due to large confidence intervals in both groups. The same trend could be observed at 21 dpi for all analyzed organs though without statistically significant

differences between the V+I and INF group. Taken together, a lower challenge strain load was found in V+I pigs versus INF pigs for all investigated organs with differences reaching significance in ICLN, ileal, and cecal mucosa at 7 dpi.

3.4. STM-Specific Production of IFN-γ, TNF-α and/or IL-17A by CD4$^+$, CD8$^+$ and CD4$^-$CD8β$^-$ T Cells

To gain a thorough understanding of the STM-specific T-cell response in the different animal groups at the two time points after treatment, lymphocytes isolated from blood, spleen, JLN, ICLN, jejunum, and ileum at necropsy were stimulated in vitro with either the STM vaccine strain or the STM challenge infection strain and subjected to intracellular cytokine staining. Cells cultivated in medium only acted as negative controls. For the identification of STM-specific cytokine production in T cells, live lymphocytes were gated on CD3$^{+/dim}$CD4$^+$ cells to identify CD4$^+$ T cells (Figure S1). Within CD3$^+$CD4$^-$ cells, CD8$^+$ T cells were identified by a gate on CD8α$^+$CD8β$^+$ cells, leaving a remaining subset with a CD4$^-$CD8α$^{+/-}$CD8β$^-$ phenotype. Subsequently, all three T-cell subsets were further analyzed for IFN-γ, TNF-α and IL-17A production. Representative FCM plots depicting cytokine-producing cells within CD4$^+$, CD8$^+$ and CD4$^-$CD8β$^-$ T cells for all treatment groups are shown in Figures S2–S4. For CD4$^+$ and CD4$^-$CD8β$^-$ T cells, seven possible cytokine-producing phenotypes were identified by Boolean combination gates: IFN-γ single-producing, TNF-α single-producing, IL-17A single-producing, IFN-γ/TNF-α co-producing, IFN-γ/IL-17A co-producing, TNF-α/IL-17A co-producing and IFN-γ/TNF-α/IL-17A triple-producing cells. Unsurprisingly, barely any IL-17A production was discovered within CD8$^+$ T cells (Figure S3). Consequently, only IFN-γ single-producing, TNF-α single-producing and IFN-γ/TNF-α co-producing CD8$^+$ T cells were further investigated.

Frequencies of cytokine-producing T cells for all analyzed phenotypes, stimulation variants, organs, treatment groups and T-cell subsets are summarized as individual graphs in Figure S5 (CD4$^+$ T cells), Figure S6 (CD8$^+$ T cells) and Figure S7 (CD4$^-$CD8β$^-$ T cells). The y-axes of these graphs are scaled individually for each cytokine-producing T-cell phenotype but kept the same for the following organ groups: blood and spleen, JLN and ICLN, jejunum and ileum. Looking at all analyzed T-cell subsets on this global level, highest frequencies for most cytokine-related phenotypes were produced by CD4$^+$ T cells. In accordance with previously published data [33] and regardless of the cytokine phenotype, frequencies of STM-specific CD4$^+$ T cells within the two intestinal sections, jejunum and ileum, largely surpassed those measured in the other organs.

3.5. Treatment-Related Contrasts for STM-Specific Cytokine-Producing CD4$^+$ T Cells

Main aim of the study was the comparison of frequencies of cytokine-producing T cells between the four treatment groups to detect differences between the effect of the vaccine and the infection on the cellular immune response of the pigs. To this end, proportions of cytokine-producing T cells for all phenotypes, organs, time points, and treatment groups were modeled in a generalized mixed model. STM-specific T-cell responses were compared between 7 dpv (VAC group) and 7 dpi (V+I and INF group), as well as 21 dpv and 21 dpi. Control animals served as reference values for either 7 dpv/dpi or 21 dpv/dpi, depending on their respective days of euthanasia.

Since the raw data indicated a dominant role for cytokine-producing CD4$^+$ T cells in the response against STM, statistical analysis was first conducted on this T-cell subset. Contrasts between groups that yielded significant *p*-values are highlighted in Figure 4 with their respective effect sizes. Darker colors indicate higher effect sizes. Stimulation during in vitro cultivation with the vaccine strain (Salmoporc, S) and with the challenge infection strain (Challenge, C) yielded largely similar results. By applying our generalized mixed model, no significant differences were detected between VAC and CON animals (Figure 4A). After challenge infection, differences between groups became more pronounced. At 7 dpi, fields with significant contrasts where relative abundances of cytokine-producing CD4$^+$ T cells in V+I animals surpassed those of the CON group were largely confined to the intestine

(Figure 4B). By contrast, cytokine production by STM-specific $CD4^+$ T cells in INF animals at 7 dpi was not only significantly higher in the gut compared to CON animals but also in systemic organs and both lymph nodes (Figure 4C). Two weeks later, at 21 dpi, frequencies for most phenotypes of cytokine-producing $CD4^+$ T cells were at lower levels in V+I and INF pigs (Figure S5). Consequently, fewer significant differences could be observed for both groups but still dominated within the contrasts between the INF and the CON group (Figure 4B,C, bottom half). Similar results but with effects into the opposite direction were obtained for comparisons of the VAC group with the V+I and the INF group (Figure 4D,E).

Overall, highest abundance of significant contrasts and highest effect sizes were obtained for STM-specific IFN-γ single-producing, TNF-α single-producing and IFN-γ/TNF-α co-producing $CD4^+$ T cells. Of note, cytokine-producing phenotypes involving IL-17A production such as IFN-γ/IL-17A co-producing, TNF-α/IL-17A co-producing and IFN-γ/TNF-α/IL-17A triple-producing STM-specific $CD4^+$ T cells were significantly higher in almost all organs from INF animals at 7 dpi when compared to either the control (Figure 4C) or the VAC group (Figure 4E). Nevertheless, effect sizes stayed below those obtained for IFN-γ single-producing, TNF-α single-producing and IFN-γ/TNF-α co-producing $CD4^+$ T cells.

When contrasting the two infected groups, V+I and INF animals, 7 days after infection, higher levels of STM-specific cytokine-producing $CD4^+$ T cells were elicited in the INF group with significant differences emerging mainly in blood and spleen as well as the two lymph nodes (Figure 4F), even encompassing all seven cytokine-producing phenotypes in the ICLN. Additionally, IFN-γ/TNF-α/IL-17A triple-producing $CD4^+$ T cells were significantly more abundant in jejunum and ileum of INF pigs in comparison to the V+I group. Although significantly higher abundances of cytokine-producing STM-specific $CD4^+$ T cells were detected in INF animals compared to V+I pigs at 7 dpi in systemic sites and intestinal lymph nodes, differences in levels of cytokine production between these groups were less pronounced by 21 dpi.

The significant contrasts found between $CD4^+$ T cells from V+I and INF animals clearly indicated an effect of the vaccination. This motivated us to scrutinize the $CD4^+$ T-cell response after vaccination in the VAC group in more detail. Indeed, a closer look at individual cytokine phenotypes revealed higher emmeans in VAC animals for STM-specific IFN-γ single-producing (Figure 5) and IFN-γ/TNF-α co-producing $CD4^+$ T cells (Figure 6) in spleen, ICLN, jejunum, and ileum at 7 dpv when compared to the control group. This was still the case for the two aforementioned phenotypes at 21 dpv in spleen (Figure S8B), ICLN (Figure S8D), jejunum (Figure S8E) and ileum (Figure S8F). Due to the large confidence intervals present in the control group, however, these differences did not reach significance at either time point. Of note, in the spleen, and to some extent in the ICLN, emmeans for IFN-γ single-producing and IFN-γ/TNF-α co-producing $CD4^+$ T cells in VAC animals were close to levels obtained in V+I animals. By contrast, and as described for the effect sizes in Figure 4C,E,F, IFN-γ/TNF-α/IL-17A triple-producing $CD4^+$ T cells were clearly outstanding in the INF group, followed by the V+I group (Figure 6).

3.6. Treatment-Related Contrasts for STM-Specific Cytokine-Producing $CD8^+$ and $CD4^-CD8\beta^-$ T Cells

In addition to $CD4^+$ T cells, proportions of STM-specific cytokine-producing phenotypes were also modeled for $CD8^+$ and $CD4^-CD8\beta^-$ T cells (Figures S9 and S10). In accordance with the raw data (Figures S6 and S7), statistical analysis of both remaining T-cell subsets generated less significant differences between groups than found for $CD4^+$ T cells. Regarding $CD8^+$ T cells, most instances of significance were found for contrasts of CON or VAC animals with the INF group at 7 dpi (Figure S9). Unlike $CD4^+$ T-cell results for the same comparisons, relative abundance of IFN-γ and/or TNF-α producing $CD8^+$ T cells were significantly higher in INF animals mostly in the spleen and both lymph nodes, but rarely in the intestine. This could also be observed for STM-specific cytokine-producing $CD4^-CD8\beta^-$ T cells though analysis of this subset overall provided a more heterogeneous

clearly indicated an effect of the vaccination. This motivated us to scrutinize the CD4+ T-cell response after vaccination in the VAC group in more detail. Indeed, a closer look at individual cytokine phenotypes revealed higher emmeans in VAC animals for STM-specific IFN-γ single-producing (Figure 5) and IFN-γ/TNF-α co-producing CD4+ T cells (Figure 6) in spleen, ICLN, jejunum, and ileum at 7 dpv when compared to the control group. This was still the case for the two aforementioned phenotypes at 21 dpv in spleen (Figure S8B), ICLN (Figure S8D), jejunum (Figure S8E) and ileum (Figure S8F). Due to the large confidence intervals present in the control group, however, these differences did not reach significance at either time point. Of note, in the spleen, and to some extent in the ICLN, emmeans for IFN-γ single-producing and IFN-γ/TNF-α co-producing CD4+ T cells in VAC animals were close to levels obtained in V+I animals. By contrast, and as described for the effect sizes in Figures 4C,E,F, IFN-γ/TNF-α/IL-17A triple-producing CD4+ T cells were clearly outstanding in the INF group, followed by the V+I group (Figure 6).

pattern (Figure S10). Similar to CD4+ T cells, STM-specific cytokine production by the CD8+ and CD4−CD8β− subsets was significantly lower in systemic organs and lymph nodes of V+I pigs at 7 dpi when compared to INF pigs. Of all phenotypes analyzed for CD8+ and CD4−CD8β− T cells, the IFN-γ/TNF-α co-producing phenotype proved to be the most prominent one with higher levels in INF animals compared to all other groups.

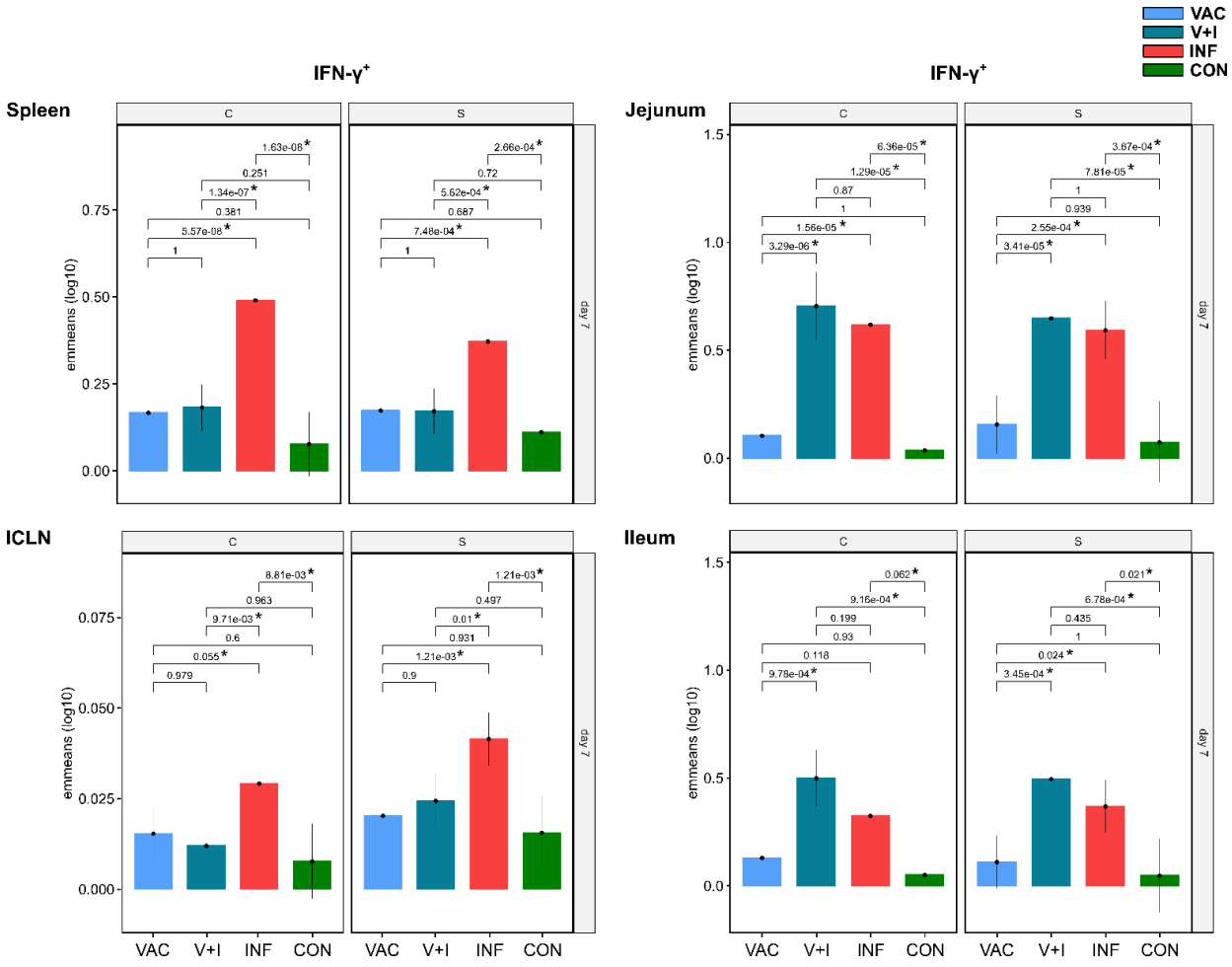

Figure 5. Estimated marginal means (emmeans) of STM-stimulated IFN-γ single-producing CD4+ T cells compared across treatment groups 7 days post vaccination/infection in spleen, ICLN, jejunum, and ileum. Samples were stimulated in vitro with STM antigen (S: Salmoporc, vaccine strain; C: challenge/infection strain). Y-axes depict emmeans of STM-stimulated IFN-γ single-producing CD4+ T cells for each treatment group on a $\log_{10}$ scale. Numbers above brackets show corresponding false discovery rate corrected p-values for contrasts between emmeans (* indicates $p < 0.1$) in the VAC (light blue), V+I (petrol), INF (red) and CON group (green). Black whiskers indicate the lower and upper 95% confidence intervals of emmeans. Multiple testing correction was applied across all comparisons (all six pairwise comparisons of the four treatment groups, for 7 and 21 dpv/dpi, for each of the three stimulation levels, six tissues, and seven phenotypes). This leads to a total multiple testing load of $6 * 2 * 3 * 6 * 7 = 1512$ comparisons.

emmeans. Multiple testing correction was applied across all comparisons (all six pairwise comparisons of the four treatment groups, for 7 and 21 dpv/dpi, for each of the three stimulation levels, six tissues, and seven phenotypes). This leads to a total multiple testing load of 6*2*3*6*7 = 1512 comparisons.

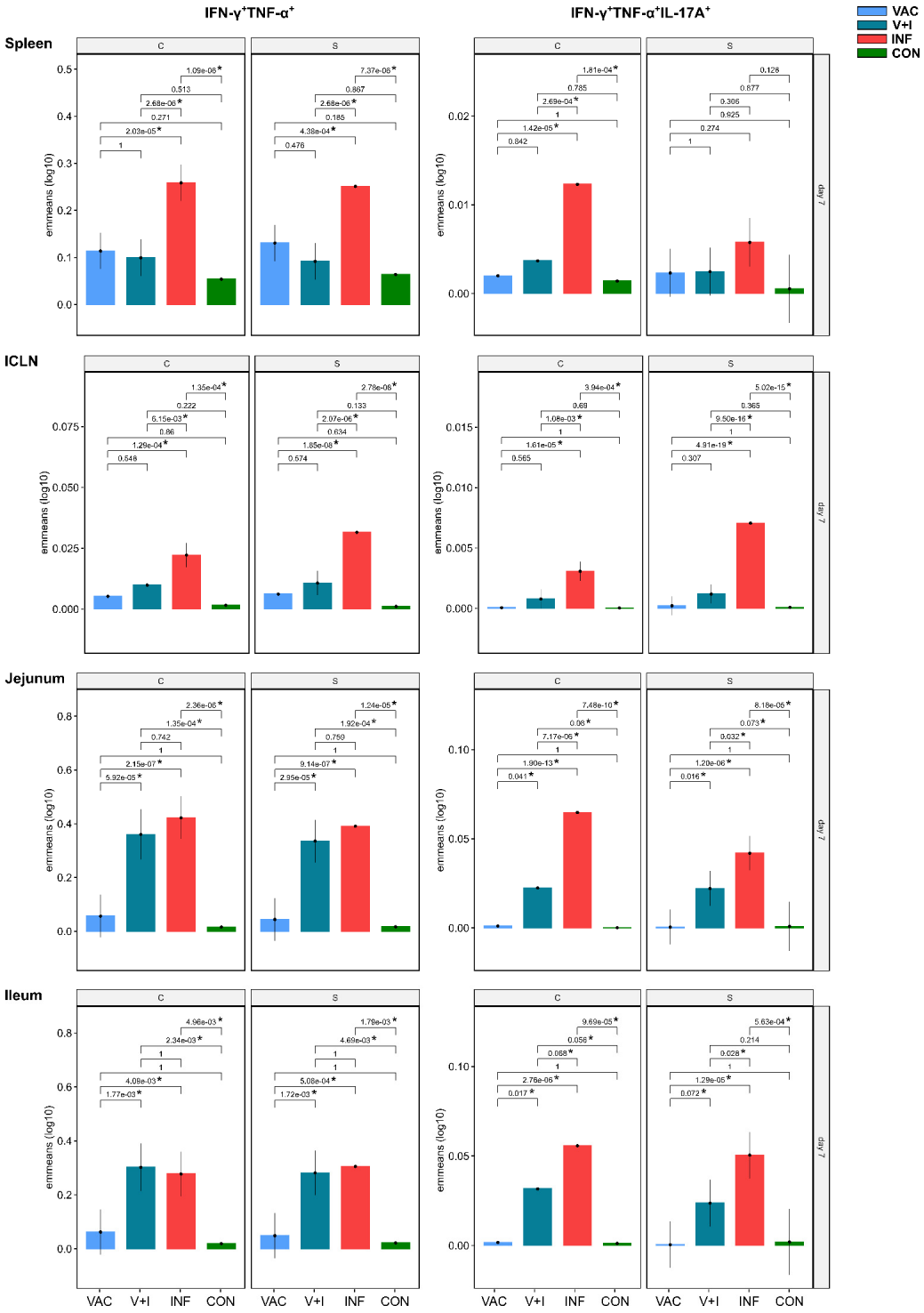

Figure 6. Estimated marginal means (emmeans) of STM-stimulated IFN-γ/TNF-α and IFN-γ/TNF-α/IL-17A co-producing CD4+ T cells compared across treatment groups 7 days post vaccination/infection in spleen, ICLN, jejunum, and ileum. Samples were stimulated in vitro with STM antigen (S: Salmoporc, vaccine strain; C: challenge/infection strain). Y-axes depict emmeans of STM-stimulated IFN-γ/TNF-α and IFN-γ/TNF-α/IL-17A co-producing CD4+ T cells for each treatment group on a $\log_{10}$ scale. Numbers above brackets show corresponding false discovery rate corrected *p*-values for contrasts between emmeans (* indicates $p < 0.1$) in the VAC (light blue), V+I (petrol), INF (red) and CON group (green). Black whiskers indicate the lower and upper 95% confidence intervals of emmeans. Multiple testing correction was applied across all comparisons (all six pairwise comparisons of the four treatment groups, separately for 7 and 21 dpv/dpi, separately for each of the three stimulation levels, six tissues, and seven phenotypes). This leads to a total multiple testing load of $6 * 2 * 3 * 6 * 7 = 1512$ comparisons.

Figure 6. Estimated marginal means (emmeans) of STM-stimulated IFN-γ/TNF-α and IFN-γ/TNF-α/IL-17A co-producing CD4+ T cells compared across treatment groups 7 days post vaccination/infection in spleen, ICLN, jejunum, and ileum. Samples were stimulated in vitro with STM antigen (S: Salmoporc, vaccine strain; C: challenge/infection strain). Y-axes depict emmeans of STM-stimulated IFN-γ/TNF-α and IFN-γ/TNF-α/IL-17A co-producing CD4+ T cells for each treatment group on a $\log_{10}$ scale. Numbers above brackets show corresponding false discovery rate corrected *p*-values for contrasts between emmeans (* indicates $p < 0.1$) in the VAC (light blue), V+I (petrol), INF (red) and CON group (green). Black whiskers indicate the lower and upper 95% confidence intervals of emmeans. Multiple testing correction was applied across all comparisons (all six pairwise comparisons of the four treatment groups, separately for 7 and 21 dpv/dpi, separately for each of the three stimulation levels, six tissues, and seven phenotypes). This leads to a total multiple testing load of $6 * 2 * 3 * 6 * 7 = 1512$ comparisons.

4. Discussion

STM is a major foodborne pathogen that continues to be highly prevalent in the swine industry. For its control in affected swine herds, vaccination is a recommended and effective measure. Up to date, several inactivated vaccines have shown a reasonable efficacy in controlled as well as in field studies [48–50]. Nevertheless, live attenuated commercial vaccines such as Salmoporc (Ceva Santé Animale, Libourne, France) as well as other live vaccine candidates [51,52] are more likely to induce a strong stimulation of the humoral and the cell-mediated immune response. This applies in particular to the Th1 response, which appears to be a key element in controlling STM infections in the mouse model [26,27,53,54] and in pigs [55]. Hence, we considered the use of the live vaccine Salmoporc and its comparison to infection as the most relevant setting to study the porcine T-cell response against STM in detail.

In a previous study, we have shown the induction of multifunctional STM-specific $CD4^+$ T cells after vaccinating pigs two times with Salmoporc followed by an infection with STM. To expand on this, we have now investigated the T-cell response in various tissues of only vaccinated (VAC), vaccinated and infected (V+I) as well as only infected (INF) pigs with untreated pigs as a control (CON) group.

To confirm successful vaccination and infection of the animals, serological and bacteriological parameters were ascertained. The vaccine strain could be isolated from the gut tissue of most VAC animals after two immunizations with the Salmoporc vaccine. After challenge infection, V+I animals exhibited significantly lower bacterial loads in ICLN, ileal, and cecal mucosa compared to the INF group. Similar reductions in challenge strain contents of vaccinated animals have been observed in other studies where pigs underwent two oral vaccinations with the Salmoporc vaccine followed by oral challenge infection [12,13,15].

Analysis of serum antibodies with the Swine *Salmonella* Ab Test by IDEXX and an in-house Ig-specific STM ELISA revealed very similar kinetics for STM-specific IgG with both tests. In contrast to S/P ratios of both STM-specific IgG and IgA, no clear vaccination-related elevation of IgM levels could be detected post immunization. Similar observations were made in other studies after oral vaccination of pigs with Salmoporc [15,19]. This finding may be due to natural IgM antibodies which bind with low affinity but are highly cross-reactive and would be present in control and treated animals alike [56]. After challenge infection, S/P ratios of STM-specific IgM increased in all infected animals with highest levels measured in INF animals. This suggests that the more virulent infection strain leads to an activation of naïve B cells that produce bona fide STM-specific IgM antibodies. A considerable elevation was also detected for STM-specific IgG and IgA with S/P ratios of V+I animals far surpassing those of INF animals at 7 dpi. Of note, overall levels of STM-specific IgA were up to 3-fold higher compared to STM-specific IgG. Antigen-specific serum IgG and IgA are mainly produced by long-lived plasma cells in the bone marrow (BM). Interestingly, it has recently been demonstrated that *Salmonella* inhibits IgG-secreting but not IgA-secreting plasma cells in the BM of mice [57]. SiiE, the *Salmonella* protein identified as responsible, seems to compete with laminin on BM stromal cells for binding of IgG-secreting plasma cells leading to their depletion [57]. STM might employ the same strategy in swine to evade humoral immunity, potentially explaining the lower increases in STM-specific serum IgG observed in our and other [15] studies.

To further investigate the T-cell immune response against STM in the pig, we looked for the production of IFN-γ, TNF-α and/or IL-17A by $CD4^+$, $CD8^+$ and $CD4^-CD8\beta^-$ T cells in the four different treatment groups. Of all investigated phenotypes, STM-specific IFN-γ/TNF-α co-producing $CD4^+$ T cells stood out with the largest number of significant contrasts between groups accompanied by considerable effect sizes, especially in spleen and intestine. Although differences in relative abundances of STM-specific $CD4^+$ T cells between VAC and CON animals did not reach significance for any cytokine phenotype, STM-specific IFN-γ/TNF-α co-producing $CD4^+$ T cells were induced at low levels in spleen, ICLN, jejunum, and ileum at both 7 and 21 dpv after vaccination which suggests

a role in protection. This corresponds to data collected in murine studies, where IFN-γ producing Th1 cells have been manifoldly reported to play a crucial role in controlling STM infection [26,27,53,54]. In fact, best correlates of protection for infections with other intracellular pathogens have been multifunctional $CD4^+$ T cells, capable of producing IFN-γ, TNF-α and IL-2 simultaneously [58–60]. Regarding the fairly low levels of STM-specific cytokine-producing $CD4^+$ T cells in response to vaccination alone, it is conceivable that the two-dose immunization also induced other relevant cell types which were not investigated in this study such as memory B cells or IgA-secreting plasma cells in the intestine. A strong IgA response in particular is desirable for protection of the epithelial barrier against mucosal pathogens and secretory IgA has been shown to have protective capacity against STM in mice [61]. IgA-secreting plasma cells specific for STM LPS have been identified in murine Peyer's patches after orogastric STM immunization and infection in the streptomycin mouse model of nontyphoidal *Salmonella* [62]; however, this has not yet been done in the pig. This is partially due to a substantial lack of tools to study porcine memory B cells and plasma cells [63] which was also the reason why no further investigations on B cells were performed in this study.

This study also identified IL-17A-producing CD4 T-cell phenotypes. Induction of STM-specific IL-17A single-producing, IFN-γ/IL-17A co-producing, TNF-α/IL-17A co-producing and IFN-γ/TNF-α/IL-17A triple-producing $CD4^+$ T cells mainly took place after infection with highest levels reached in INF animals. IL-17A is a key component of mucosal immunity by promoting the recruitment of neutrophils and guarding the integrity of the epithelial barrier [64]. However, IL-17A production is also associated with immunopathology and pathogenic subsets of Th17 cells have been described [65,66]. Our findings suggest that the more virulent infection strain supports the induction of Th17 cells in STM-infected pigs. Due to the chosen low infection dose, which was controlled by the immune system of the INF pigs, these cells may have had a beneficial role in the immune response. However, it is conceivable that at higher doses of infection such cells also contribute to immunopathology. In this context, the restriction of Th17-associated phenotypes to the gut tissue in V+I animals in comparison to INF animals might be interpreted as another beneficial outcome of the vaccination. Indeed, these cells with their inflammatory potential may also under field conditions stay local and therefore contribute to an efficient immune response yet could contribute to a more systemic inflammation in infected animals.

Of key interest in the study was the comparison of the T-cell response between V+I and INF pigs after challenge infection to evaluate possible protective effects of the vaccine. Interestingly, relative abundances of STM-specific cytokine-producing $CD4^+$ T cells at 7 dpi were significantly higher in INF animals than in V+I animals for numerous cytokine phenotypes in blood, spleen and JLN, even extending to all seven phenotypes in the ICLN. Significantly higher levels in the gut samples of INF animals were limited to IL-17A containing phenotypes, such as STM-specific TNF-α/IL-17A co-producing and IFN-γ/TNF-α/IL-17A triple-producing $CD4^+$ T cells. These findings correspond to microbiological results where bacterial loads in V+I pigs were reduced in the two analyzed lymph nodes and intestinal sections when compared to the INF group. Since significant differences at 7 dpi between the two infected groups mainly centered on systemic organs and lymph nodes, it can be speculated that vaccine-induced STM-specific effector/memory $CD4^+$ T cells contribute to a reduction of STM colonization both locally and via homing to the gut thus helping to contain the pathogen in the intestine and hindering systemic dissemination. However, as outlined above, other vaccine-induced immune mechanisms such as local IgA may also have contributed to this reduction in STM colonization.

Apart from $CD4^+$ T cells, we examined other potentially relevant T-cell subsets such as $CD8^+$ T cells and $CD4^-CD8\beta^-$ T cells. Although frequencies of STM-specific cytokine-producing $CD8^+$ T cells were lower than those measured for the $CD4^+$ or the $CD4^-CD8\beta^-$ T-cell subset, TNF-α-single and IFN-γ/TNF-α co-producing cells were significantly higher in INF pigs in comparison to the other treatment groups at 7 dpi, mainly in systemic organs

and mesenteric lymph nodes. Reports on $CD8^+$ T cells in mice have mostly assigned them a modest role in protection against *Salmonella* [67,68]. In contrast, strong $CD8^+$ T-cell responses have been observed in humans after oral immunization with a live attenuated *S.* Typhi vaccine and in a challenge infection model with wild type *S*. Typhi [69,70]. However, it must be noted that the experimental layout for T-cell re-stimulation used in our study is not ideal for $CD8^+$ T cells. The MHC class I presentation necessary for stimulation of $CD8^+$ T cells requires presence of the antigen in the cytoplasm of APCs which is usually not achieved when using inactivated antigen.

Although relative abundance of cytokine-producing $CD4^-CD8\beta^-$ T cells also prevailed in INF pigs when compared to the other groups, depiction of significant contrasts yielded a scattered response pattern across phenotypes and organs. This might be explained by the different cell types possibly present in the $CD3^+CD4^-CD8\beta^-$ gate such as NKT cells, MAIT cells and γδ T cells. Although a few studies addressed the involvement of γδ T cells, it has been suggested that they contribute to host defense in *Salmonella* infection in mice [71,72], where they have been described as IL-17A-producers in spleen and intestinal mucosa [30,73]. Similarly, in chickens, another γδ-high species such as the pig, increased numbers of $CD8\alpha^{+high}$ γδ T cells were observed in blood, spleen and cecum after STM infection along with enhanced expression levels of IFN-γ mRNA in these cells [74]. Further staining assays that include a marker directed against TCR-γδ are necessary to clarify their role during STM infection in the pig.

In conclusion, our data suggest an important role of $CD4^+$ T cells in protective immune responses against STM in swine with possible additional contributions from B cells, $CD8^+$ T cells and γδ T cells. Vaccination of pigs with the live attenuated STM vaccine Salmoporc significantly reduces bacterial colonization in ICLN, ileal and cecal mucosa and stimulates local immunity in the gut thus impeding spread of STM to lymph nodes and systemic organs.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/vaccines9080845/s1, Figure S1: Gating strategy for FCM analyses of $CD4^+$, $CD8^+$ and $CD4^-CD8\beta^-$ T cells, Figure S2: Representative raw data for FCM analysis of $CD4^+$ T cells, Figure S3: Representative raw data for FCM analysis of $CD8^+$ T cells, Figure S4: Representative raw data for FCM analysis of $CD4^-CD8\beta^-$ T cells, Figure S5: Frequencies of STM-stimulated cytokine-producing $CD4^+$ T cells in blood (A), spleen (B), JLN (C), ICLN (D), jejunum (E) and ileum (F), Figure S6: Frequencies of STM-stimulated cytokine-producing $CD8^+$ T cells in blood (A), spleen (B), JLN (C), ICLN (D), jejunum (E) and ileum (F), Figure S7: Frequencies of STM-stimulated cytokine-producing $CD4^-CD8\beta^-$ T cells in blood (A), spleen (B), JLN (C), ICLN (D), jejunum (E) and ileum (F), Figure S8: Estimated marginal means of STM-stimulated IFN-γ/TNF-α/IL-17A producing $CD4^+$ T cells in blood compared across treatment groups on day 7 and day 21 post vaccination or infection, Figure S9: Contrasts of STM-stimulated cytokine-producing $CD8^+$ T cells between treatment groups at 7 and 21 days post vaccination or infection in various tissues, Figure S10: Contrasts of STM-stimulated cytokine-producing $CD4^-CD8\beta^-$ T cells between treatment groups at 7 and 21 days post vaccination or infection in various tissues.

Author Contributions: Conceptualization, methodology, supervision, project administration, S.S. (Sven Springer), T.T., V.F., A.L., A.S. and W.G.; formal analysis, S.S. (Selma Schmidt) and M.D.; investigation, S.S. (Selma Schmidt), H.K., M.S., K.H.M., M.R.S., M.K., E.V., S.D., C.K., J.S. and S.S. (Sven Springer); writing—original draft preparation, S.S. (Selma Schmidt); writing—review and editing, M.D., S.S. (Sven Springer), T.T., A.L. and W.G. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: S.S. (Sven Springer), T.T. and V.F. are employed by Ceva Innovation Center GmbH. The authors declare that this study received funding from Ceva Innovation Center GmbH (formerly part of IDT Biologika GmbH), Dessau-Roßlau, Germany. The co-authors employed by the funder were involved in study design, generation and interpretation of the results as outlined in the "author contributions" statement. However, this did not influence the scientific integrity of the study and the presented findings. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Materials: T-cell cytokine response in *Salmonella* Typhimurium vaccinated versus infected pigs

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Figure S1. Gating strategy for FCM analyses of $CD4^+$, $CD8^+$ and $CD4^-CD8\beta^-$ T cells. Intracellular cytokine staining was performed on lymphocytes isolated from several tissues following overnight *in vitro* stimulation with STM antigen. The gating strategy for FCM analyses of $CD4^+$, $CD8^+$ and $CD4^-CD8\beta^-$ T cells is shown for one representative animal (Sw#89, V+I group, SD50) in one organ (ileum-derived LPL, re-stimulation with the vaccine strain). Cells were gated according to their light scatter properties and further subgated for live cells (VDeFluor506 negative). After exclusion of doublets, a gate was set on $CD3^{+/dim}CD4^+$ T cells and remaining cells with a $CD8\beta^+$ phenotype were excluded by an additional gate. Subsequently, gates were set on $CD3^+CD8\alpha^+CD8\beta^+$ T cells and $CD3^+CD8\alpha^{+/-}CD8\beta^-$ T cells. Finally, IFN-γ-, TNF-α- and IL-17A-producing cells were identified for all three T-cell subsets and subjected to a Boolean analysis.

Figure S2. Representative raw data for FCM analysis of $CD4^+$ T cells. Intracellular cytokine staining was performed on lymphocytes isolated from several tissues following overnight *in vitro* stimulation with STM antigen. Co-production of IFN-γ/TNF-α, IFN-γ/L-17A and TNF-α/IL-17A in $CD4^+$ T cells is shown for blood, spleen, ICLN and ileum for samples that were stimulated with the vaccine strain. Representative data from one pig of each treatment group is shown: CON (Sw#6), VAC (Sw#65), V+I (Sw#89) and INF (Sw#52). Approximately $1x10^5$ cells are displayed for blood and spleen, $2x10^5$ cells for ICLN and $5x10^4$ cells for the ileum.

Figure S3. Representative raw data for FCM analysis of $CD8^+$ T cells. Intracellular cytokine staining was performed on lymphocytes isolated from several locations following overnight *in vitro* stimulation with STM antigen. Co-production of IFN-γ/TNF-α, IFN-γ/L-17A and TNF-α/IL-17A in $CD8^+$ T cells is shown for blood, spleen, ICLN and ileum for samples that were stimulated with the vaccine strain. Representative data is shown from one pig per treatment group: CON (Sw#6), VAC (Sw#65), V+I (Sw#89) and INF (Sw#52). Approximately $5x10^4$ cells are displayed for blood and spleen, $1x10^5$ cells for ICLN and ileum.

Figure S4. Representative raw data for FCM analysis of $CD4^-CD8\beta^-$ T cells. Intracellular cytokine staining was performed on lymphocytes isolated from several locations following overnight *in vitro* stimulation with STM antigen. Co-production of IFN-γ/TNF-α, IFN-γ/L-17A and TNF-α/IL-17A in $CD4^-CD8\beta^-$ T cells is shown for blood, spleen, ICLN and ileum for samples that were stimulated with the vaccine strain. Representative data is shown from one pig per treatment group: CON (Sw#6), VAC (Sw#65), V+I (Sw#89) and INF (Sw#52). Approximately $1x10^5$ cells are displayed for the blood, $3x10^5$ cells for the spleen, $3x10^4$ cells for ICLN and $6x10^4$ cells for the ileum.

Figure S5. Frequencies of STM-stimulated cytokine-producing $CD4^+$ T cells in blood (A), spleen (B), JLN (C), ICLN (D), jejunum (E) and ileum (F). $CD4^+$ T cells were gated within live lymphocytes and further analyzed for IFN-γ, TNF-α and IL-17A production by Boolean gating. Individual graphs indicate percentages of cytokine-producing $CD4^+$ T cells from individual animals of the VAC (light and dark blue), the V+I (light and dark petrol) and the INF (light and dark red) group within total $CD4^+$ T cells. Animals were euthanized either 7 days post vaccination/infection (7 dpv/dpi, circles, lighter color) or 21 days post vaccination/infection (21 dpv/dpi, rectangles, darker color). All data obtained from control pigs (CON) is displayed in green triangles on the right

of each graph. Cells were stimulated with the vaccine strain (S, Salmoporc) or the challenge infection strain (C, Challenge) or cultivated in medium-only (M, Medium). Black bars indicate the median and whiskers show the interquartile range. Y-axes are scaled individually per cytokine-producing phenotype but consistent within related organ groups: blood+spleen, JLN+ICLN and jejunum+ileum.

Figure S6. Frequencies of STM-stimulated cytokine-producing $CD8^+$ T cells in blood (A), spleen (B), JLN (C), ICLN (D), jejunum (E) and ileum (F). $CD8^+$ T cells were gated within live lymphocytes and further analyzed for IFN-γ, TNF-α and IL-17A production by Boolean gating. Individual graphs indicate percentages of cytokine-producing $CD8^+$ T cells from individual animals of the VAC (light and dark blue), the V+I (light and dark petrol) and the INF (light and dark red) group within total $CD8^+$ T cells. Animals were euthanized either 7 days post vaccination/infection (7 dpv/dpi, circles, lighter color) or 21 days post vaccination/infection (21 dpv/dpi, rectangles, darker color). All data obtained from control pigs (CON) is displayed in green triangles on the right of each graph. Cells were stimulated with the vaccine strain (S, Salmoporc) or the challenge infection strain (C, Challenge) or cultivated in medium-only (M, Medium). Black bars indicate the median and whiskers show the interquartile range. Y-axes are scaled individually per cytokine-producing phenotype but consistent within related organ groups: blood+spleen, JLN+ICLN and jejunum+ileum.

Figure S7. Frequencies of STM-stimulated cytokine-producing $CD4^-CD8\beta^-$ T cells in blood (A), spleen (B), JLN (C), ICLN (D), jejunum (E) and ileum (F). $CD4^-CD8\beta^-$ T cells were gated within live lymphocytes and further analyzed for IFN-γ, TNF-α and IL-17A production by Boolean gating. Individual graphs indicate percentages of cytokine-producing $CD4^-CD8\beta^-$ T cells from individual animals of the VAC (light and dark blue), the V+I (light and dark petrol) and the INF (light and dark red) group within total $CD4^-CD8\beta^-$ T cells. Animals were euthanized either 7 days post vaccination/infection (7 dpv/dpi, circles, lighter color) or 21 days post vaccination/infection (21 dpv/dpi, rectangles, darker color). All data obtained from control pigs (CON) is displayed in green triangles on the right of each graph. Cells were stimulated with the vaccine strain (S, Salmoporc) or the challenge infection strain (C, Challenge) or cultivated in medium-only (M, Medium). Black bars indicate the median and whiskers show the interquartile range. Y-axes are scaled individually per cytokine-producing phenotype but consistent within related organ groups: blood+spleen, JLN+ICLN and jejunum+ileum.

Figure S8. Estimated marginal means (emmeans) of STM-stimulated IFN-γ/TNF-α/IL-17A producing $CD4^+$ T cells in blood compared across treatment groups on day 7 and day 21 post vaccination or infection. Samples were stimulated *in vitro* with STM antigen (S: Salmoporc, vaccine strain; C: challenge/infection strain) or cultivated in medium-only (M: Medium). (A) Y-axes depict emmeans of STM-stimulated IFN-γ/TNF-α/IL-17A producing $CD4^+$ T cells for each treatment group on a $\log_{10}$ scale. Numbers above brackets show corresponding *p*-values calculated from contrasts between emmeans in the VAC (light blue), V+I (petrol), INF (red) and CON group (green). Black whiskers indicate the lower and upper 95% confidence intervals of emmeans. Multiple testing correction was applied across all comparisons (all six pairwise comparisons of the four treatment groups, separately for 7 and 21 dpv/dpi, separately for each of the three stimulation levels, six tissues and seven phenotypes). This leads to a total multiple testing load of 6*2*3*6*7=1512 comparisons. (B-F) Same as (A) but for cytokine-producing $CD4^+$ T cells derived from spleen (B), JLN (C), ICLN (D), jejunum (E) and ileum (F).

Figure S9. Contrasts of STM-stimulated cytokine-producing $CD8^+$ T cells between treatment groups at 7 and 21 days post vaccination (dpv) or infection (dpi) in various tissues. Results are shown as effect size heatmaps. Samples were stimulated *in vitro* with STM antigen (S: Salmoporc, vaccine strain; C: challenge/infection strain). White boxes represent non-significant differences between groups. Boxes with significant differences are color-coded and contain numbers with corresponding effect sizes aka contrasts between estimated marginal means on a $\log_{10}$ level (dark blue: > 0.1, blue: 0.1 to 0.01, light blue: < 0.01; dark red: < -0.1, red: -0.1 to -0.01, light red: > -0.01). Boxes in blue indicate STM-stimulated cytokine-producing $CD8^+$ T cells are more abundant in the group

that is stated first in the comparison. Red boxes indicate higher abundances in the second group. Differences were considered significant at a multiple testing corrected 10% false discovery rate cut-off across all pairwise comparisons (7 and 21 dpv/dpi, four treatment groups, six tissues and three phenotypes).

Figure S10. Contrasts of STM-stimulated cytokine-producing $CD4^{-}CD8\beta^{-}$ T cells between treatment groups at 7 and 21 days post vaccination (dpv) or infection (dpi) in various tissues. Results are shown as effect size heatmaps. Samples were stimulated *in vitro* with STM antigen (S: Salmoporc, vaccine strain; C: challenge/infection strain). White boxes represent non-significant differences between groups. Boxes with significant differences are color-coded and contain numbers with corresponding effect sizes aka contrasts between estimated marginal means on a $\log_{10}$ level (dark blue: > 0.1, blue: 0.1 to 0.01, light blue: < 0.01; dark red: < -0.1, red: -0.1 to -0.01, light red: > -0.01). Boxes in blue indicate STM-stimulated cytokine-producing $CD4^{-}CD8\beta^{-}$ T cells are more abundant in the group that is stated first in the comparison. Red boxes indicate higher abundances in the second group. Differences were considered significant at a multiple testing corrected 10% false discovery rate cut-off across all pairwise comparisons (7 and 21 dpv/dpi, four treatment groups, six tissues and seven phenotypes).

Figure S1

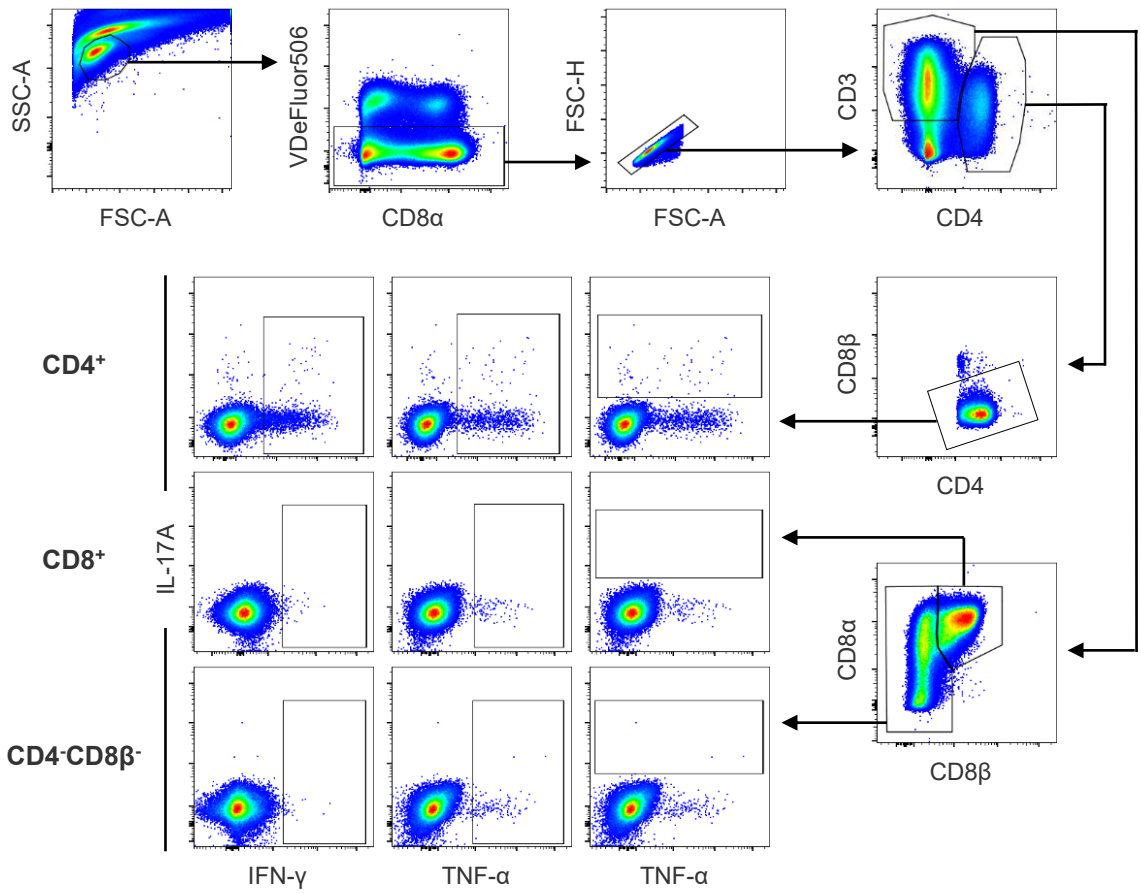

SSC-A
FSC-A
VDeFluor506
CD8α
FSC-H
FSC-A
CD3
CD4
CD4+
CD8+
CD4-CD8β-
IL-17A
IFN-γ
TNF-α
TNF-α
CD8β
CD4
CD8α
CD8β

Figure S2: CD4⁺ T cells

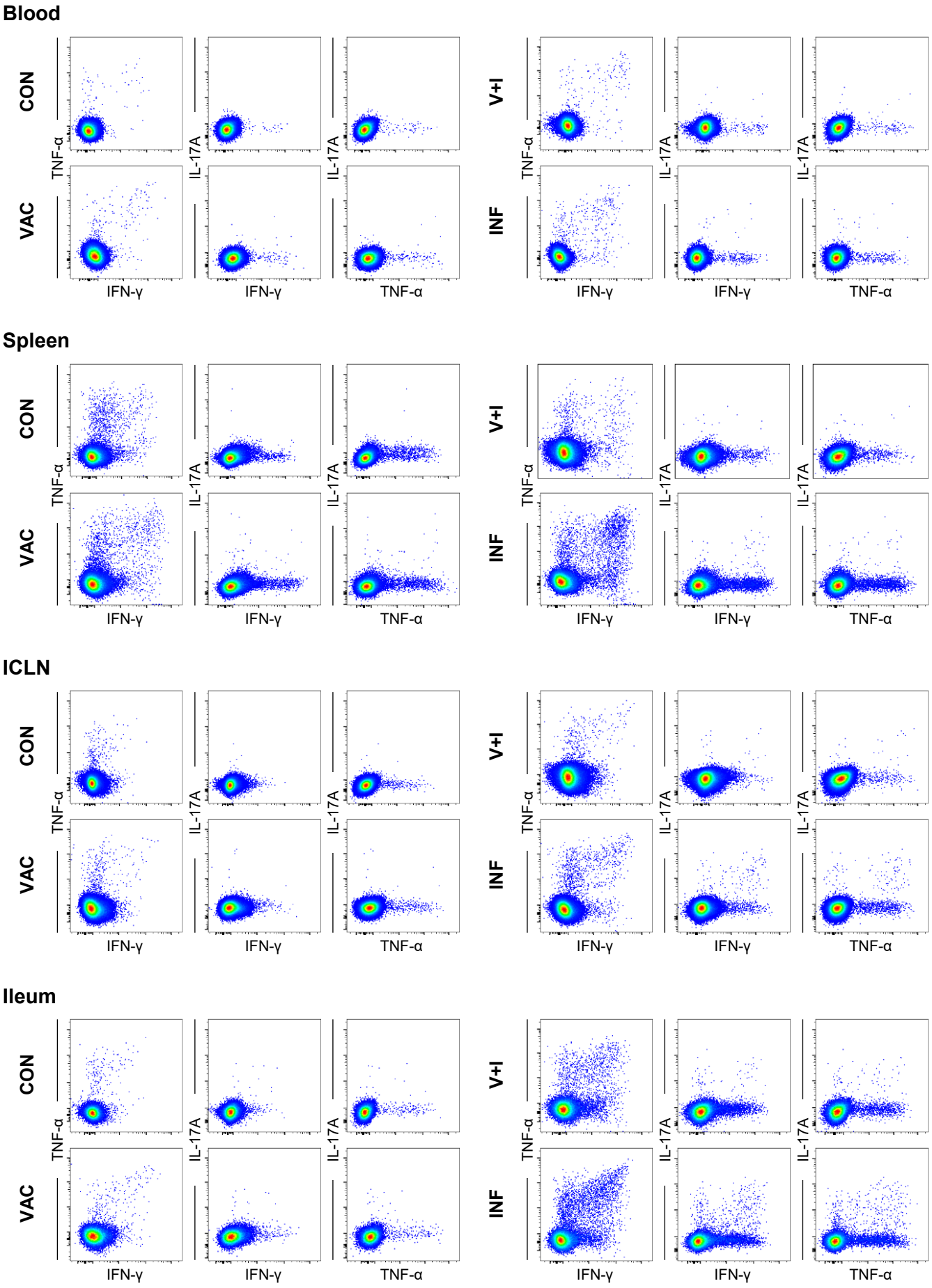


Figure S3: CD8⁺ T cells

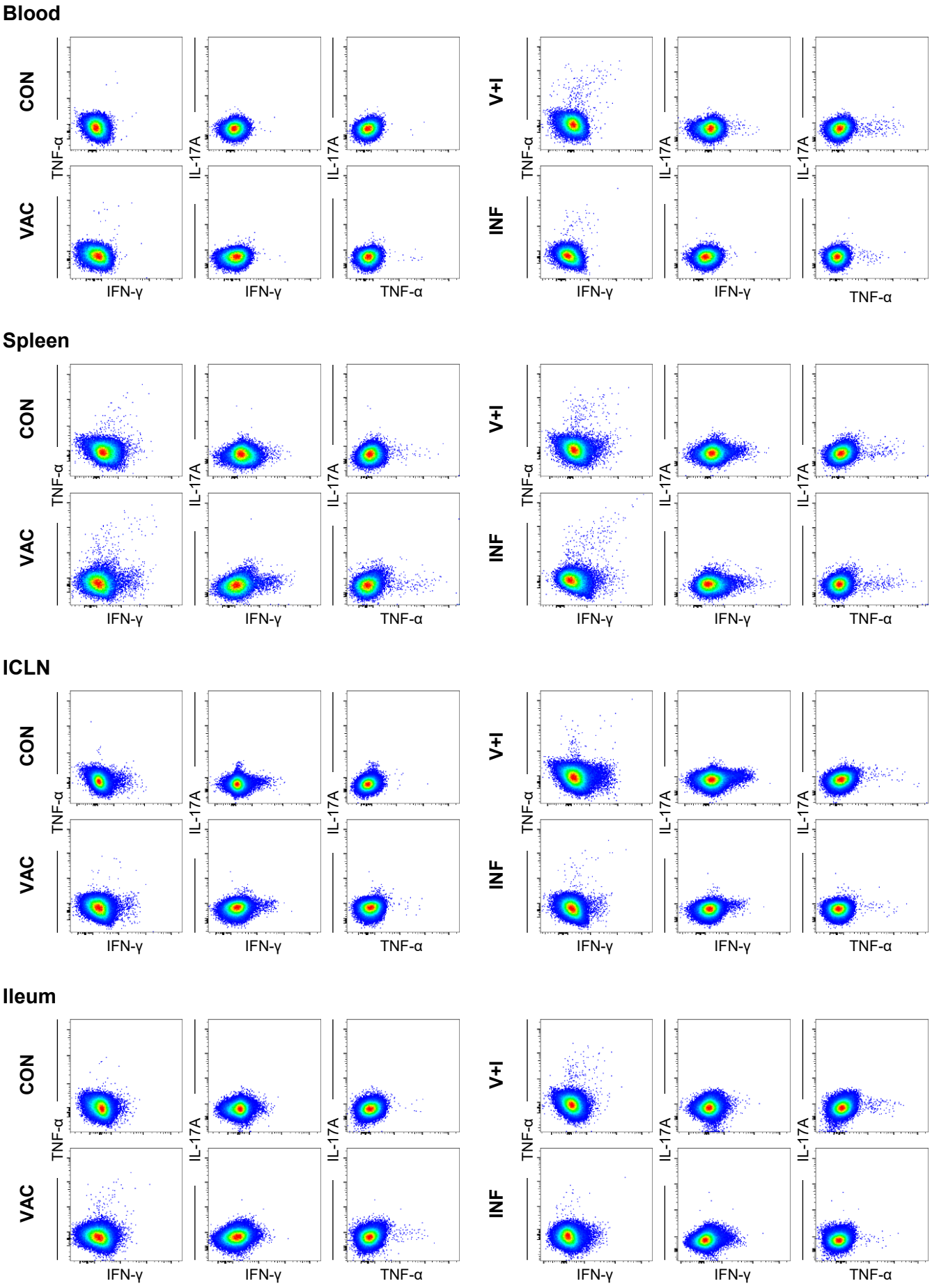


Figure S4: CD4^{-}CD8β^{-} T cells

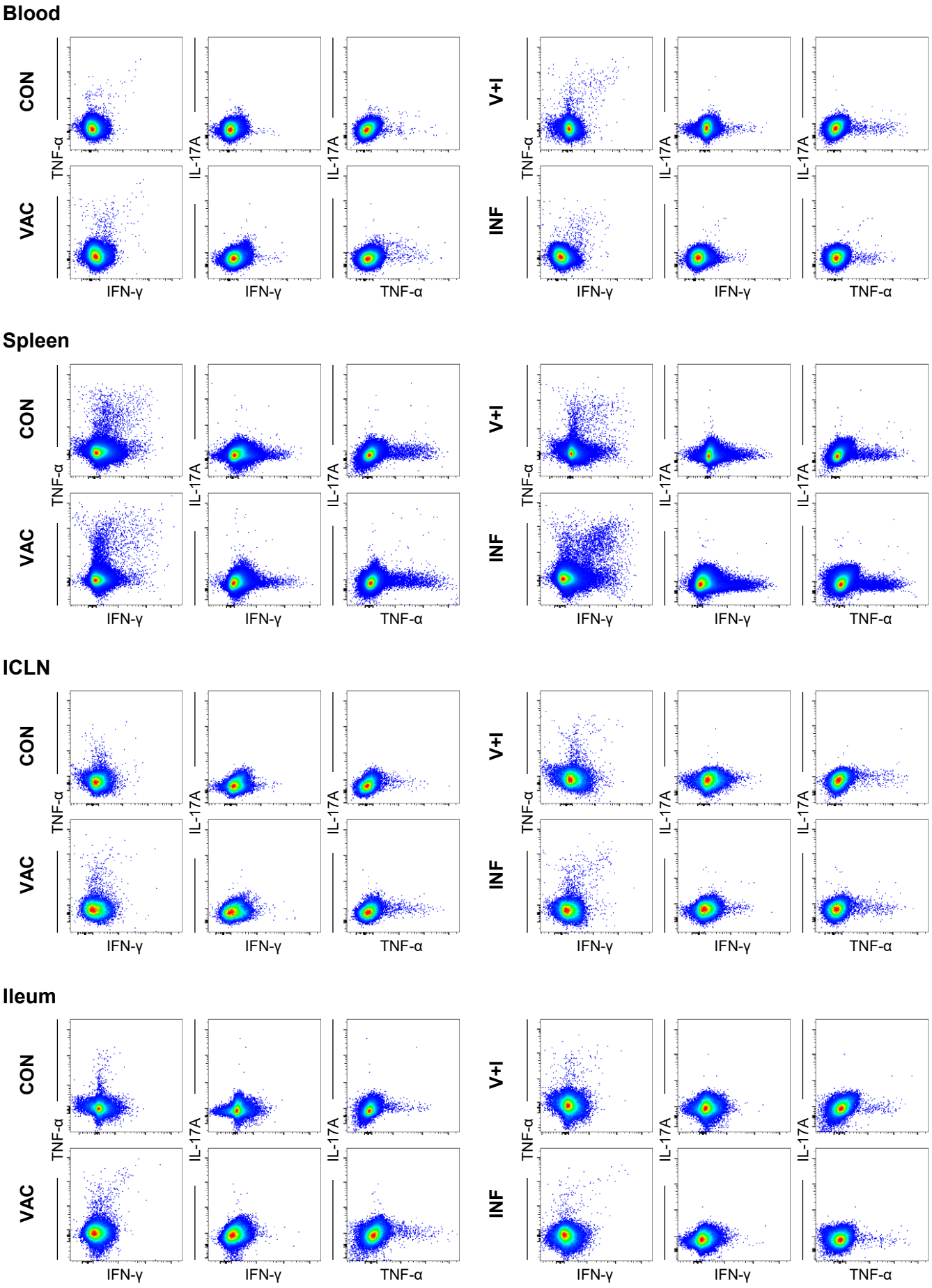


Figure S5A: CD4⁺ T cells; Blood

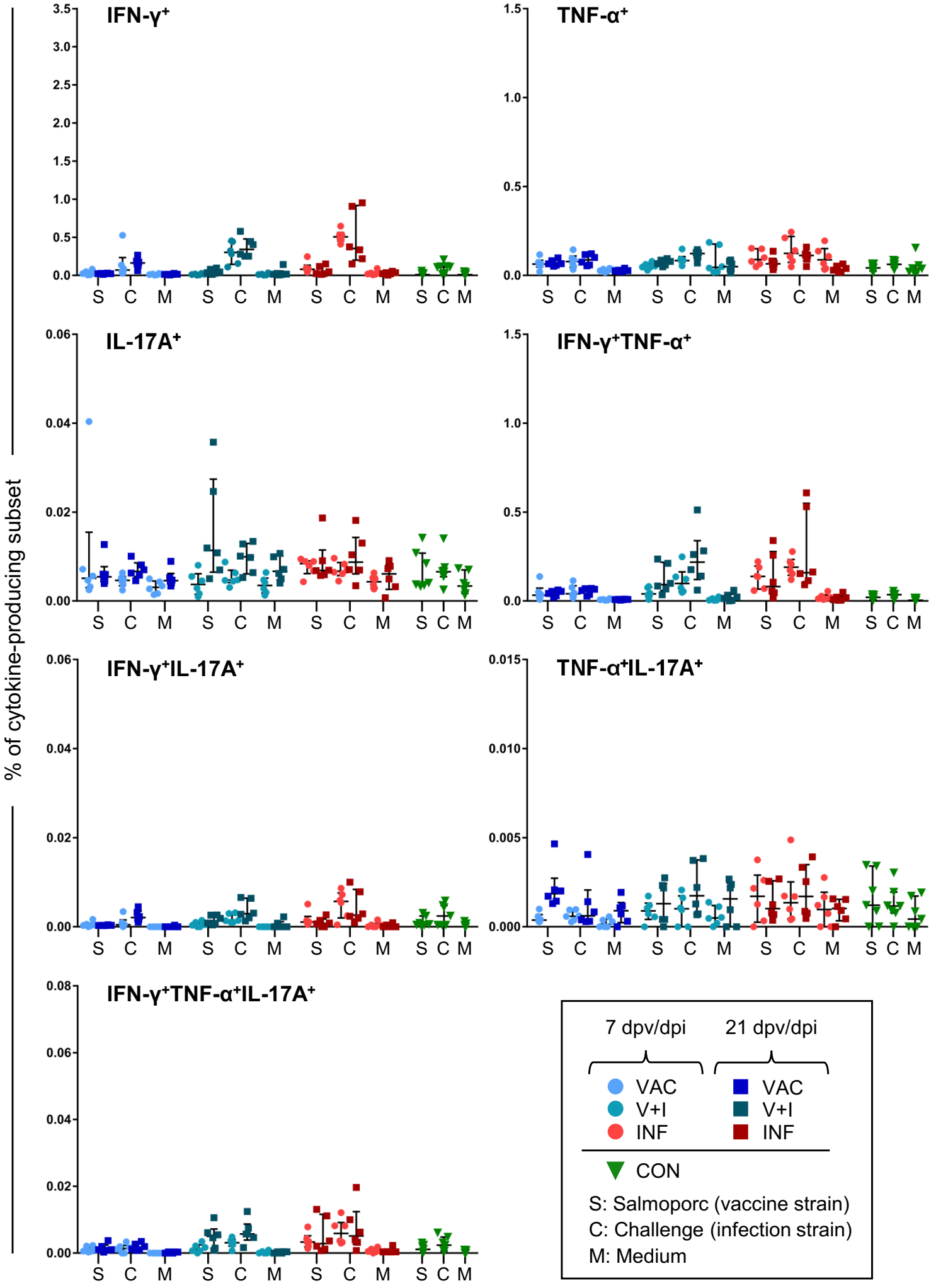


Figure S5B: CD4⁺ T cells; Spleen

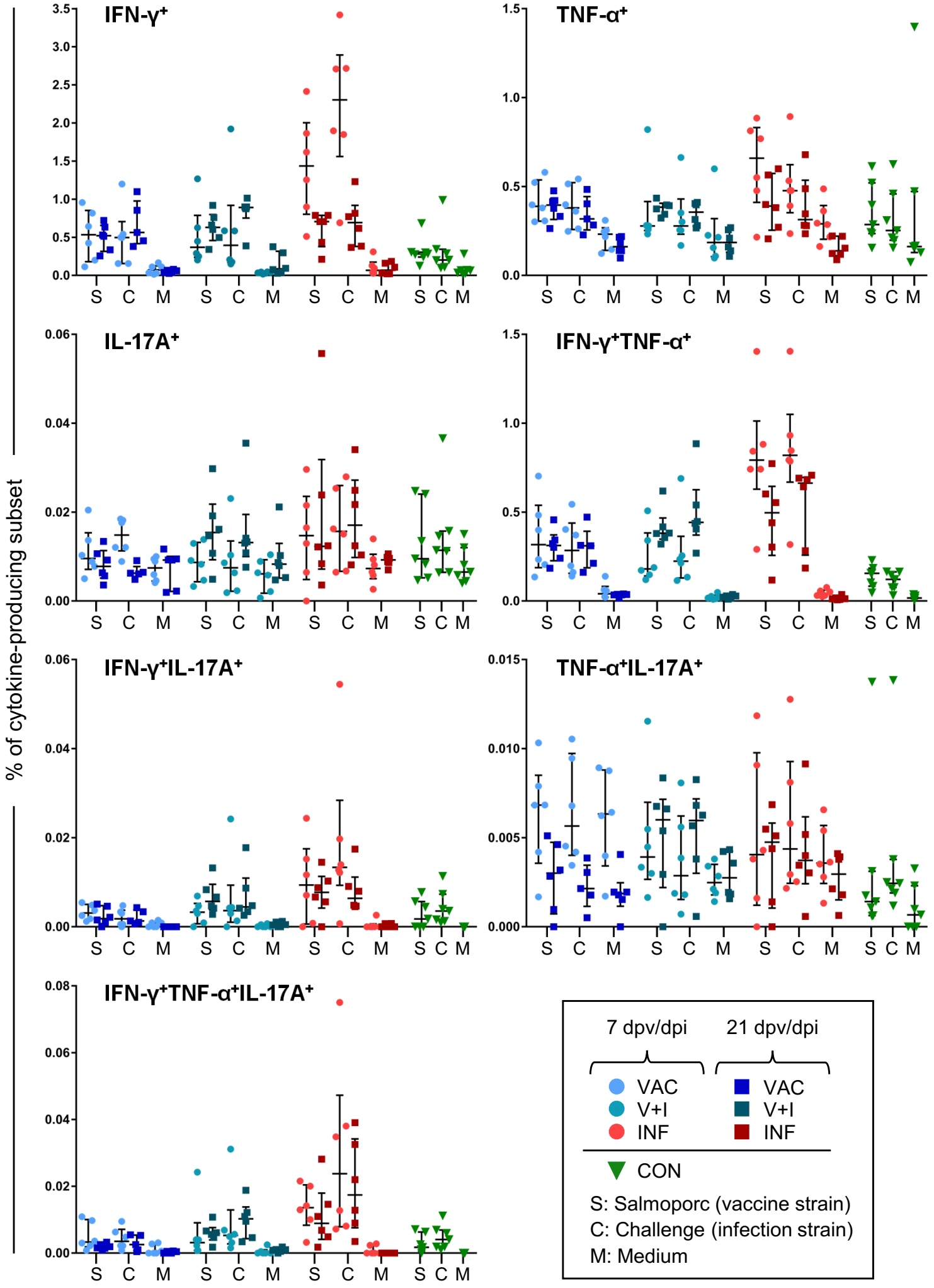


Figure S5C: CD4⁺ T cells; JLN

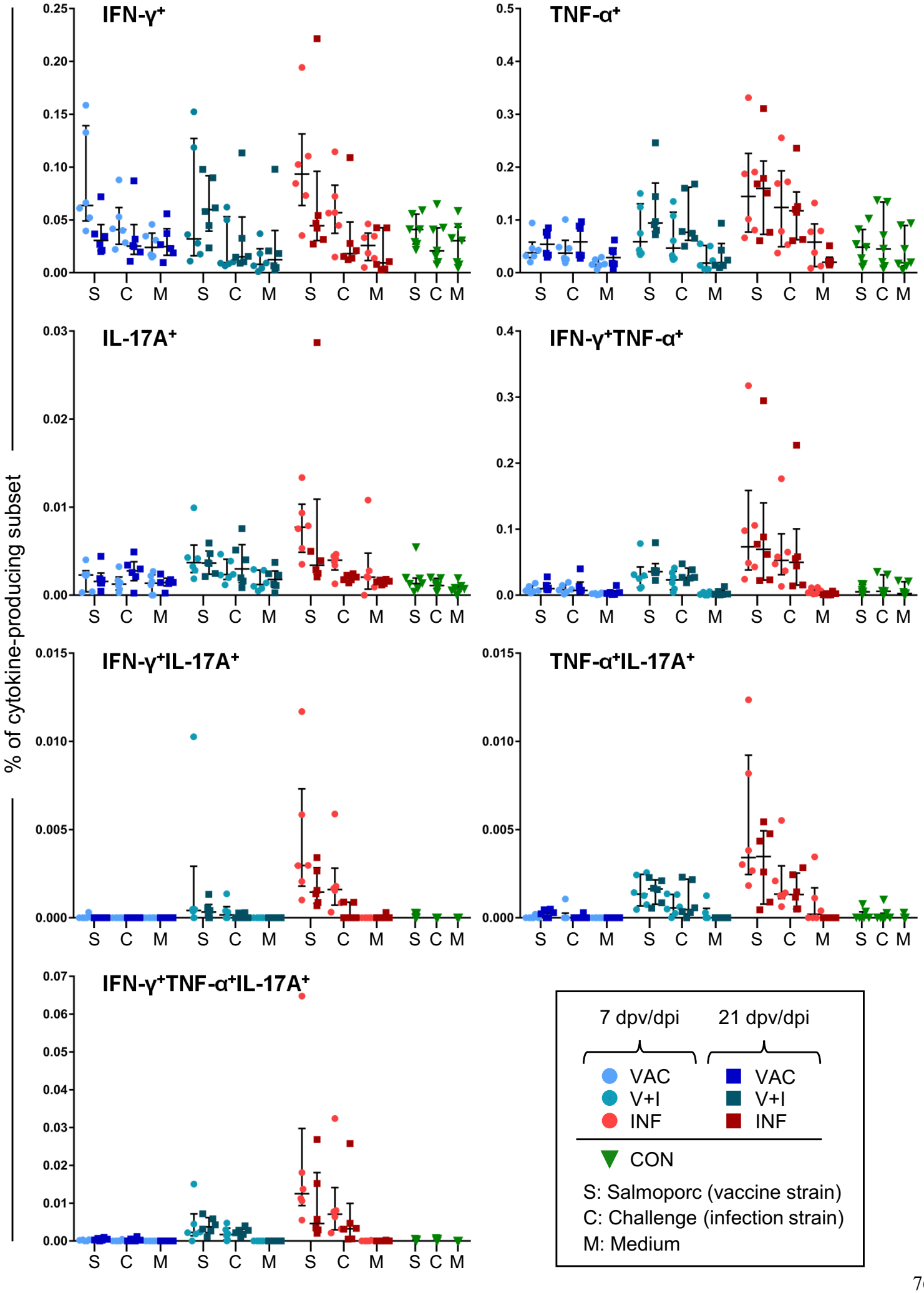


Figure S5D: CD4⁺ T cells; ICLN

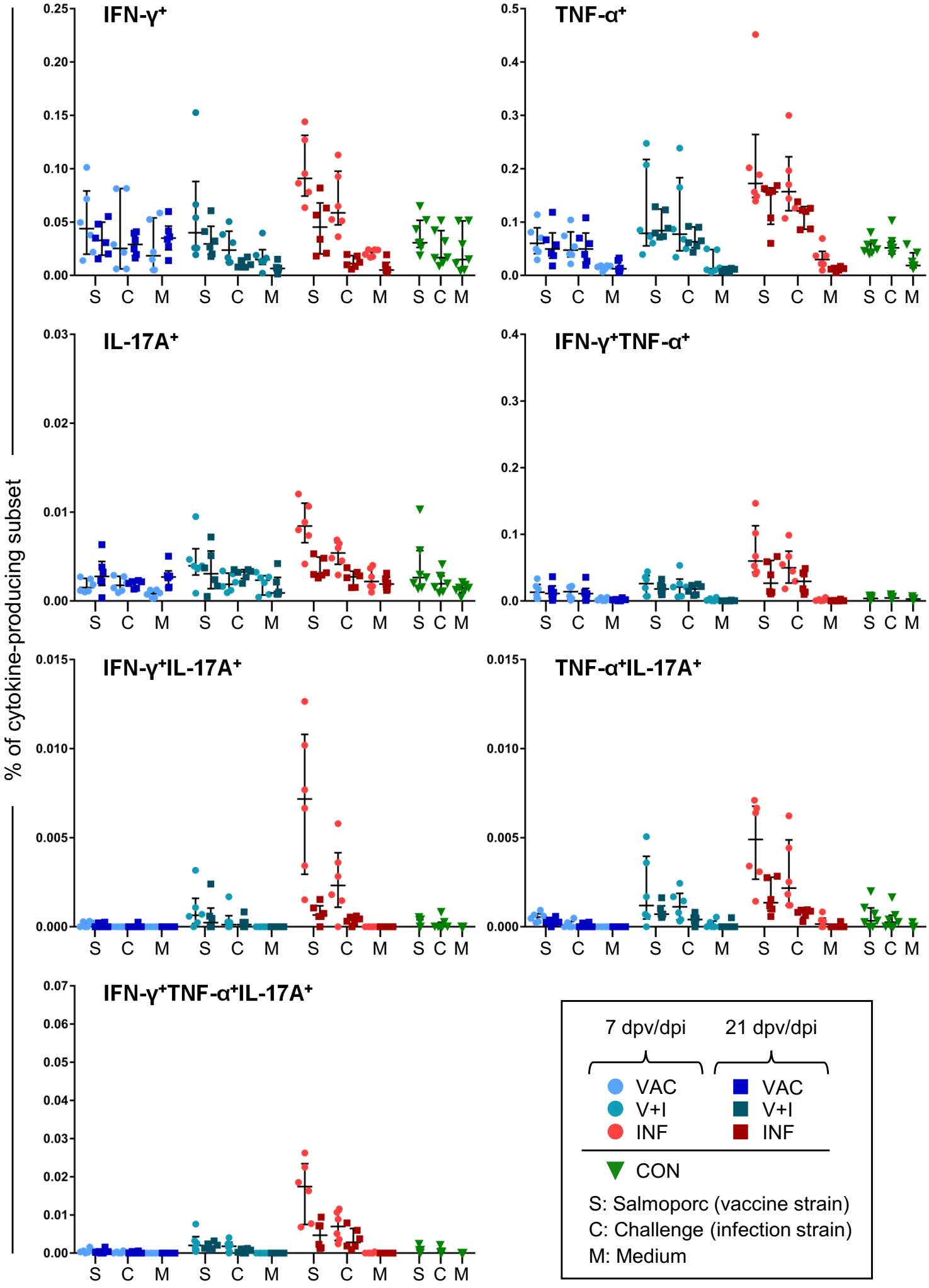


Figure S5E: CD4⁺ T cells; Jejunum

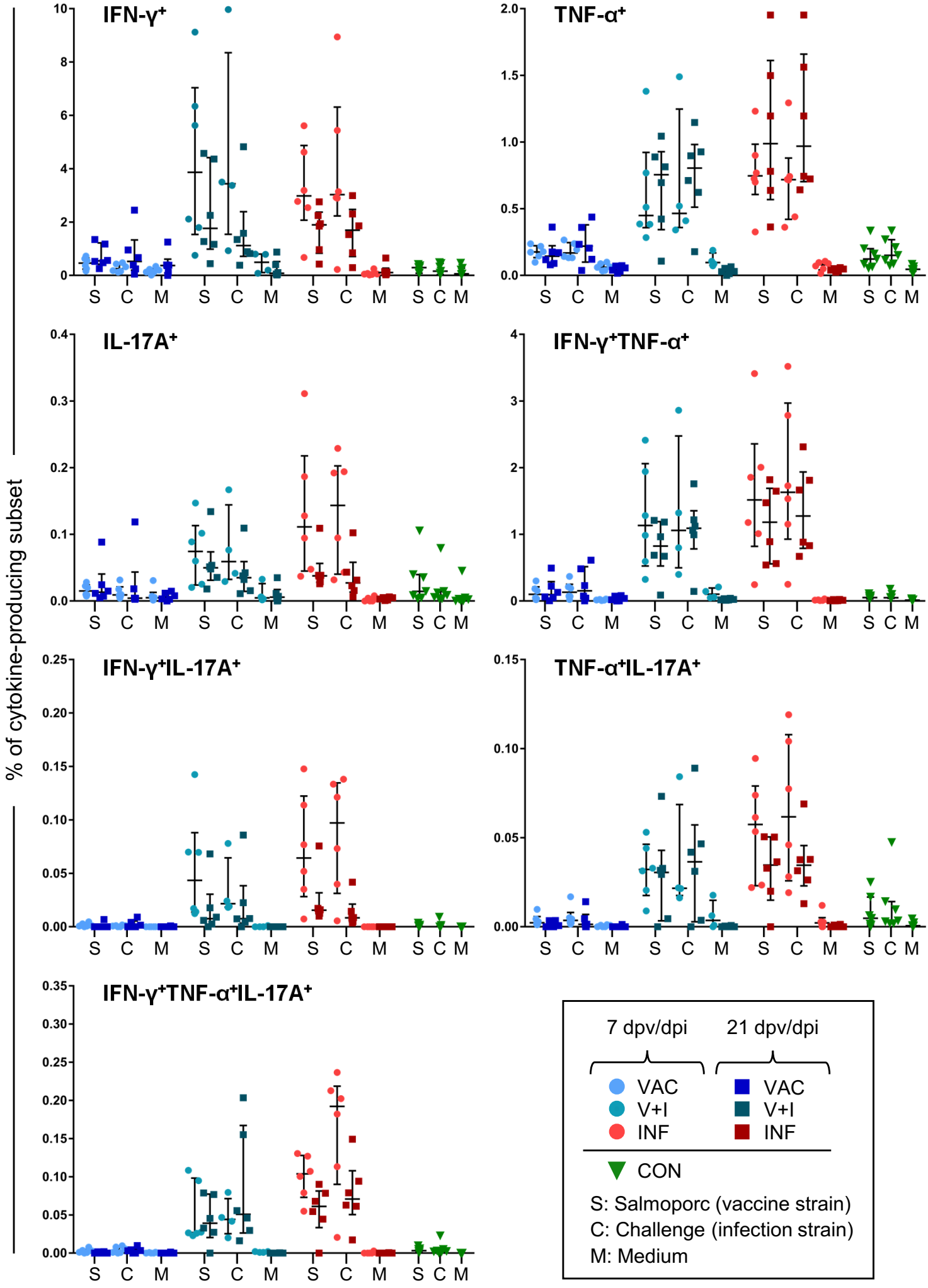


Figure S5F: CD4⁺ T cells; Ileum

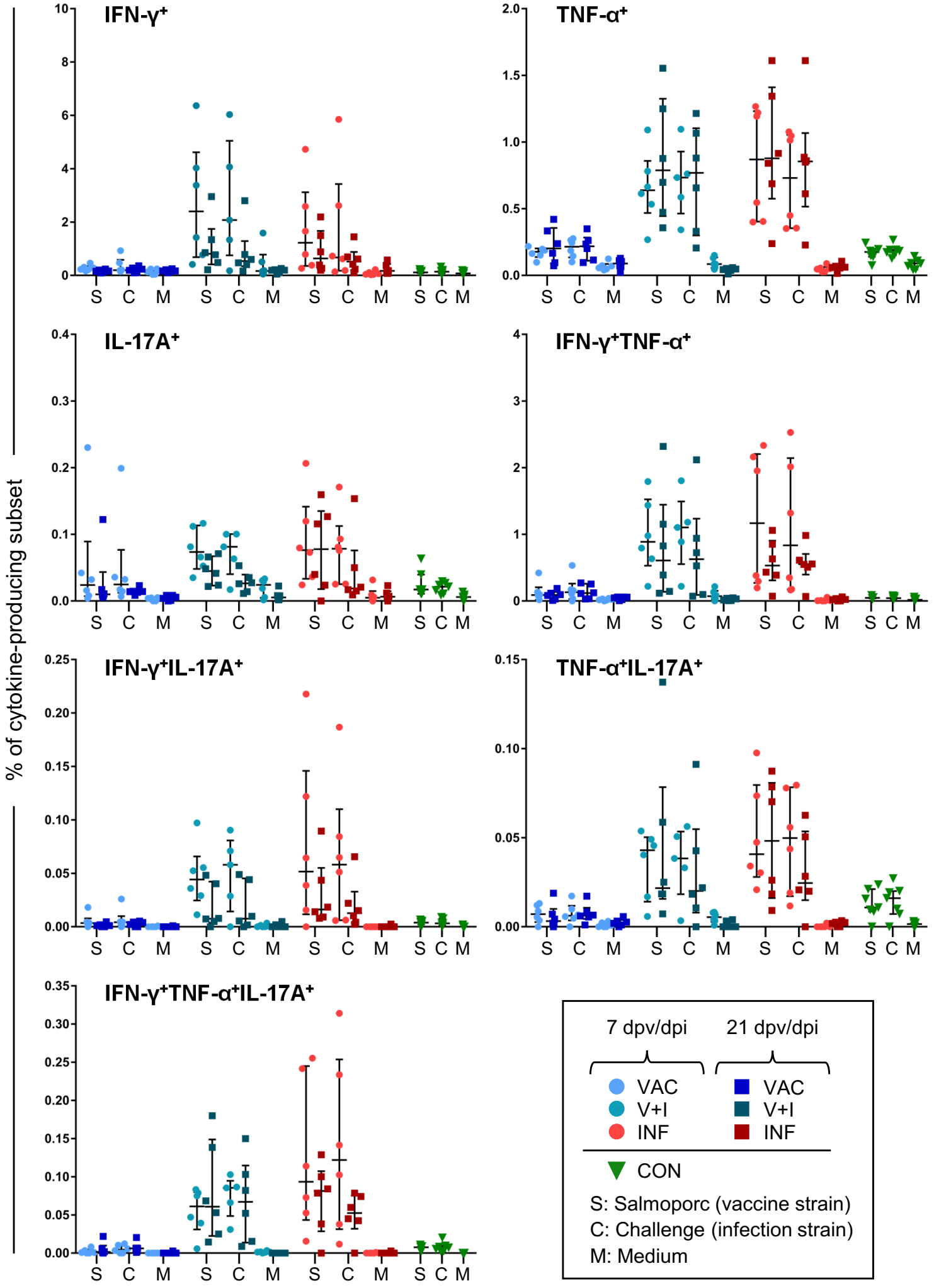


Figure S6A: CD8⁺ T cells; Blood

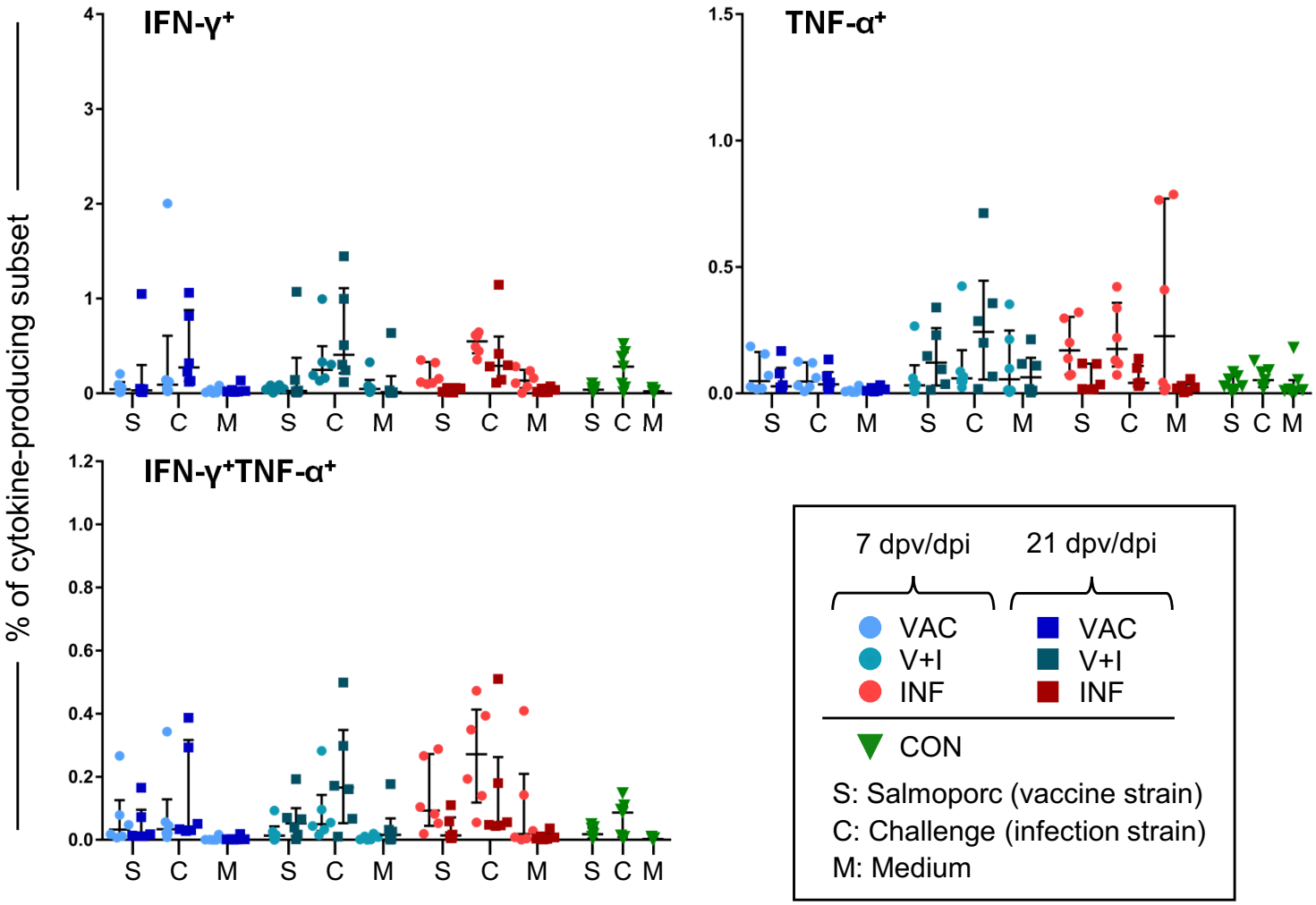


Figure S6B: CD8⁺ T cells; Spleen

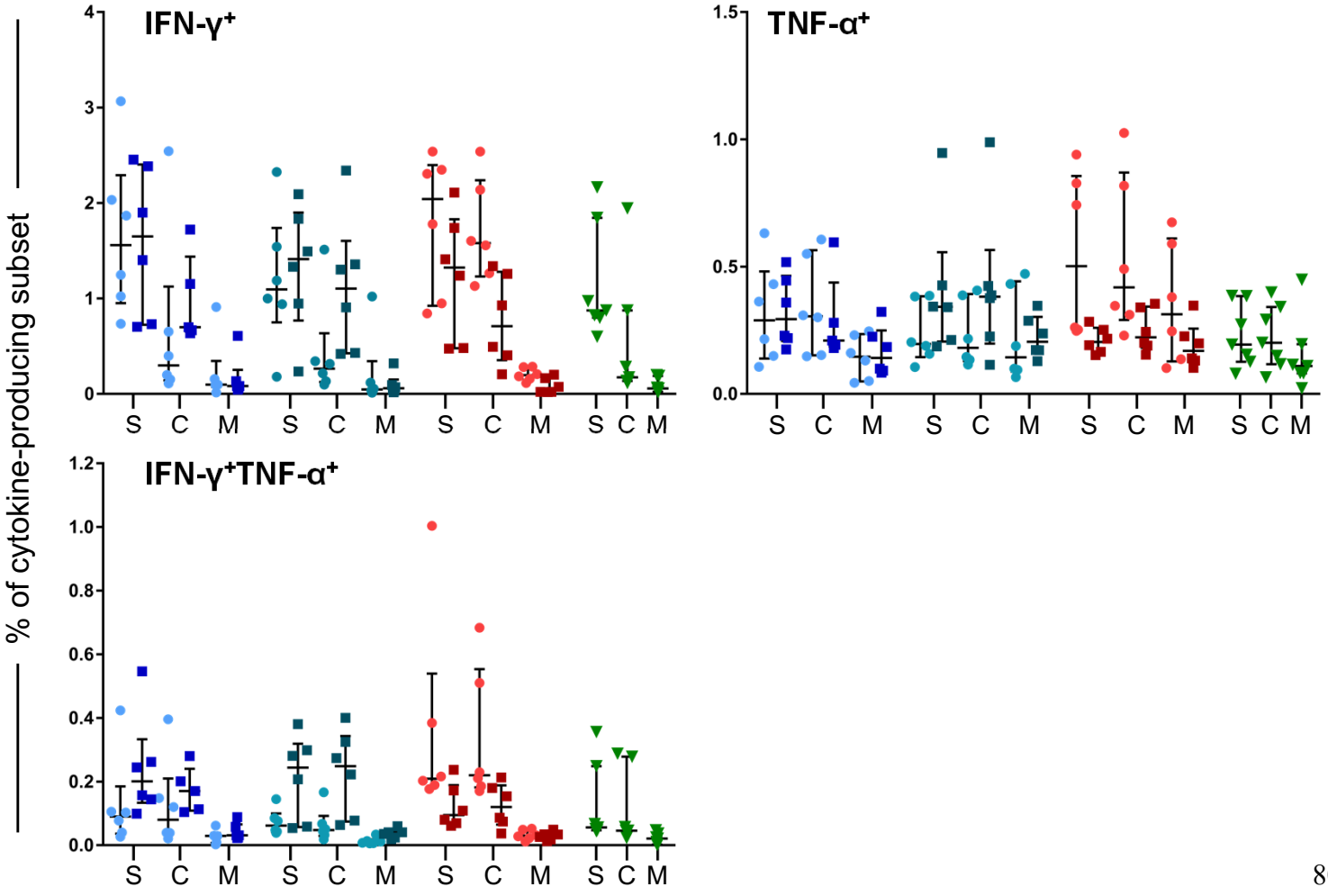


Figure S6C: CD8⁺ T cells; JLN

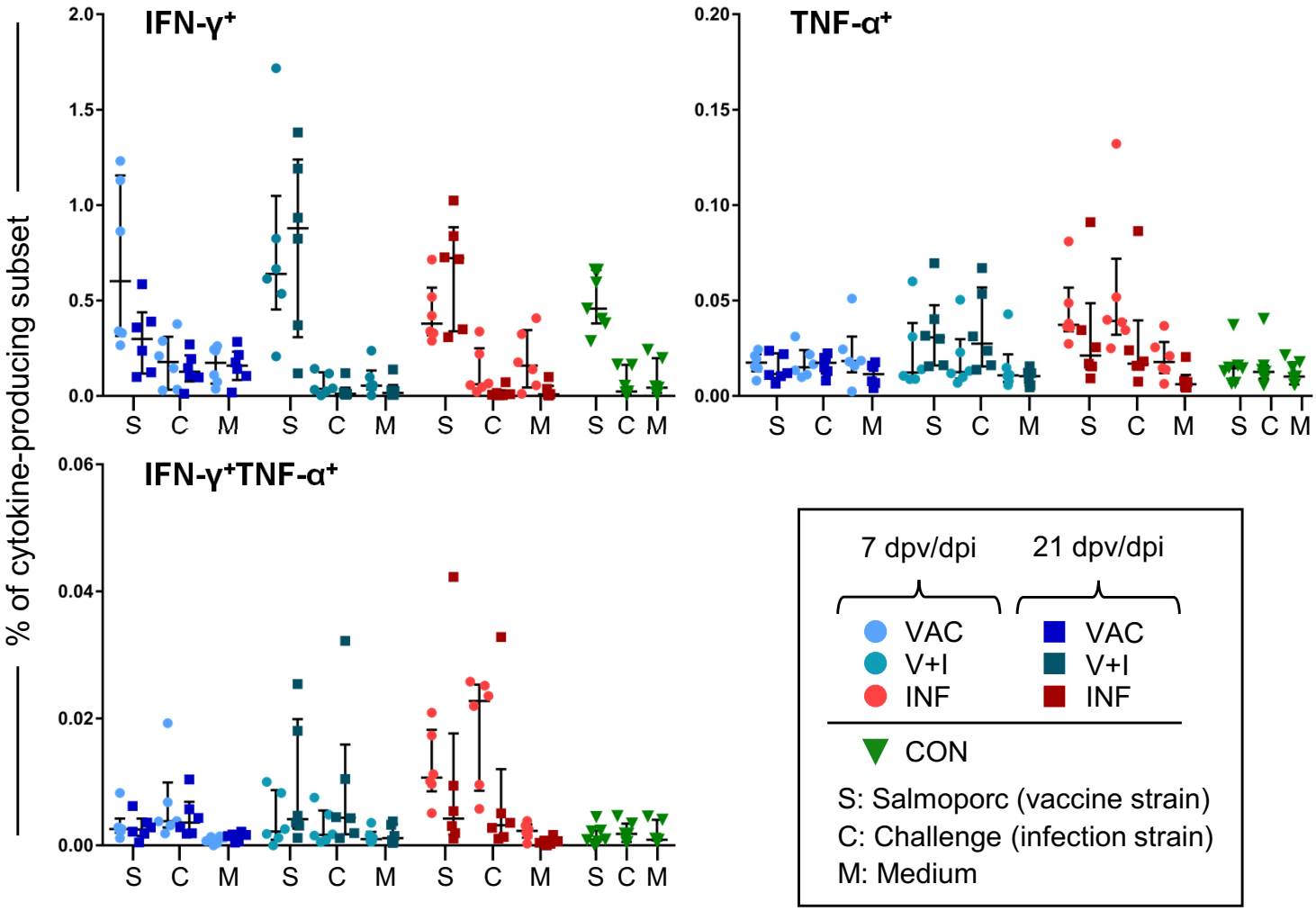


Figure S6D: CD8⁺ T cells; ICLN

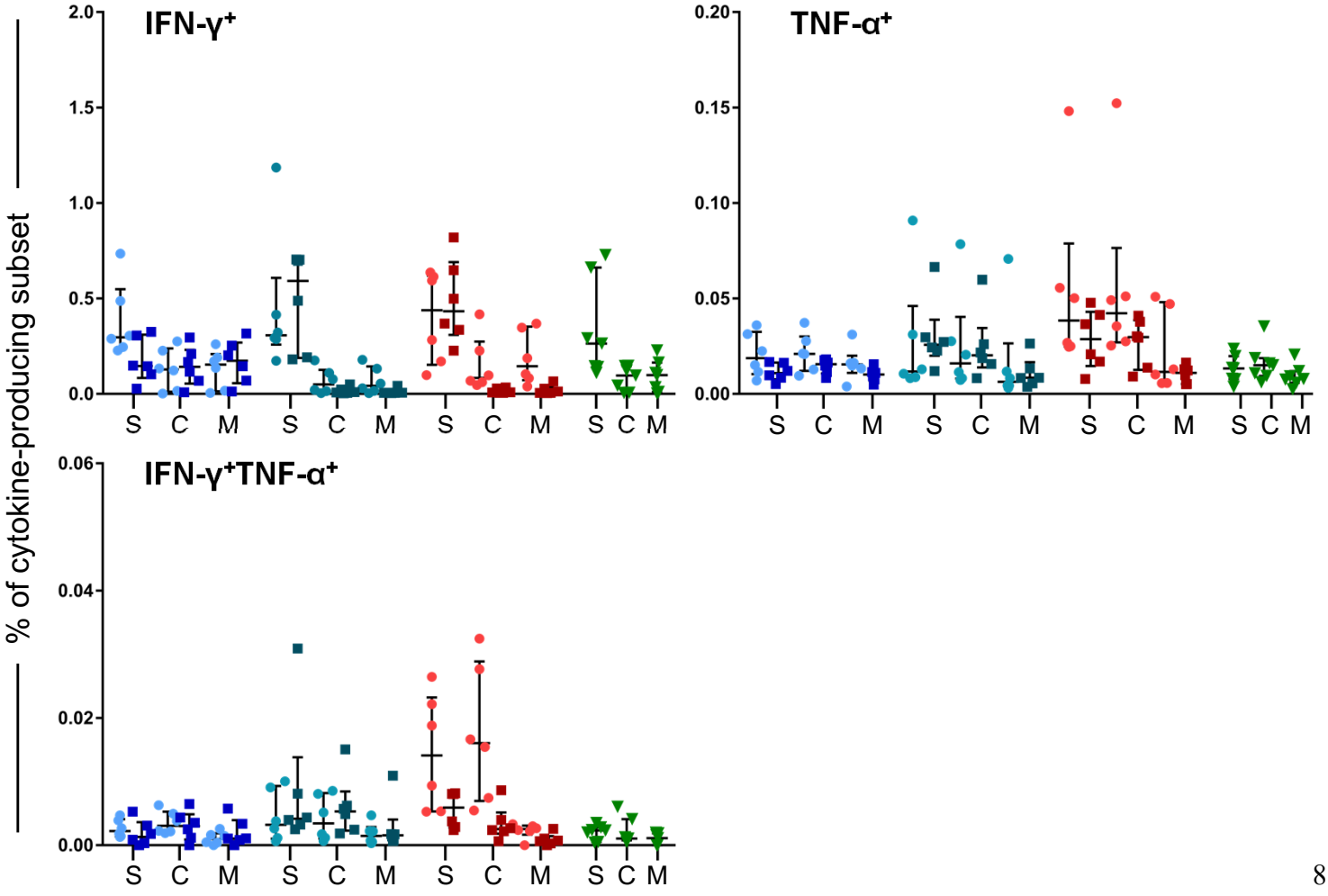


Figure S6E: CD8⁺ T cells; Jejunum

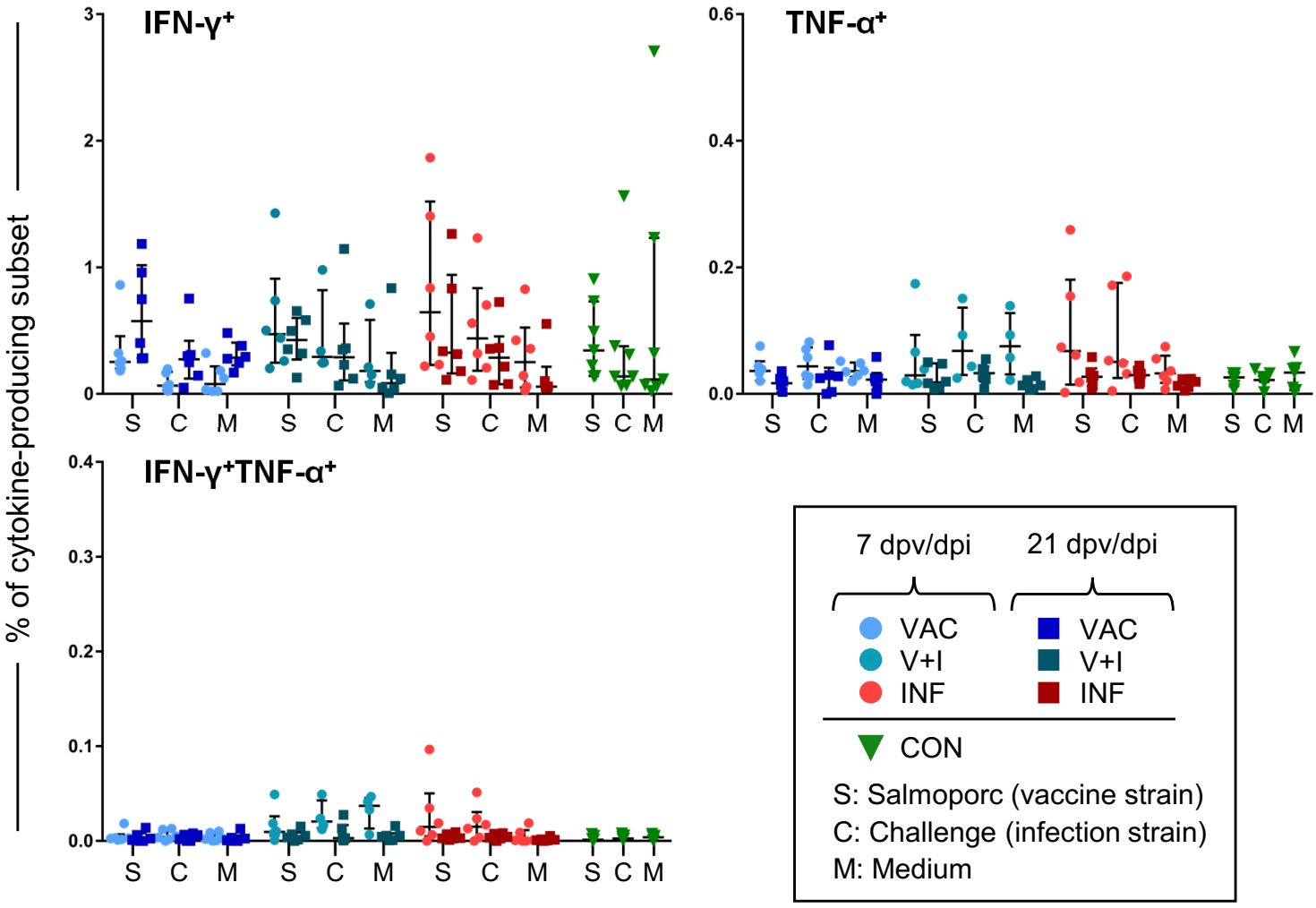


Figure S6F: CD8⁺ T cells; Ileum

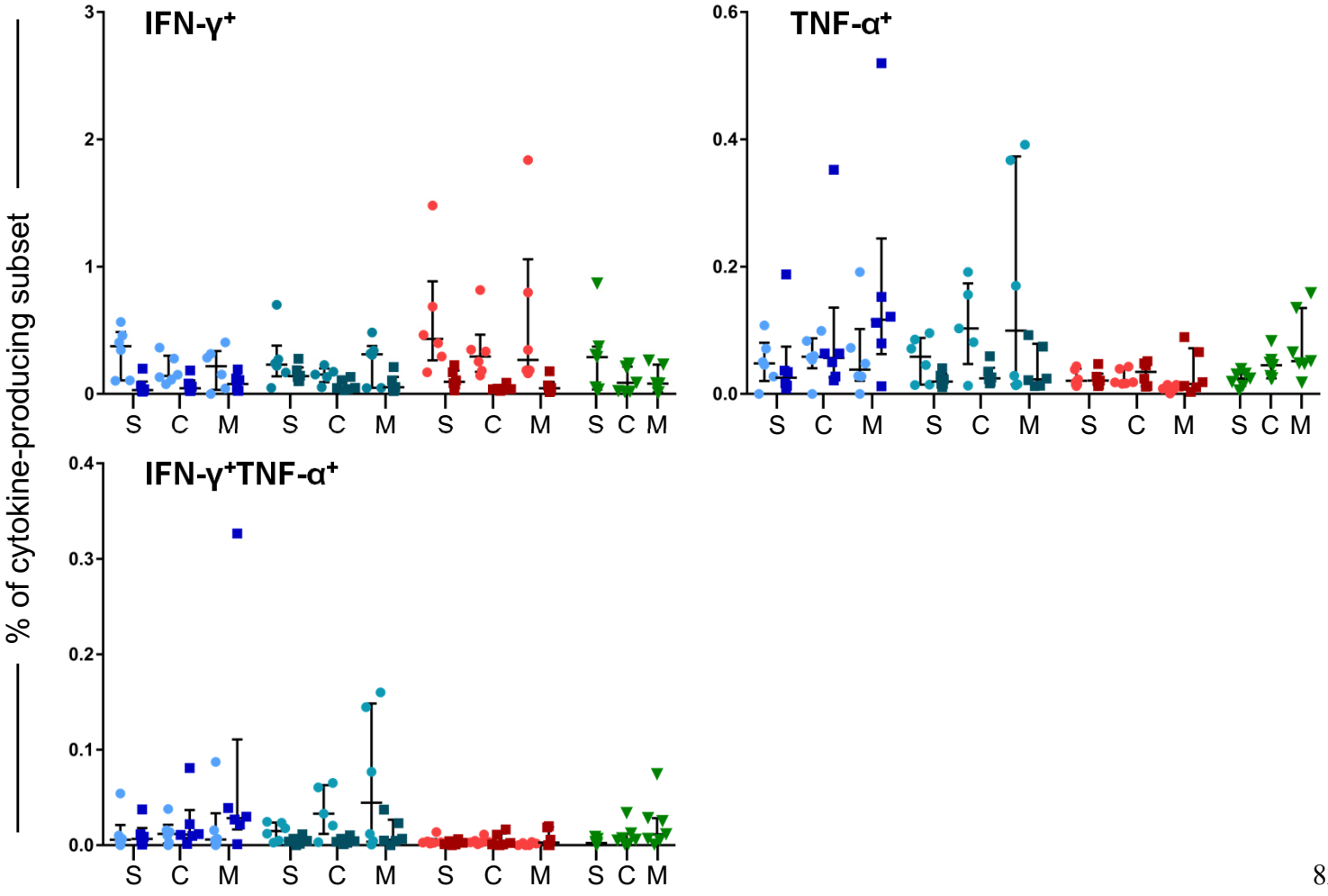


Figure S7A: CD4$^-$CD8β$^-$ T cells; Blood

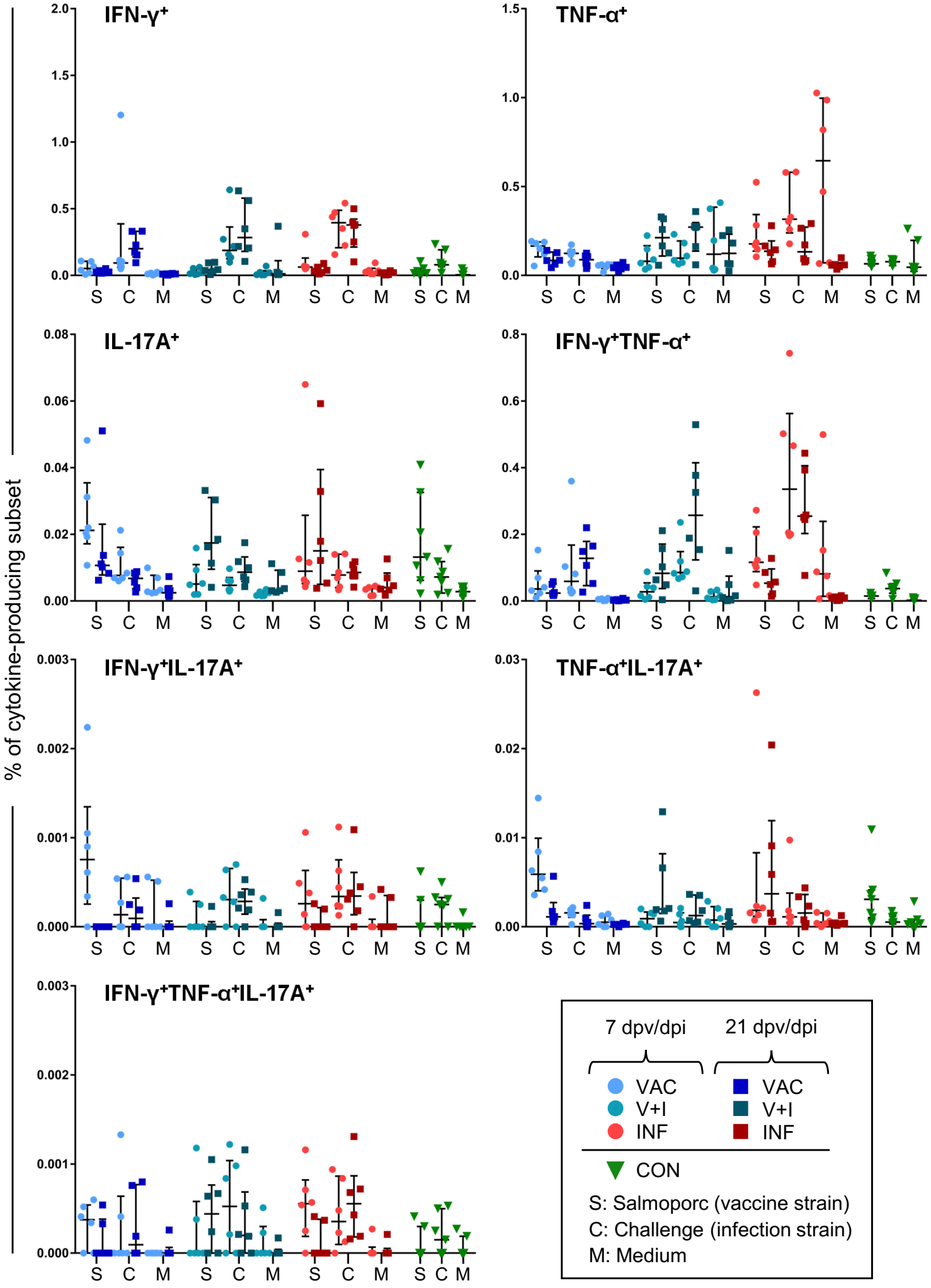


Figure S7B: CD4$^-$CD8β$^-$ T cells; Spleen

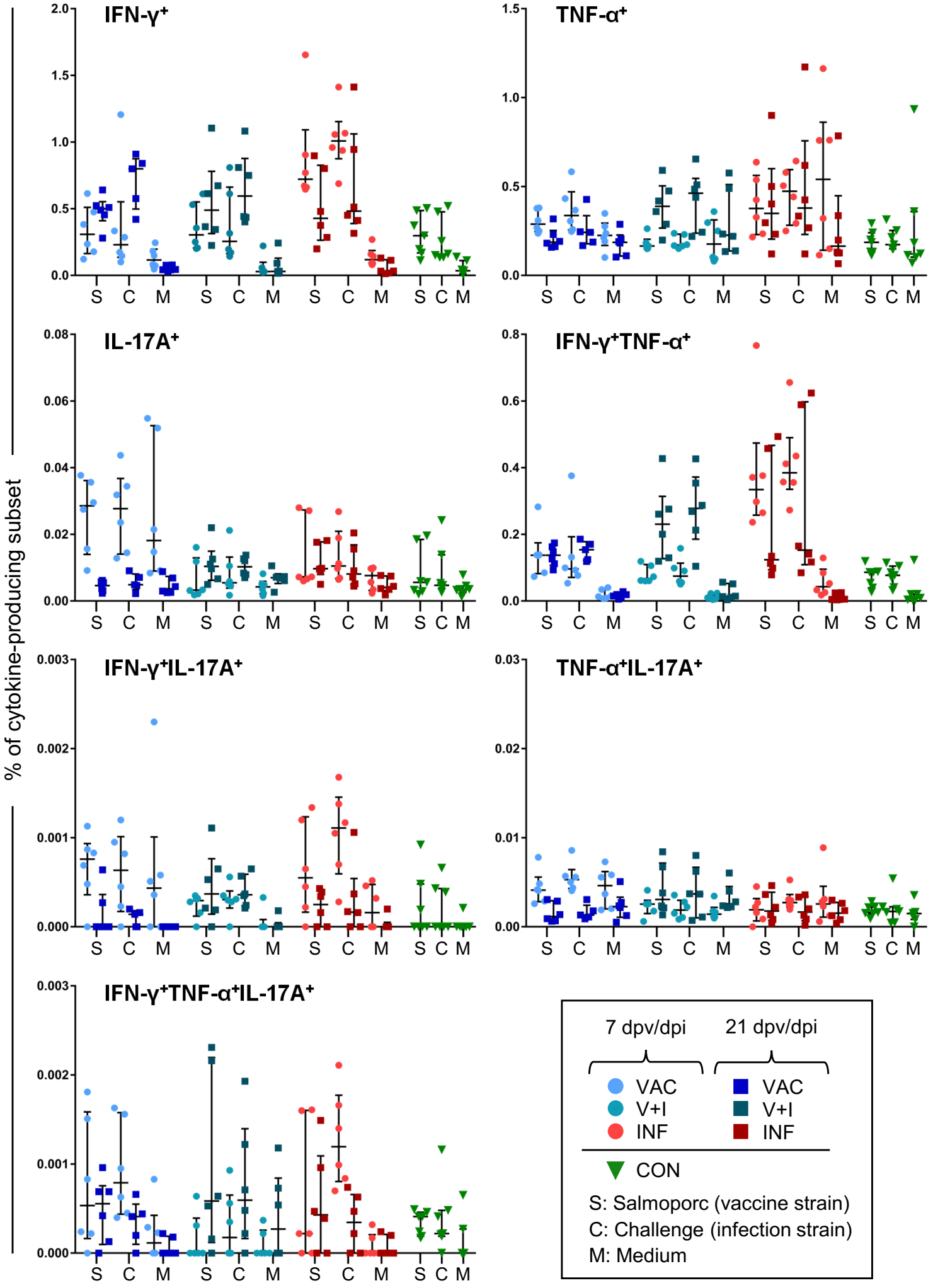


Figure S7C: CD4$^-$CD8β$^-$ T cells; JLN

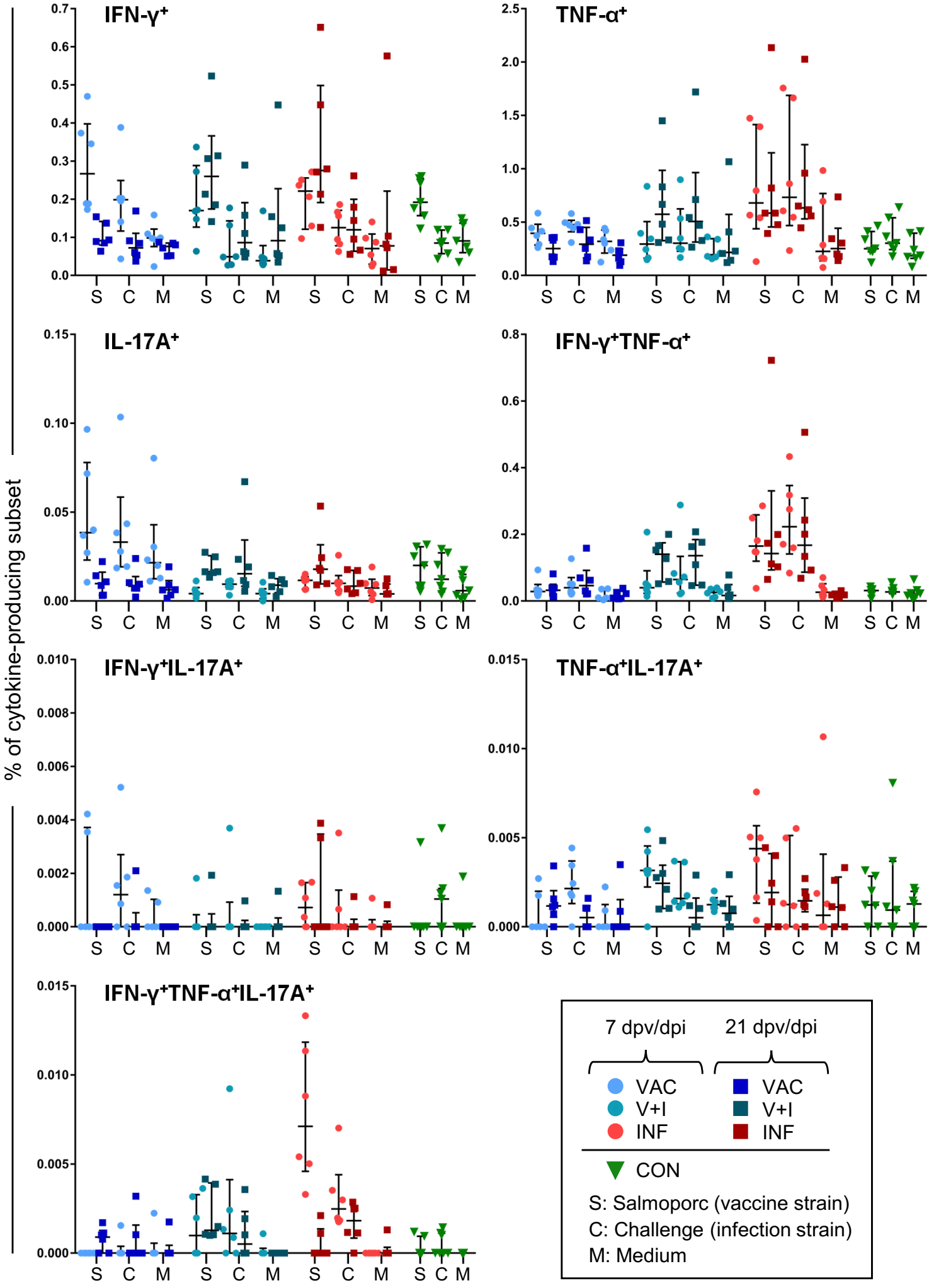


Figure S7D: CD4$^-$CD8β$^-$ T cells; ICLN

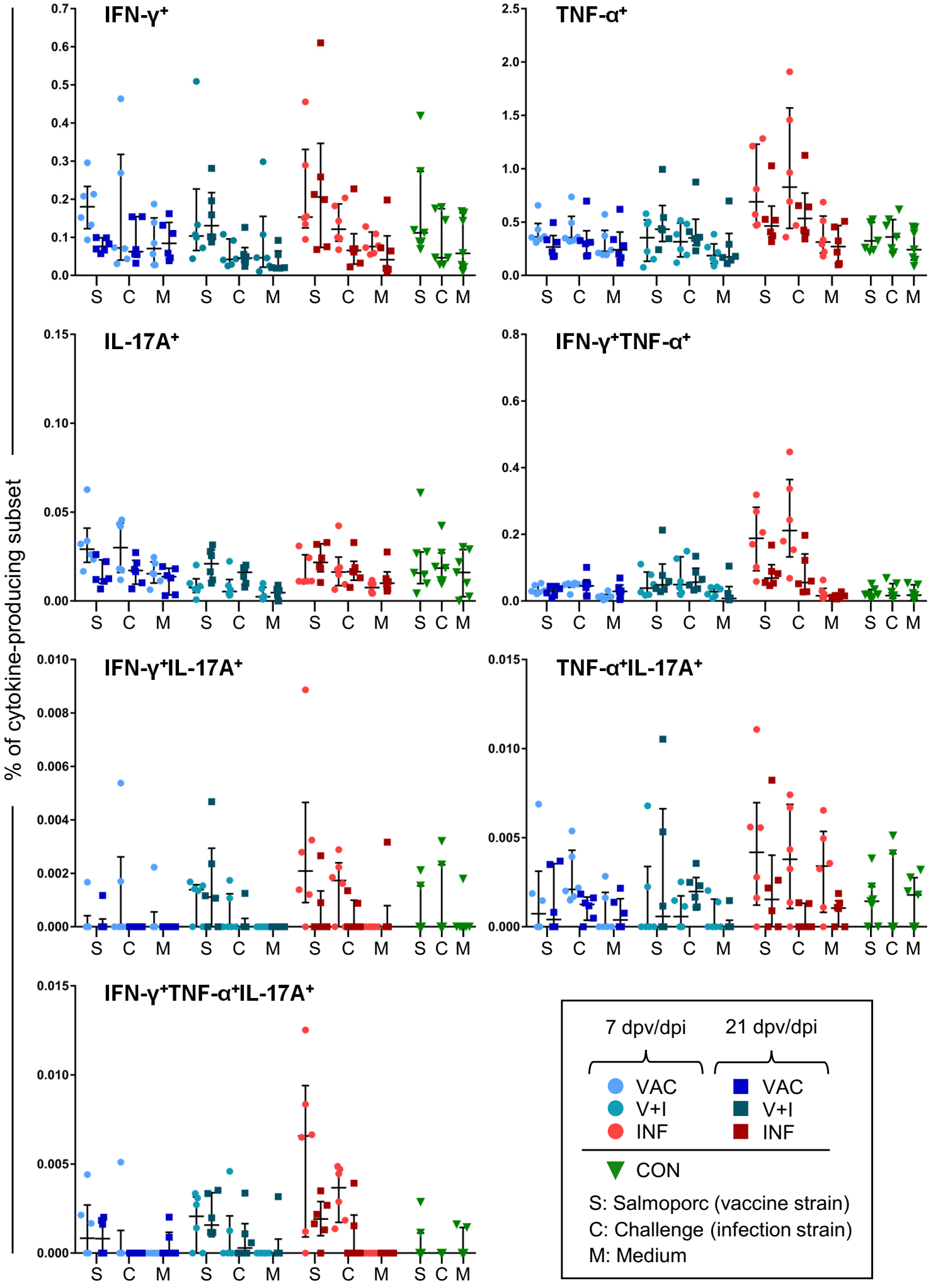


Figure S7E: CD4$^-$CD8β$^-$ T cells; Jejunum

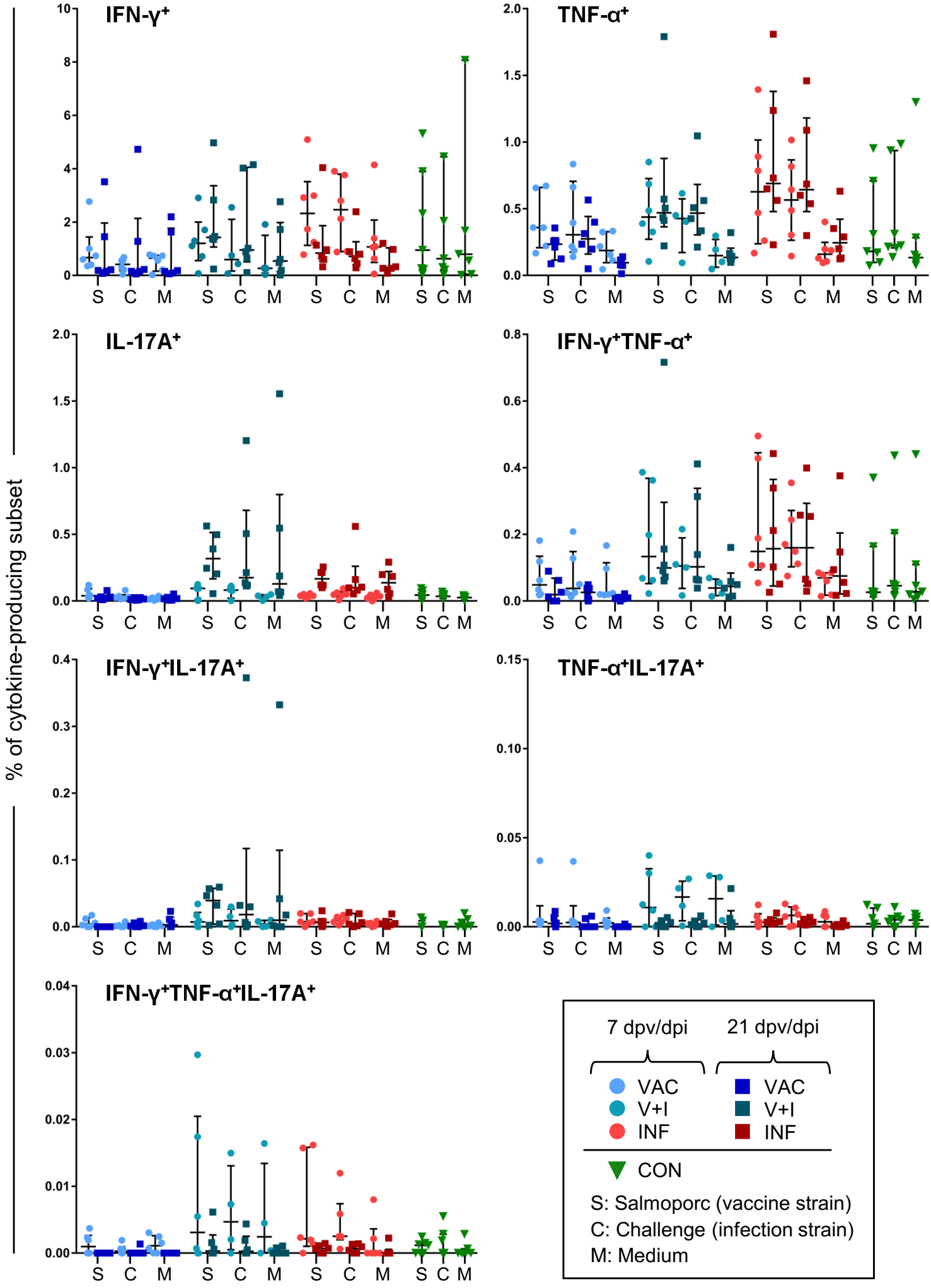


Figure S7F: CD4$^-$CD8β$^-$ T cells; Ileum

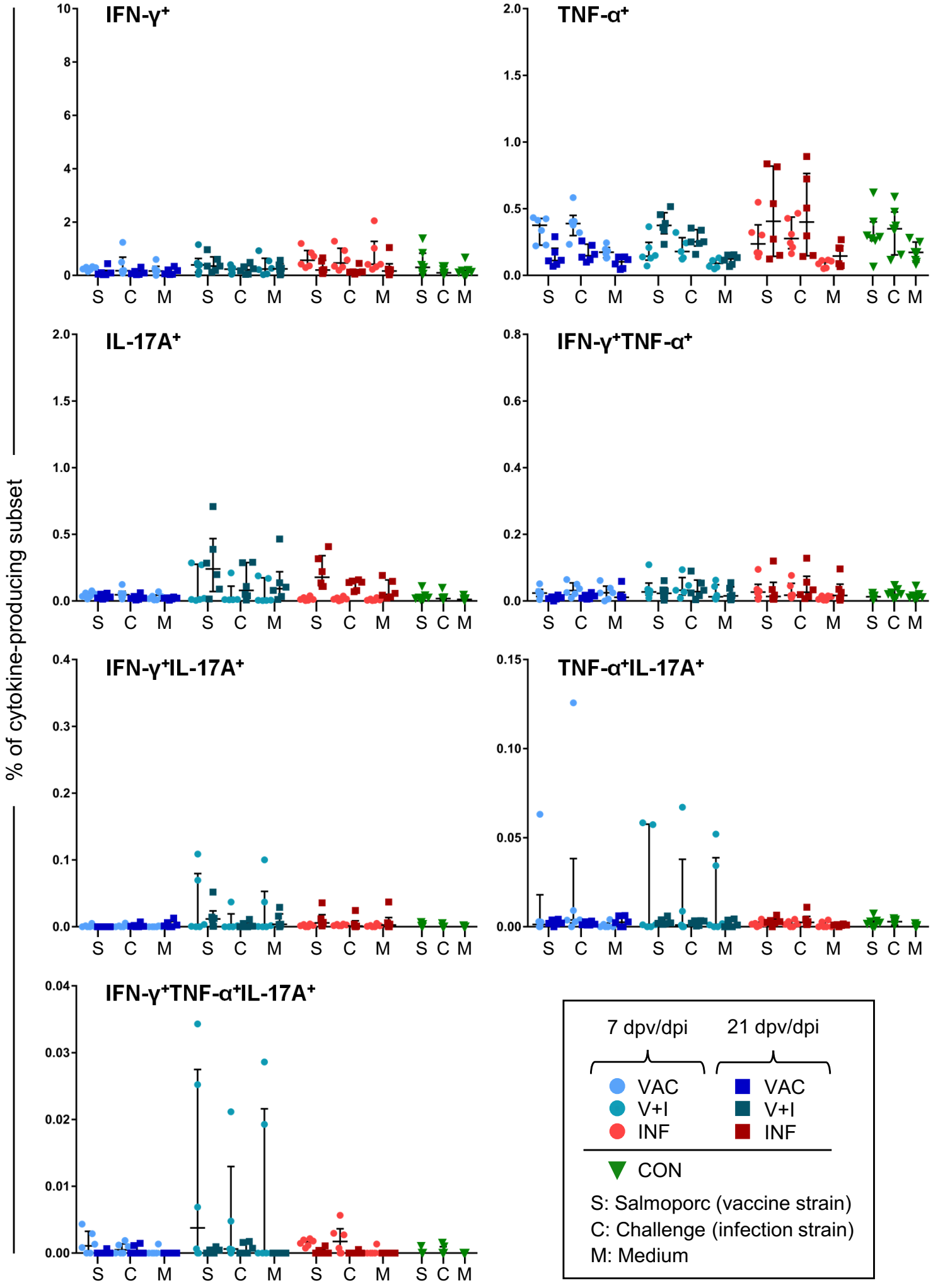
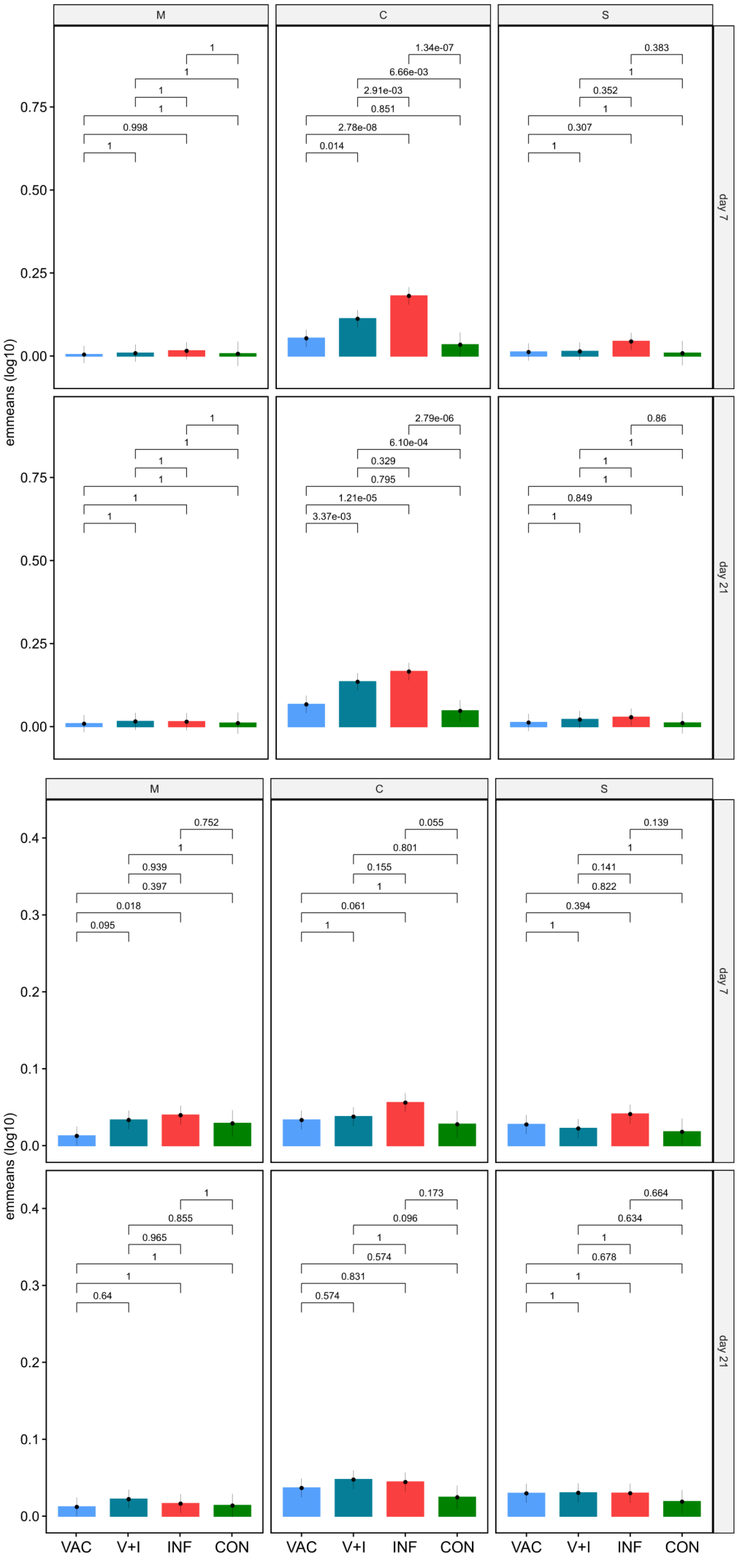


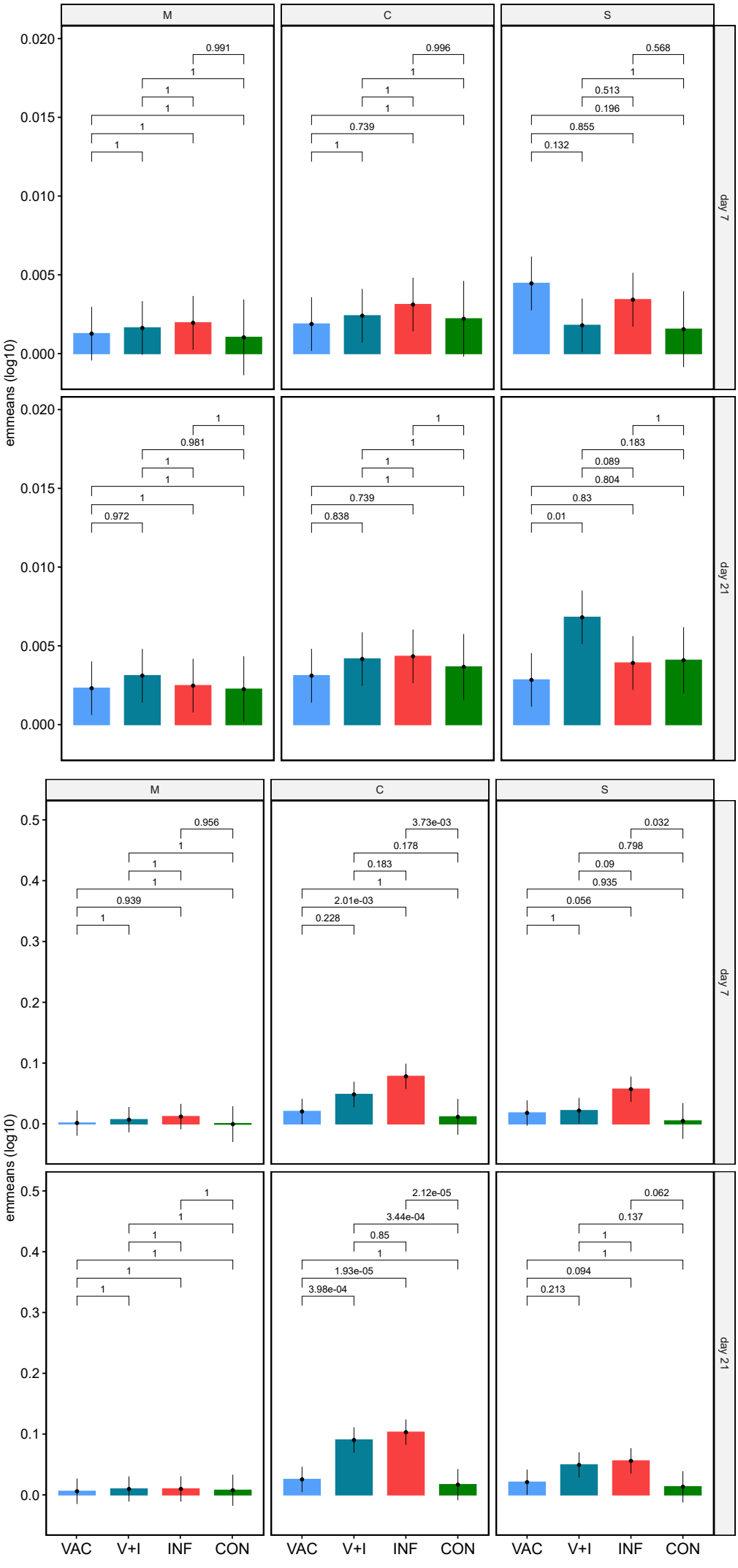
Figure S8A: CD4$^+$ T cells; Blood



S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

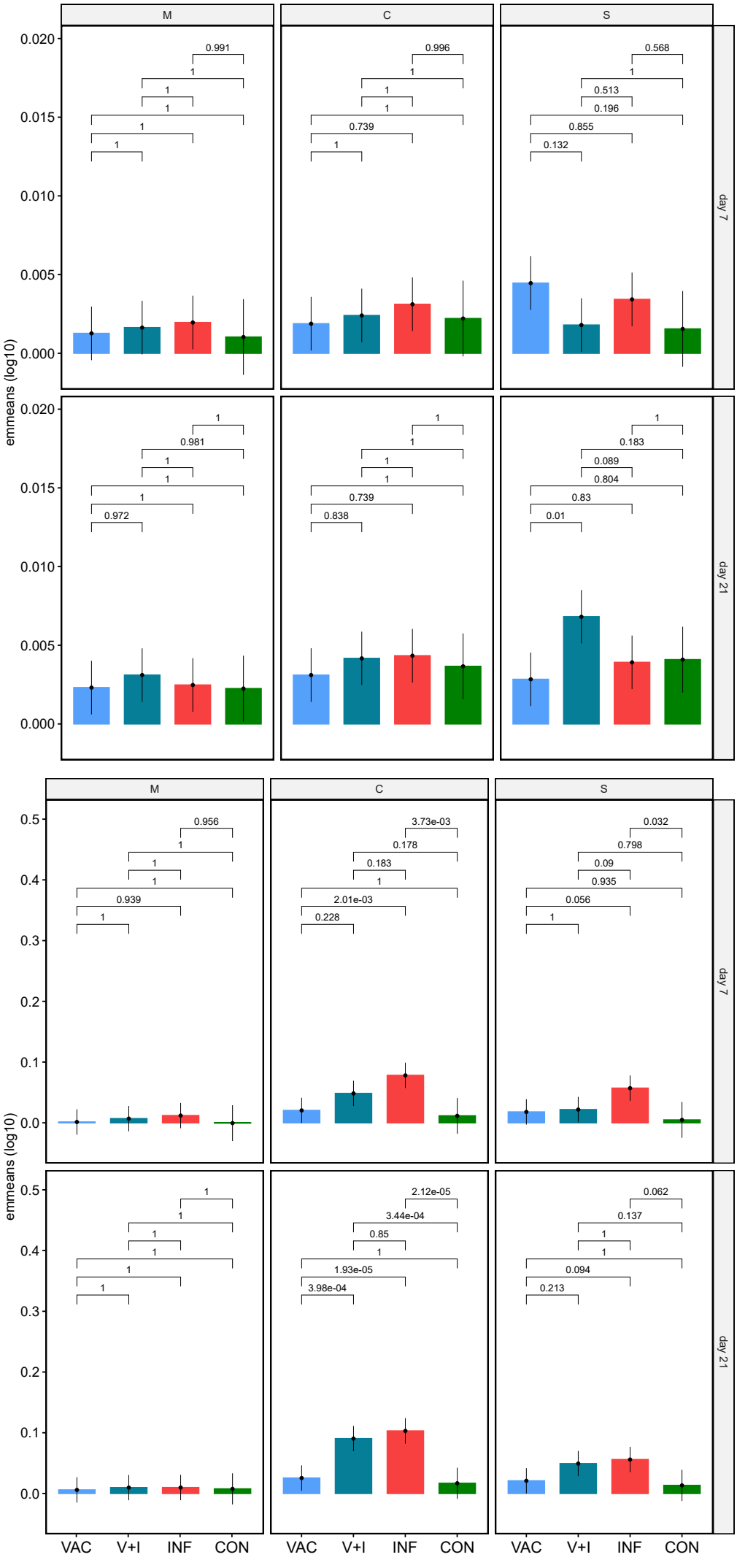
Figure S8A: CD4$^+$ T cells; Blood



S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8A: CD4$^+$ T cells; Blood

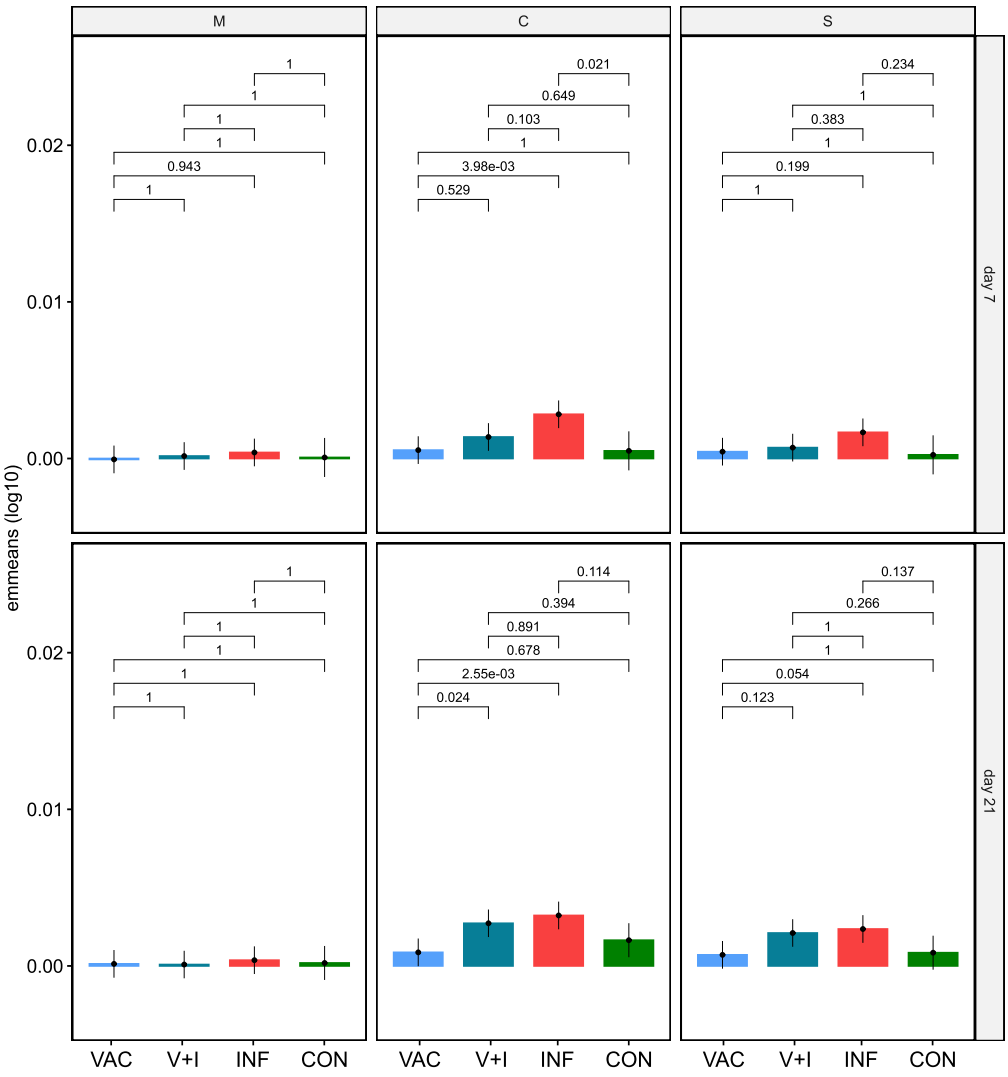


S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8A: CD4$^+$ T cells; Blood

IFN-γ$^+$TNF-α$^+$IL-17A$^+$

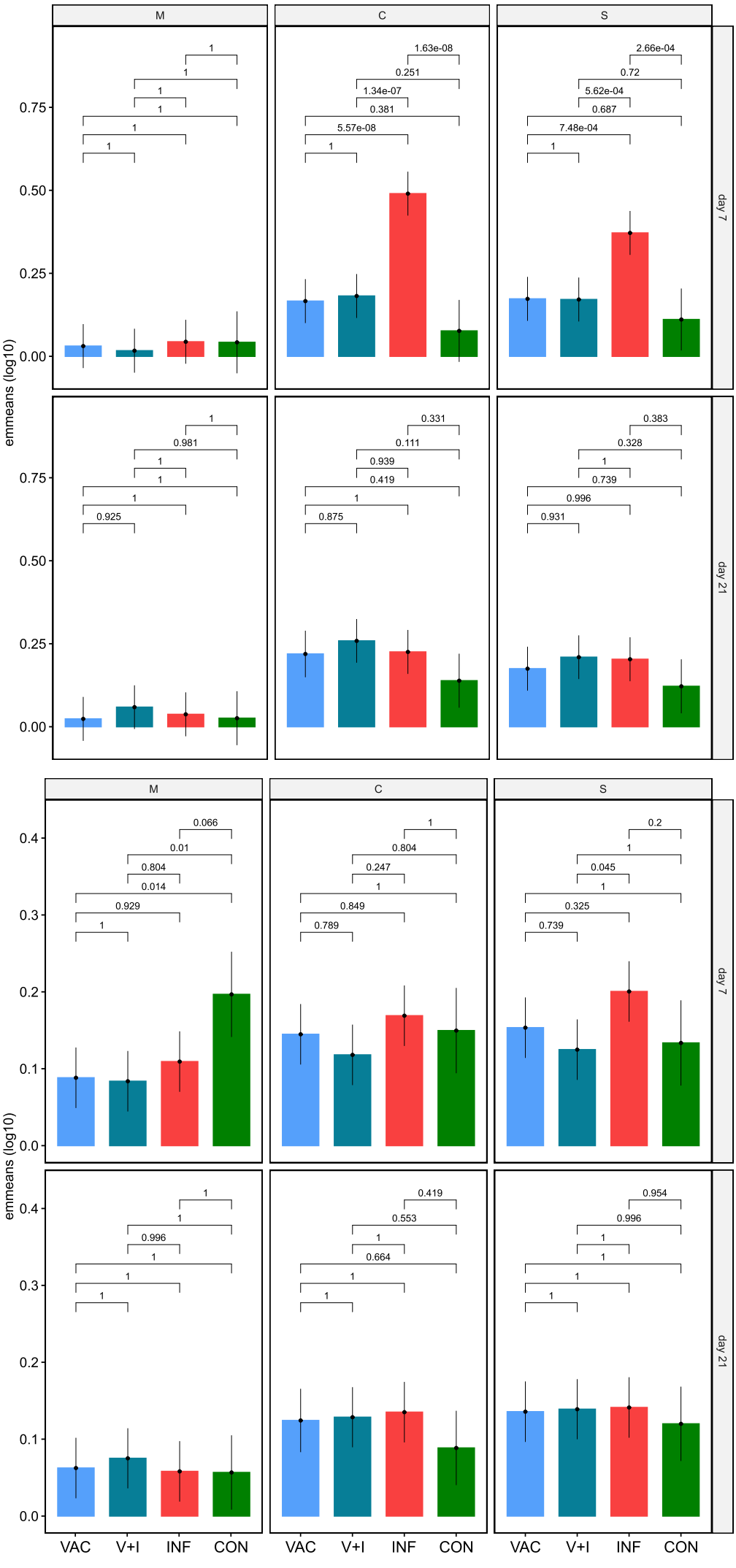


VAC
V+I
INF
CON

S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

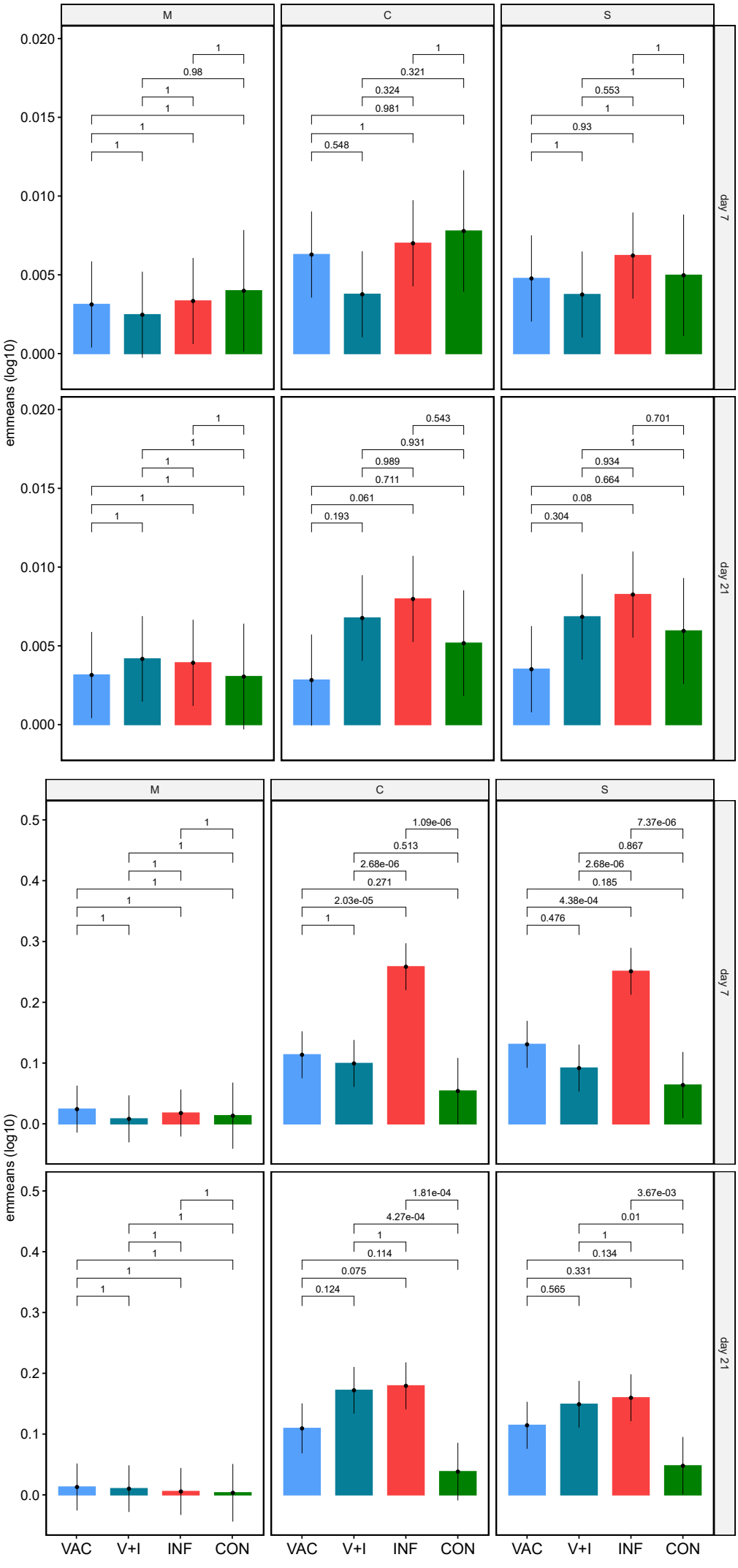
Figure S8B: CD4⁺ T cells; Spleen



S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

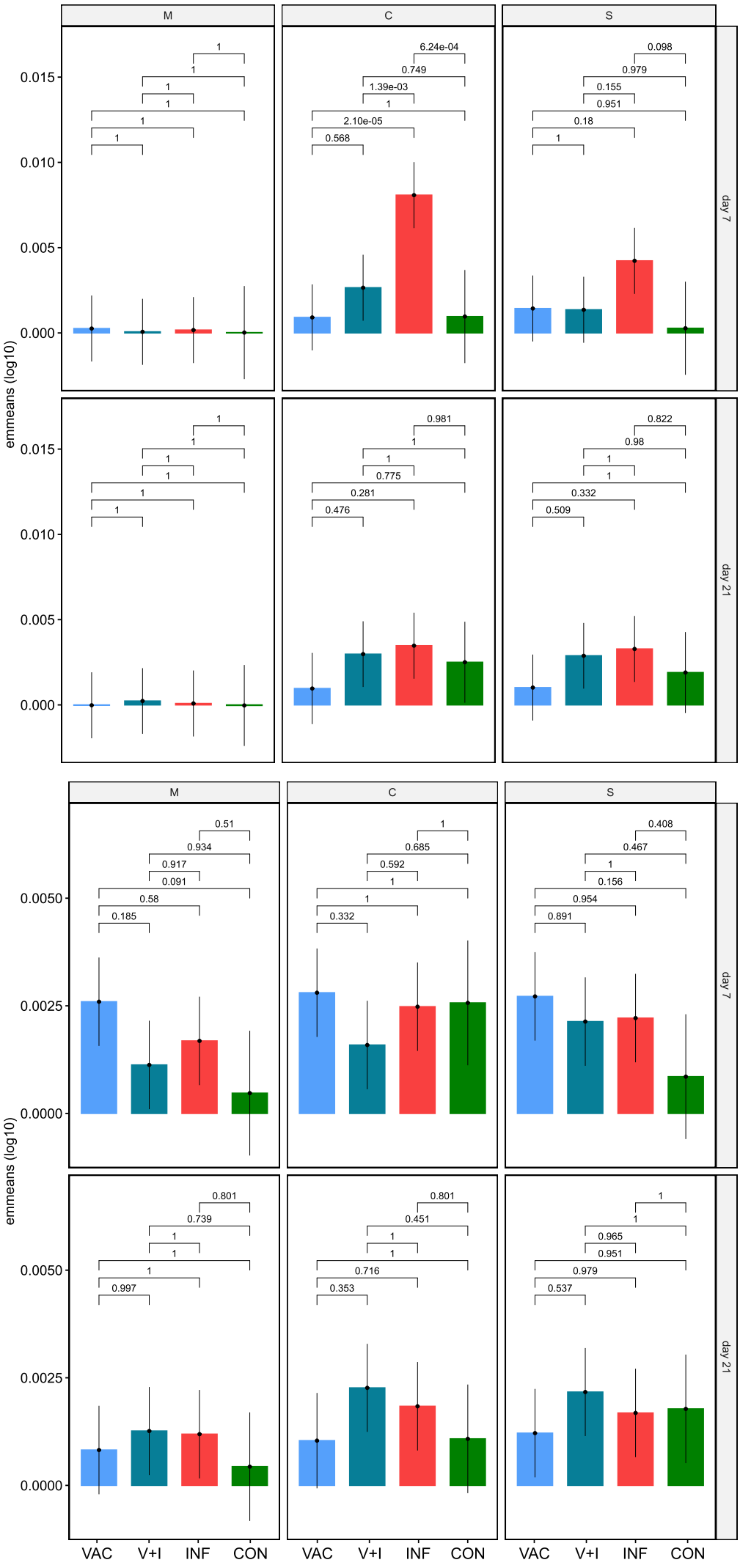
Figure S8B: CD4⁺ T cells; Spleen



S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8B: CD4⁺ T cells; Spleen

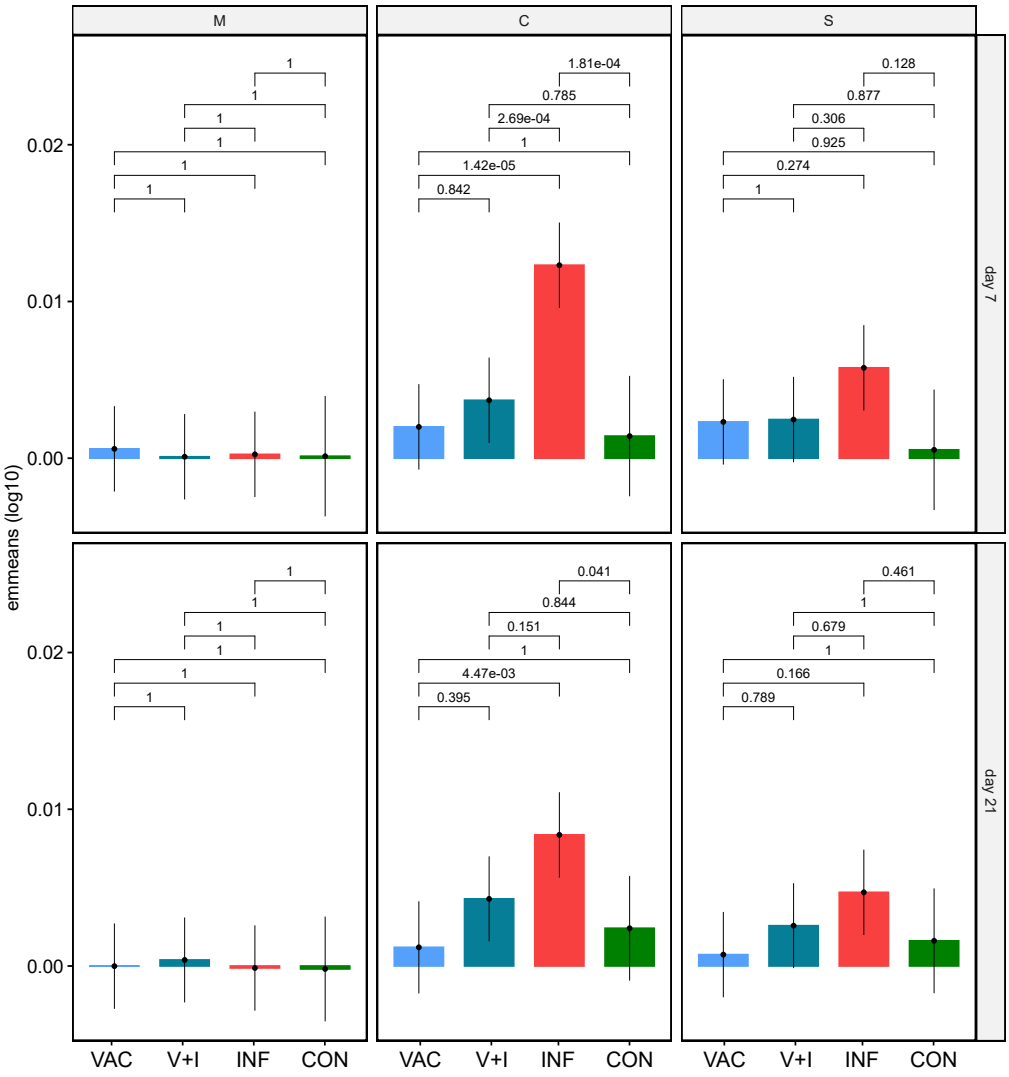


S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8B: CD4⁺ T cells; Spleen

IFN-γ⁺TNF-α⁺IL-17A⁺

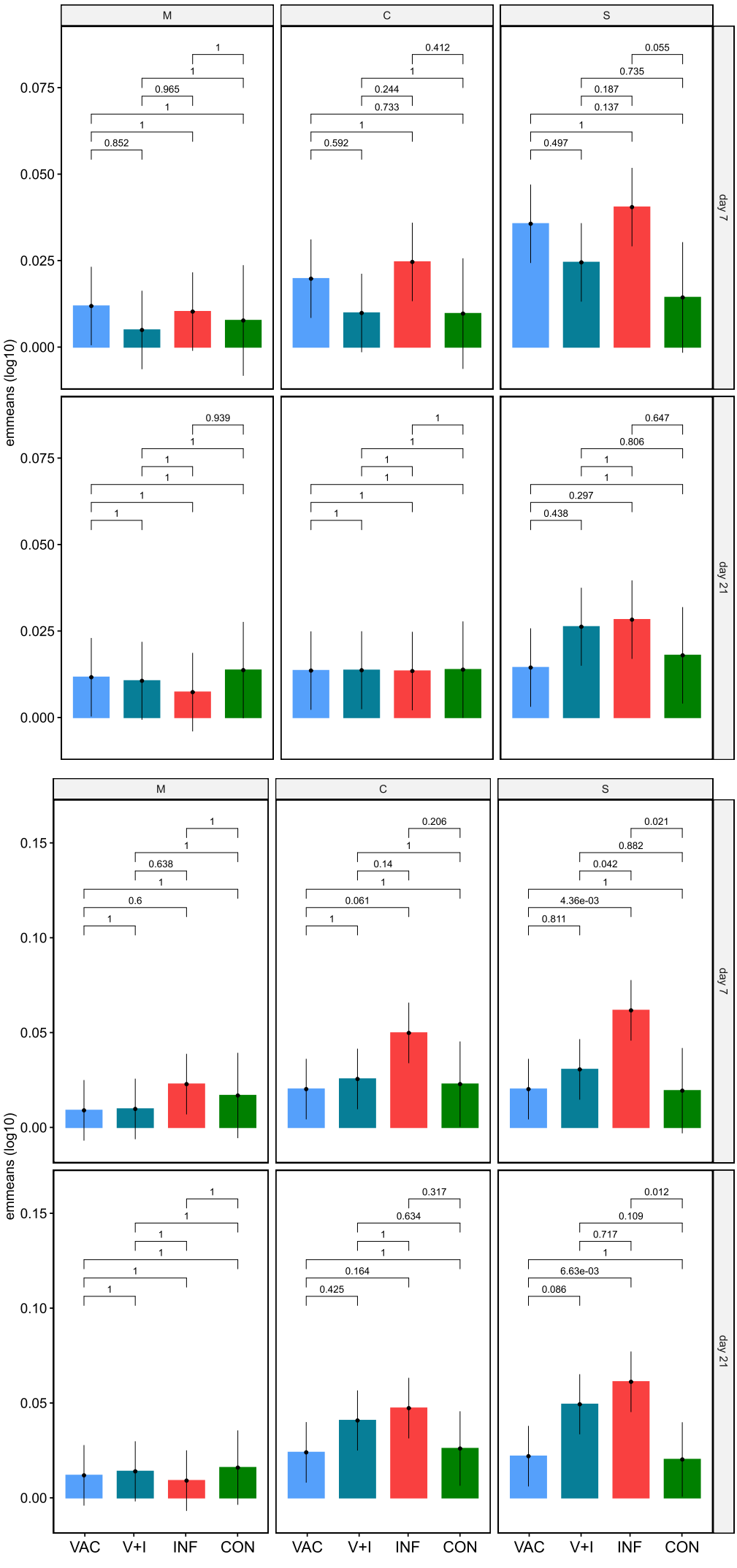


VAC
V+I
INF
CON

S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

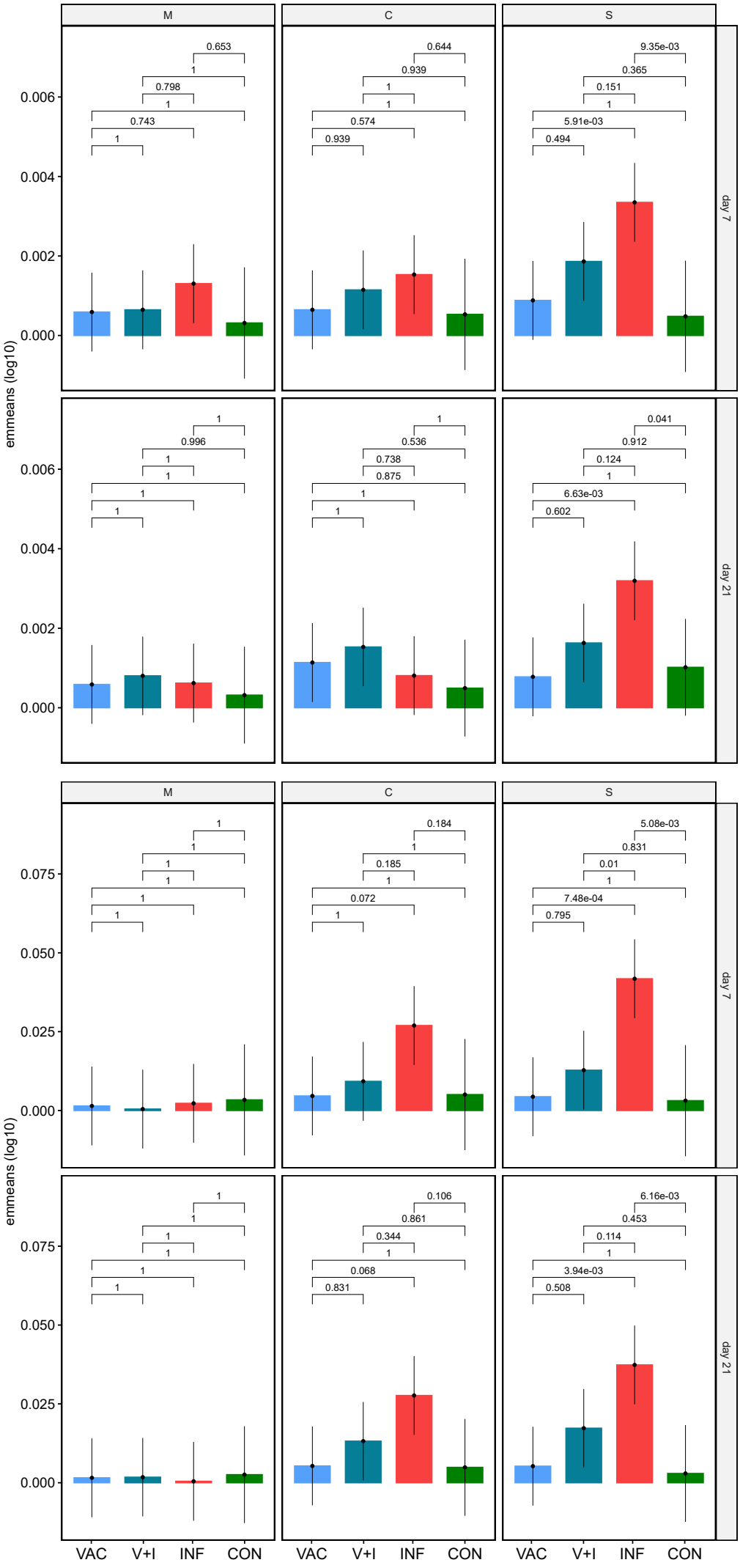
Figure S8C: CD4⁺ T cells; JLN



S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8C: CD4⁺ T cells; JLN



VAC
V+I
INF
CON

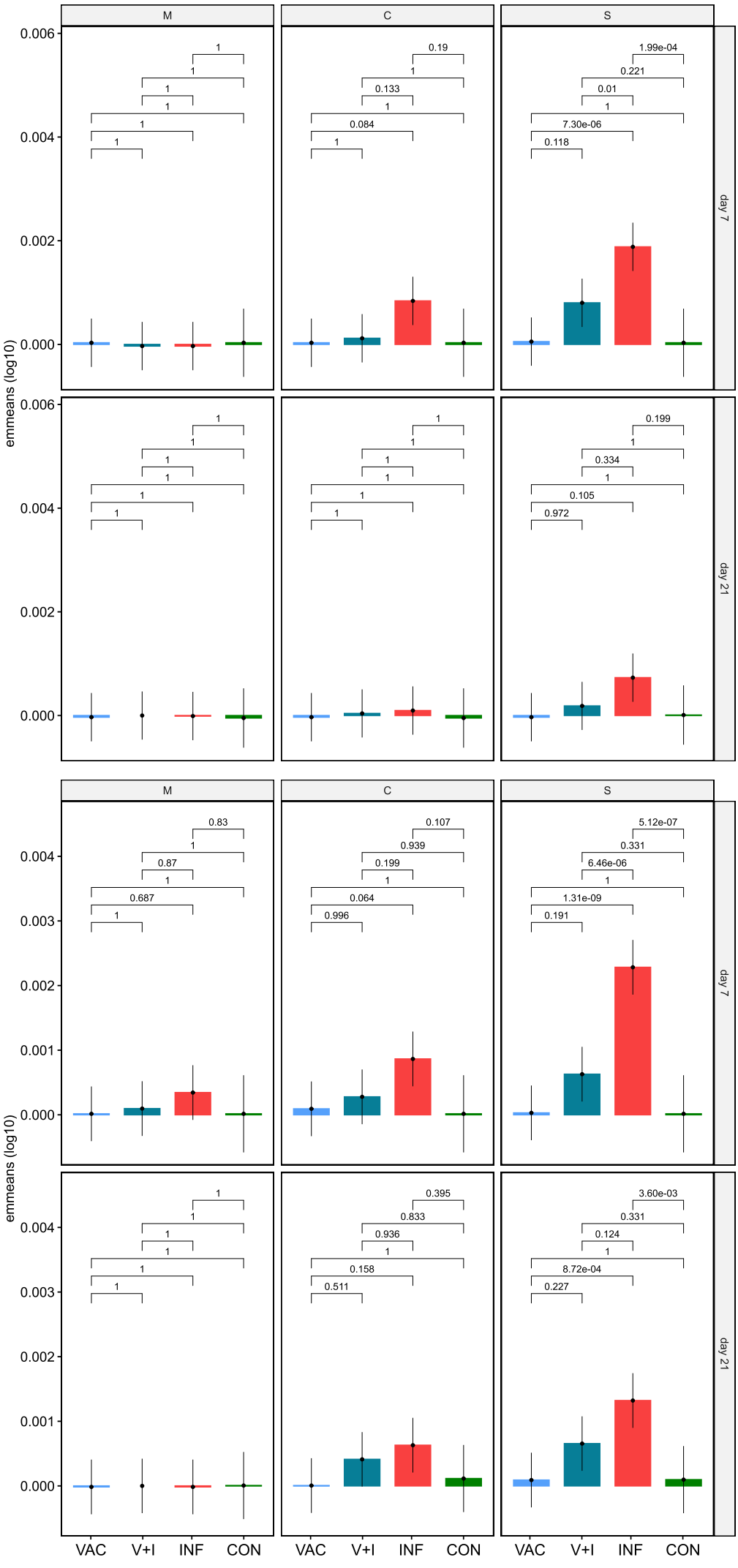
S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8C: CD4⁺ T cells; JLN

IFN-γ⁺IL-17A⁺

TNF-α⁺IL-17A⁺



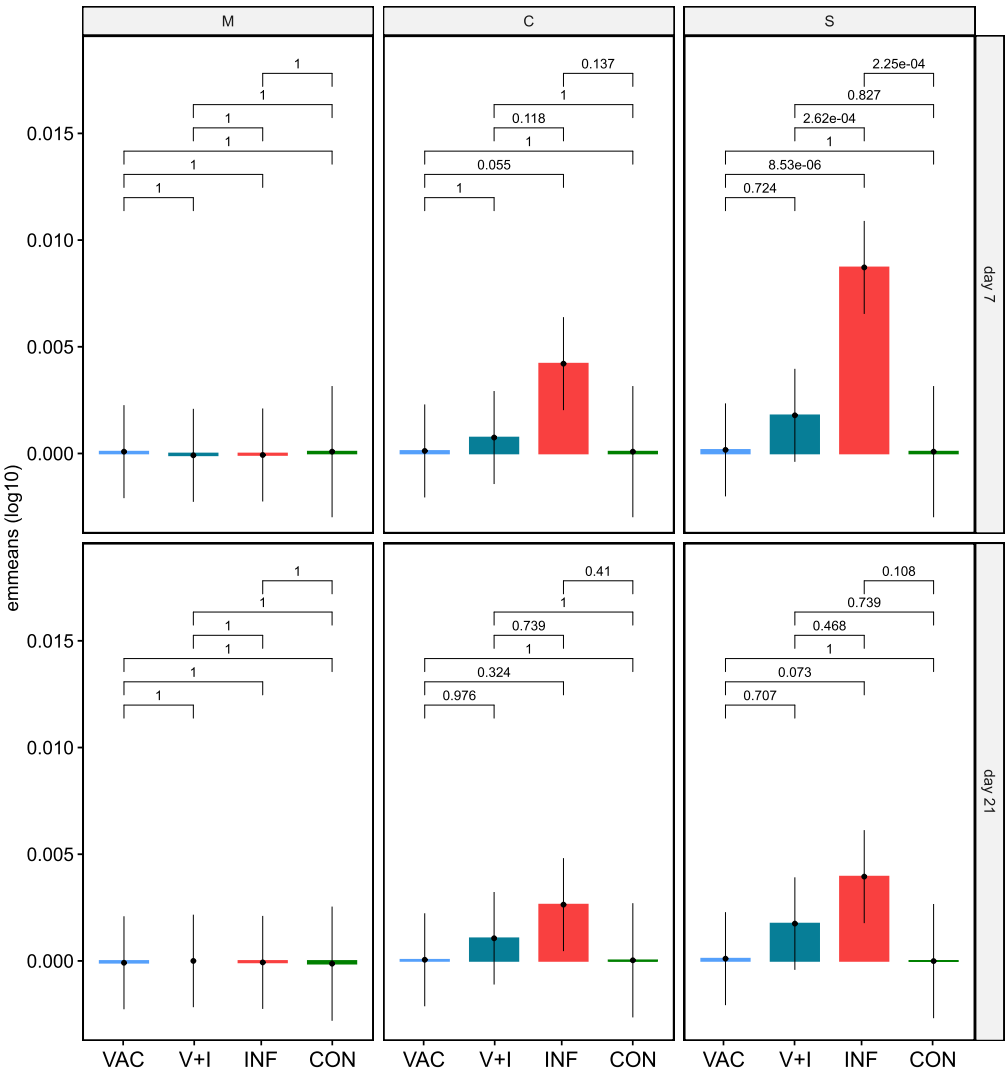
VAC
V+I
INF
CON

S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8C: CD4⁺ T cells; JLN

IFN-γ⁺TNF-α⁺IL-17A⁺

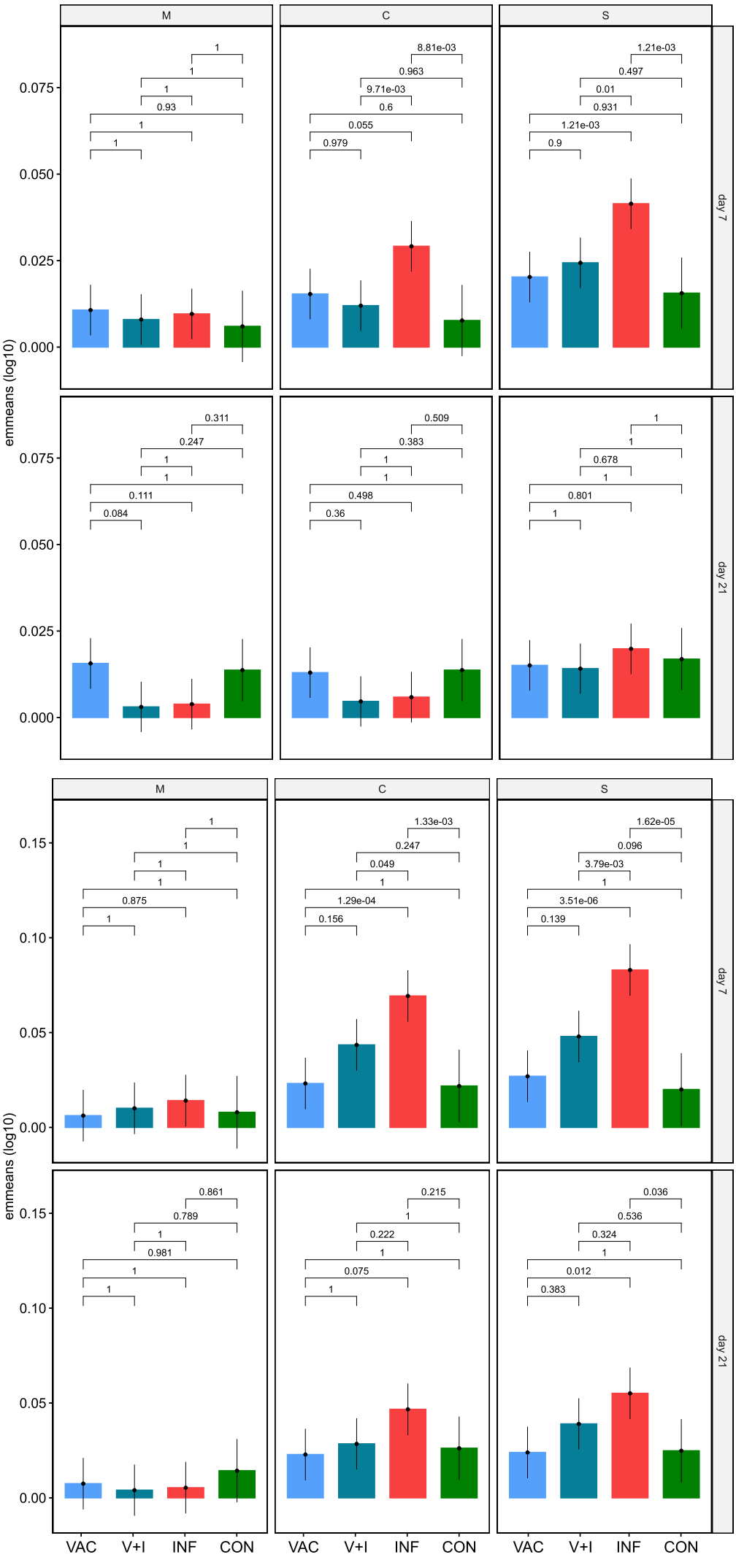


VAC
V+I
INF
CON

S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

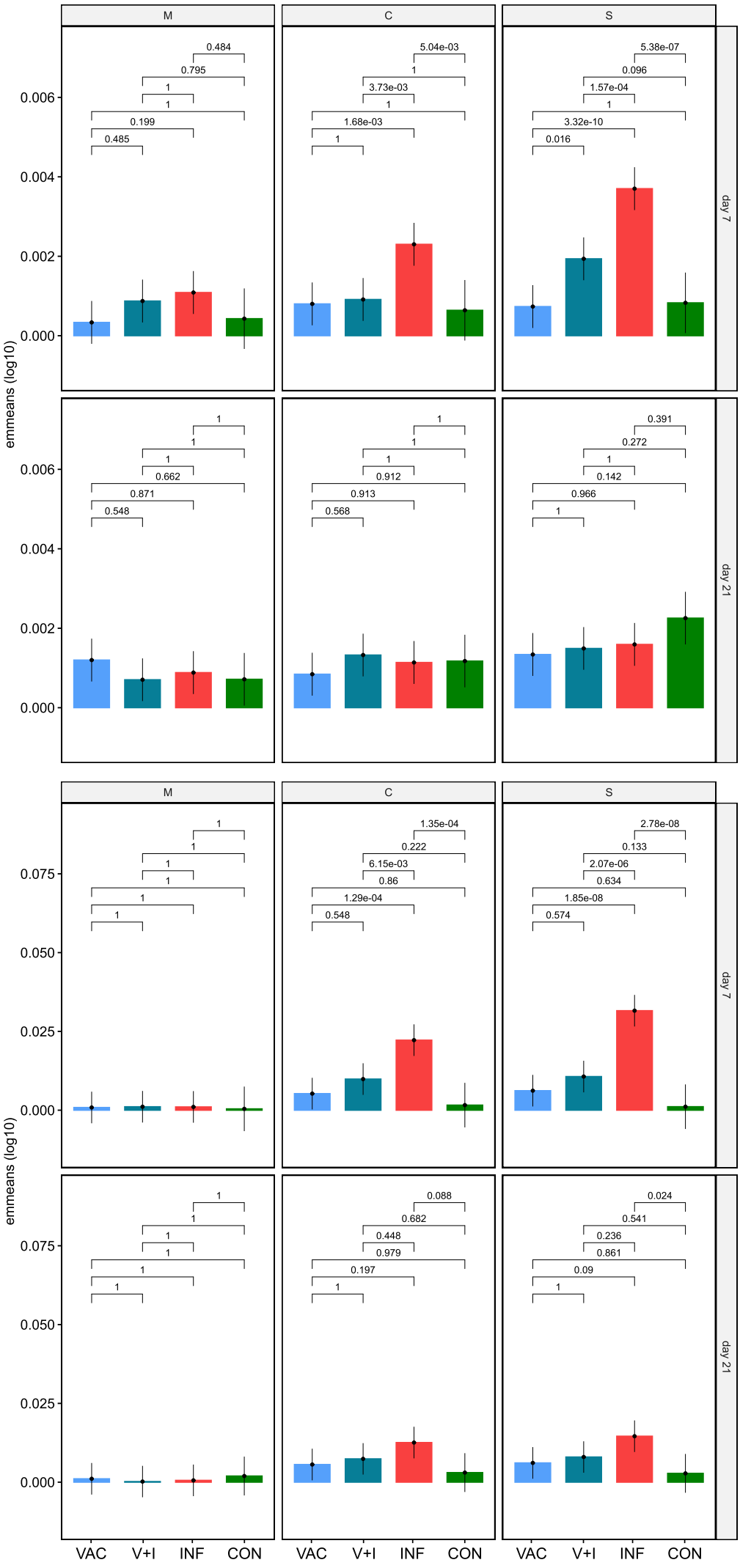
Figure S8D: CD4⁺ T cells; ICLN



S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8D: CD4⁺ T cells; ICLN

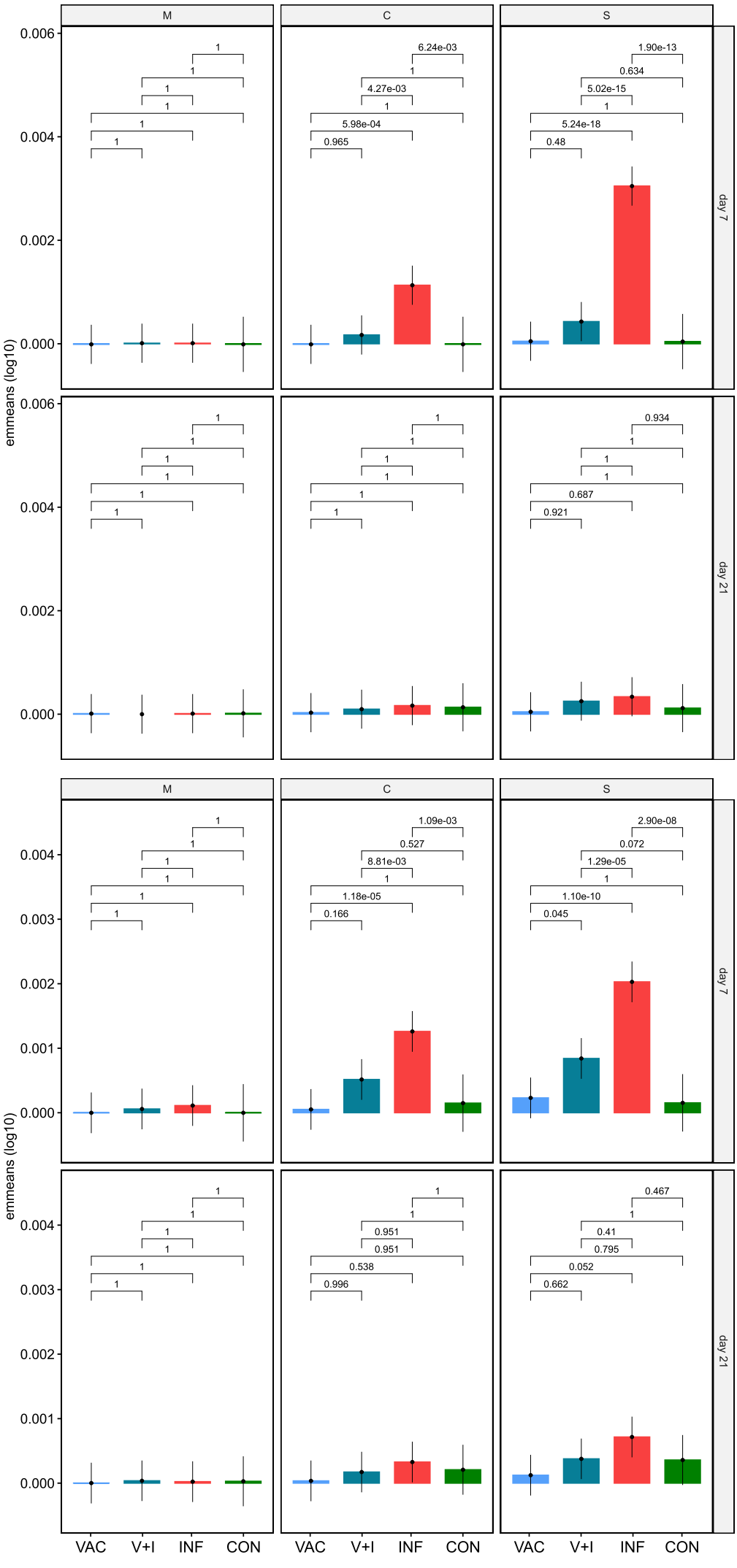


VAC
V+I
INF
CON

S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8D: CD4⁺ T cells; ICLN

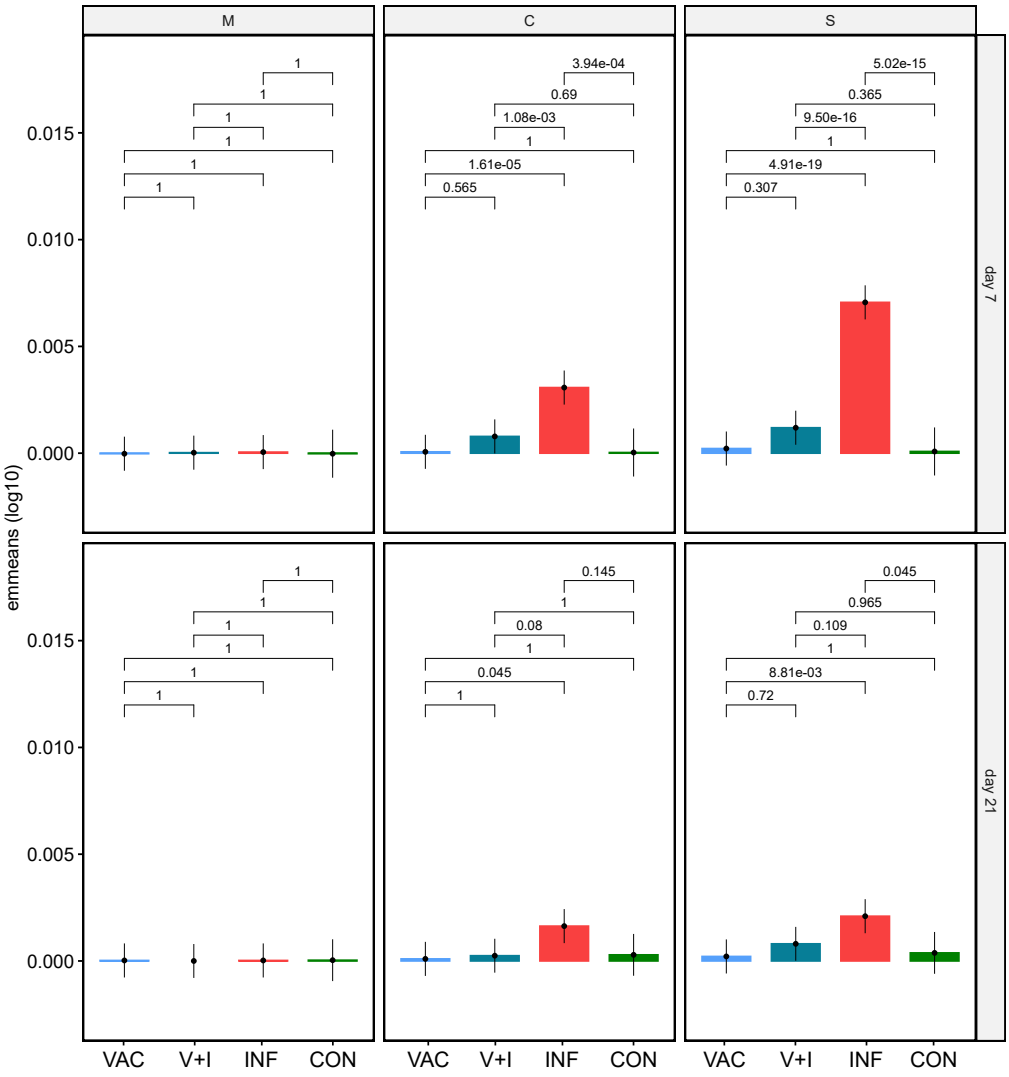


S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8D: CD4⁺ T cells; ICLN

IFN-γ⁺TNF-α⁺IL-17A⁺



VAC
V+I
INF
CON

S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8E: CD4⁺ T cells; Jejunum

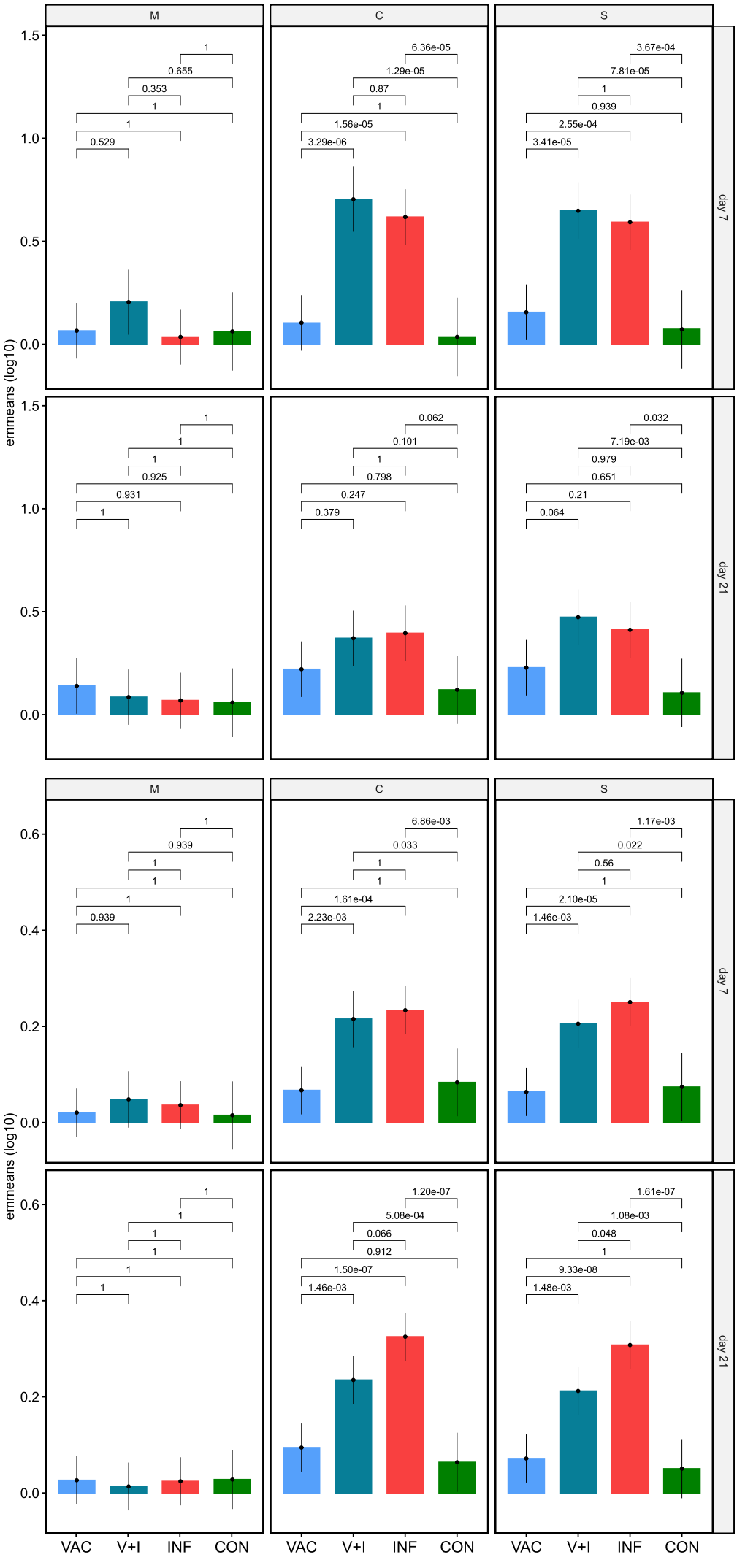
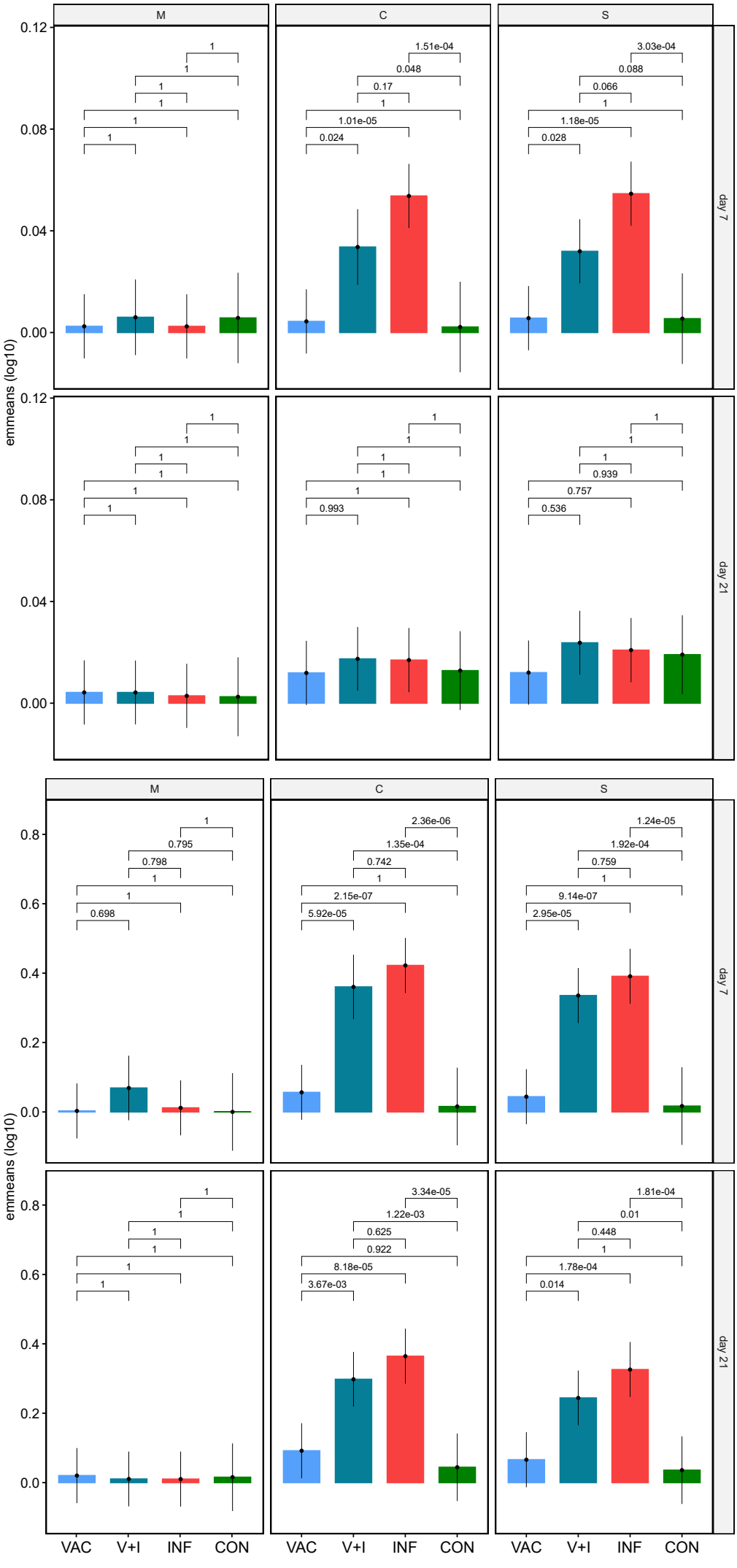


Figure S8E: CD4⁺ T cells; Jejunum

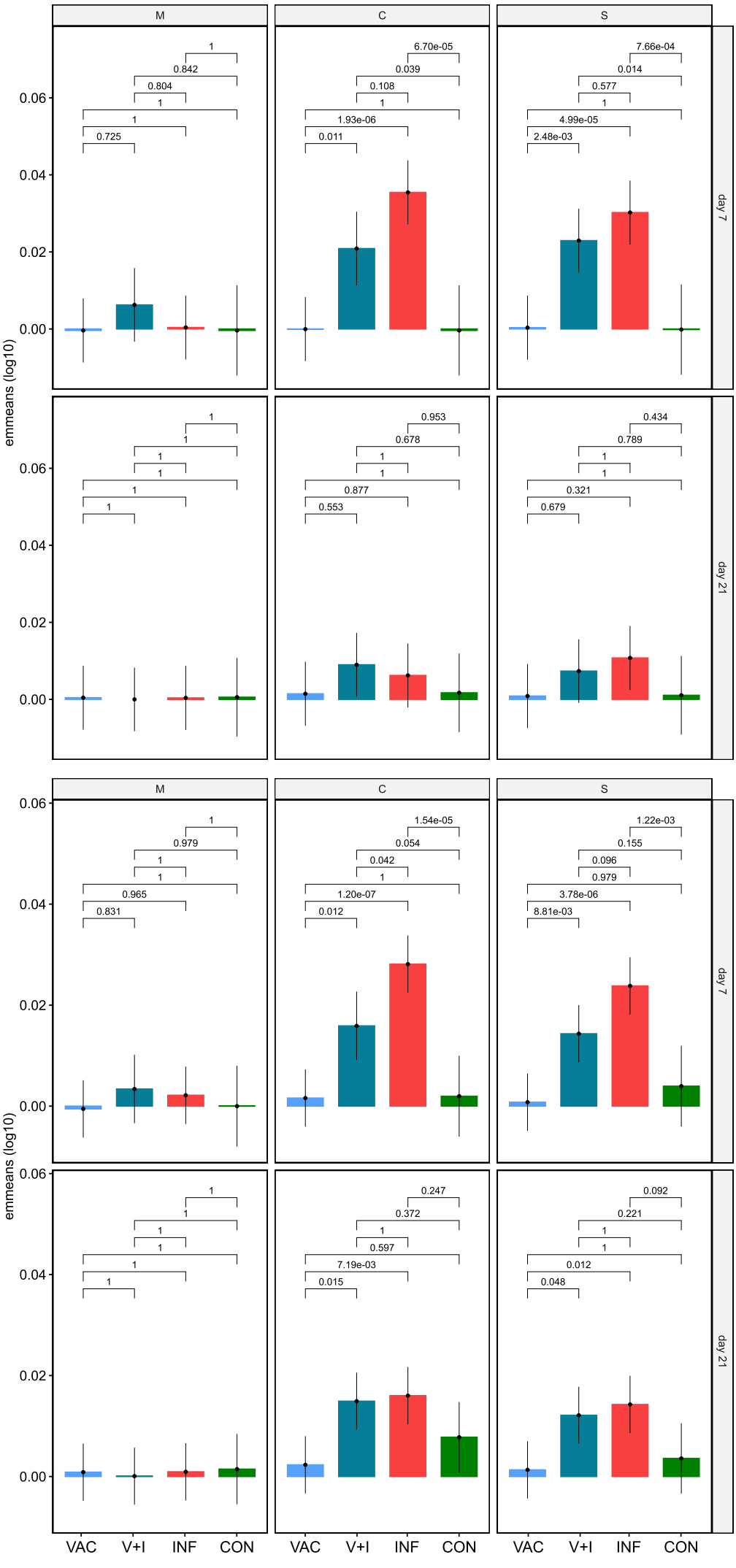


VAC
V+I
INF
CON

S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8E: CD4⁺ T cells; Jejunum

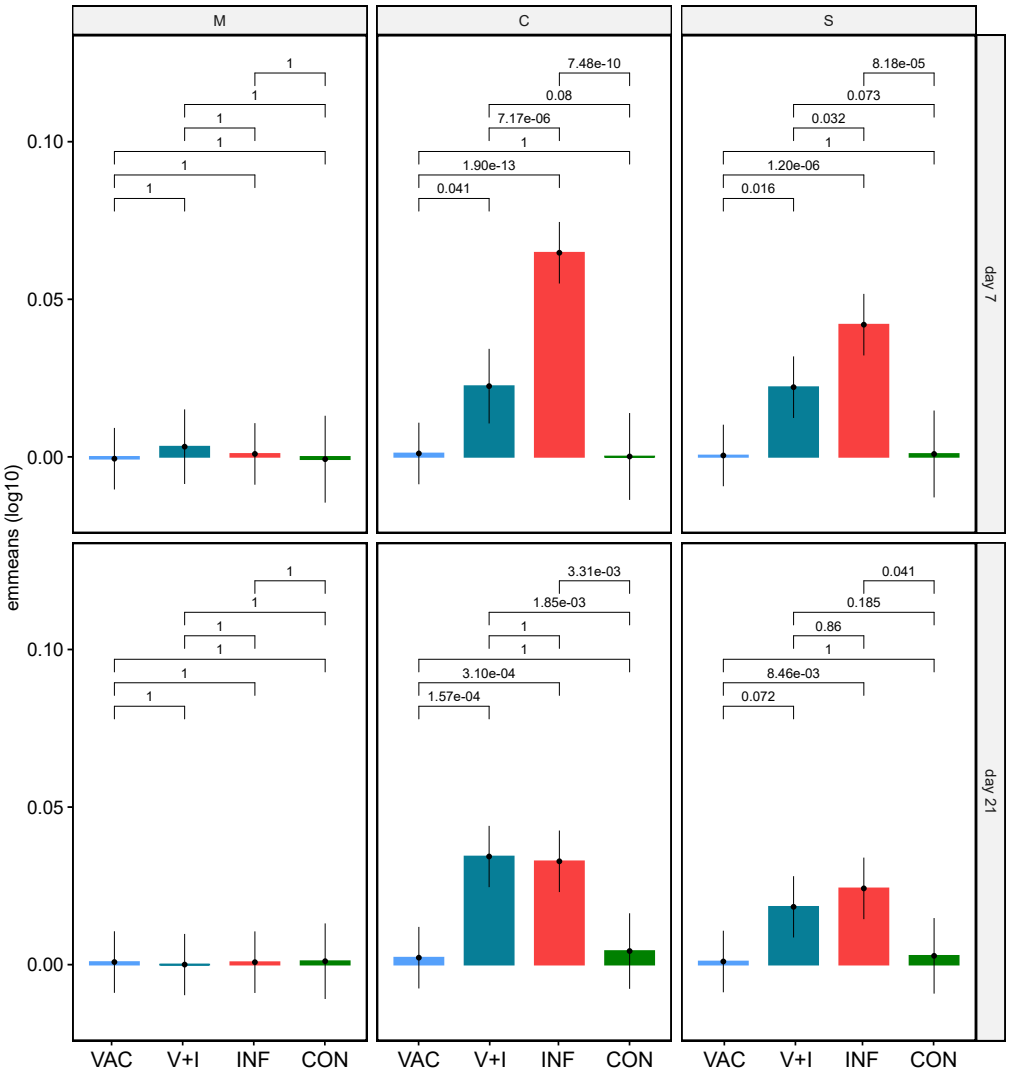


S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8E: CD4⁺ T cells; Jejunum

IFN-γ⁺TNF-α⁺IL-17A⁺

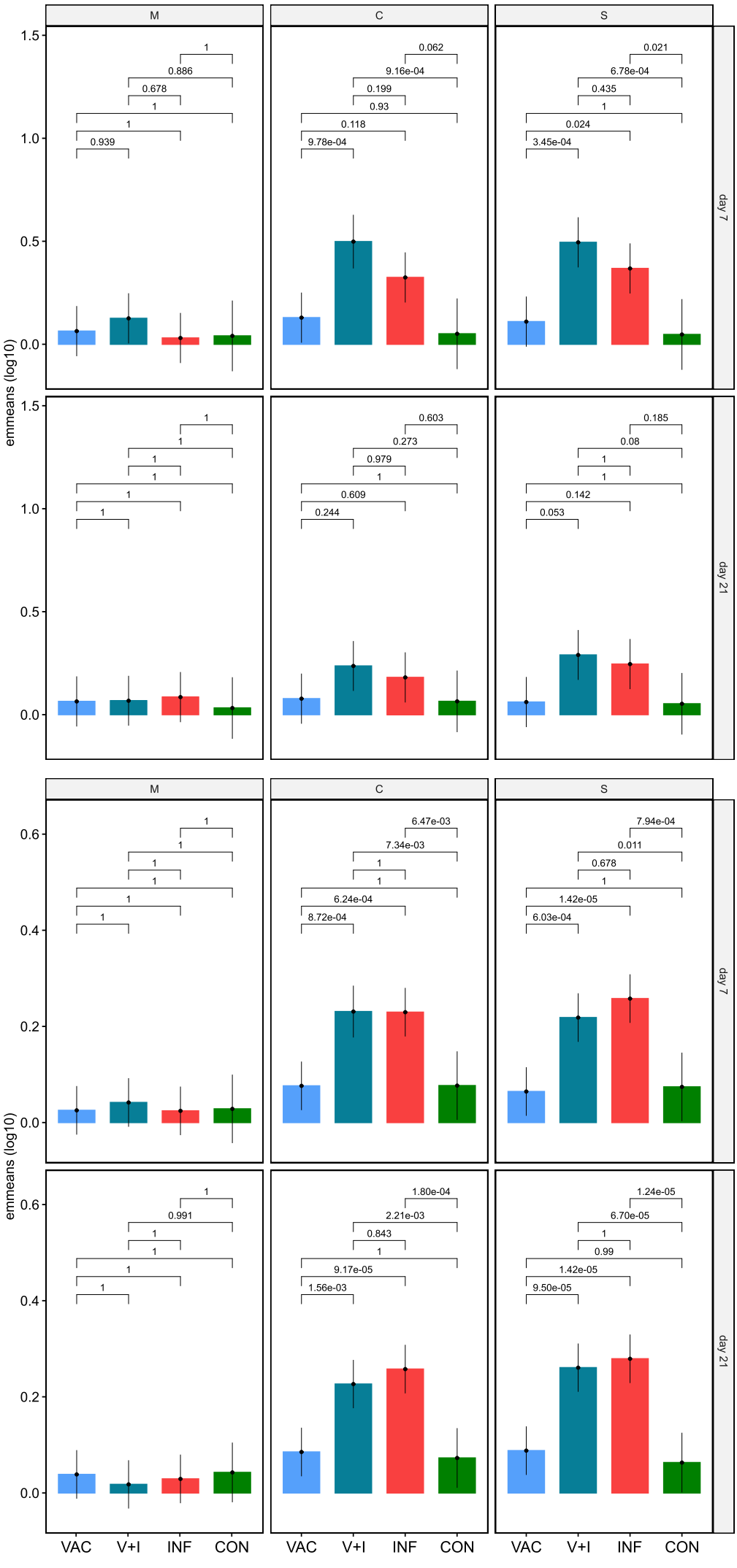


VAC
V+I
INF
CON

S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

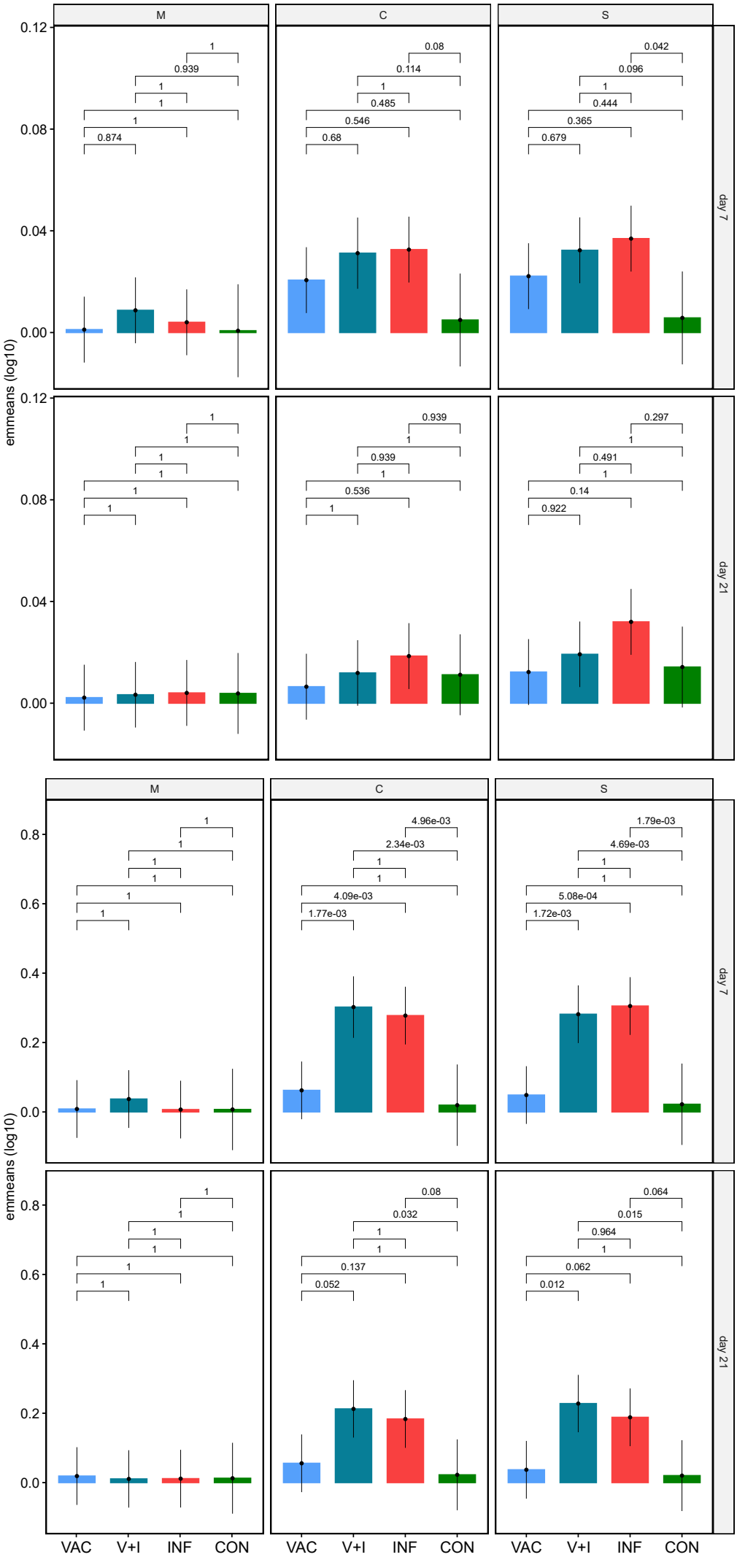
Figure S8F: CD4⁺ T cells; Ileum



S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

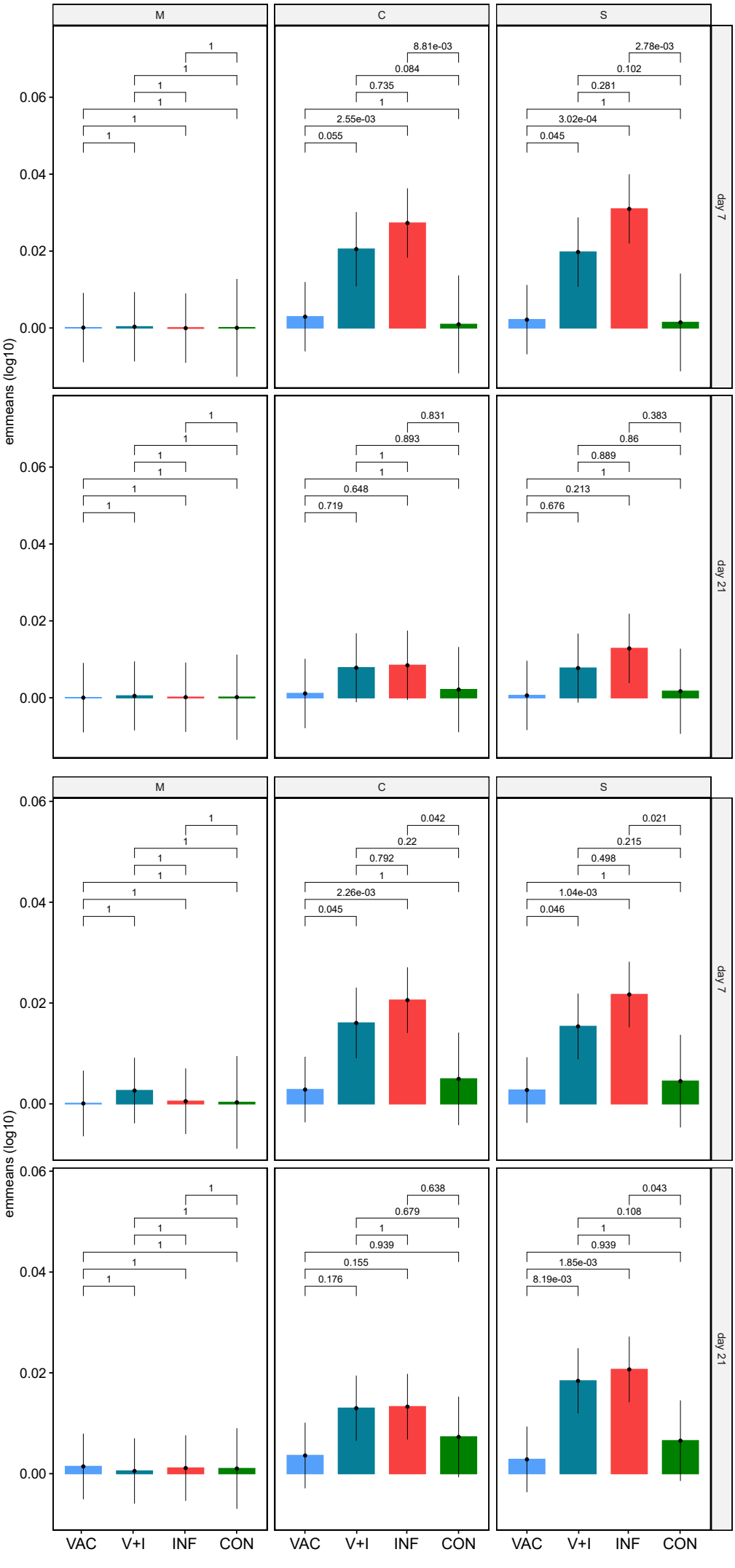
Figure S8F: CD4⁺ T cells; Ileum



S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8F: CD4⁺ T cells; Ileum



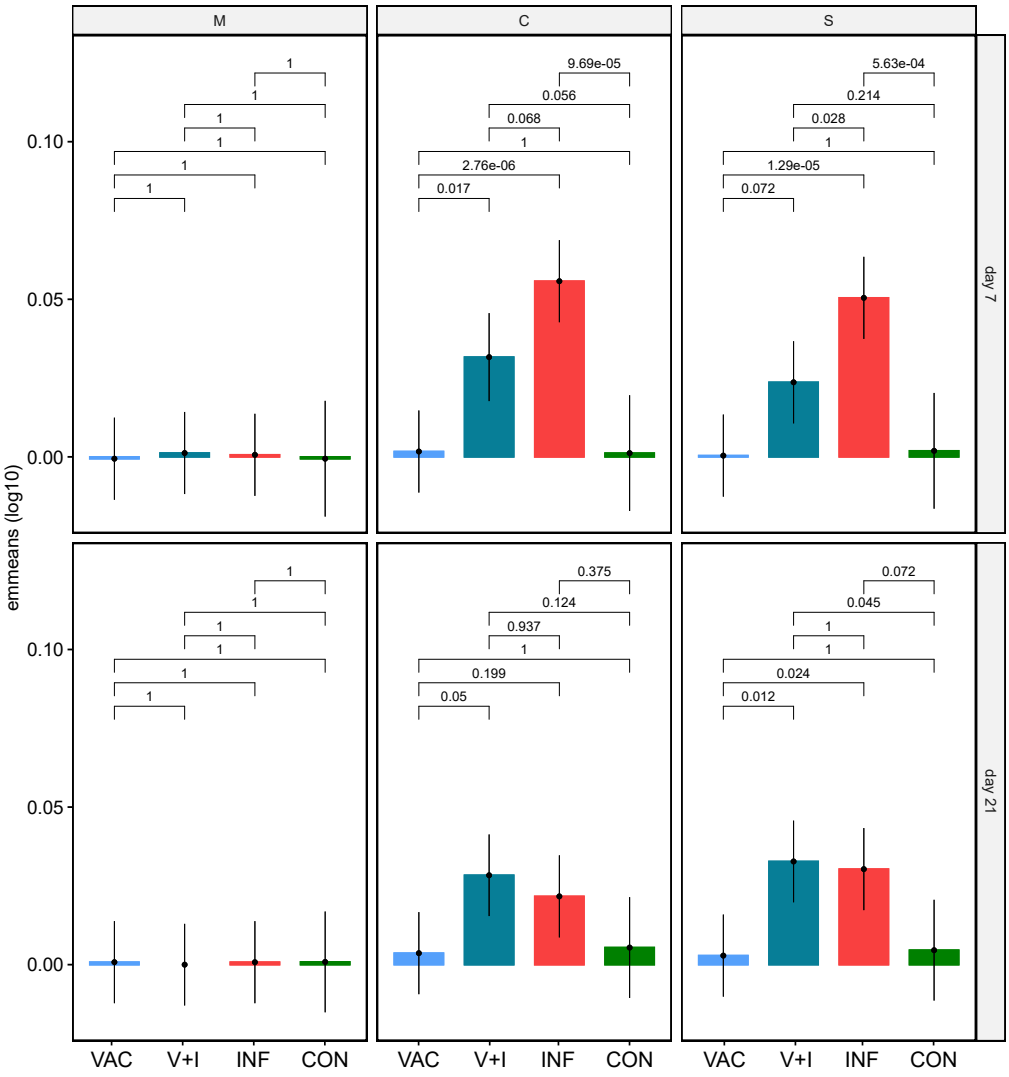
VAC
V+I
INF
CON

S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S8F: CD4⁺ T cells; Ileum

IFN-γ⁺TNF-α⁺IL-17A⁺

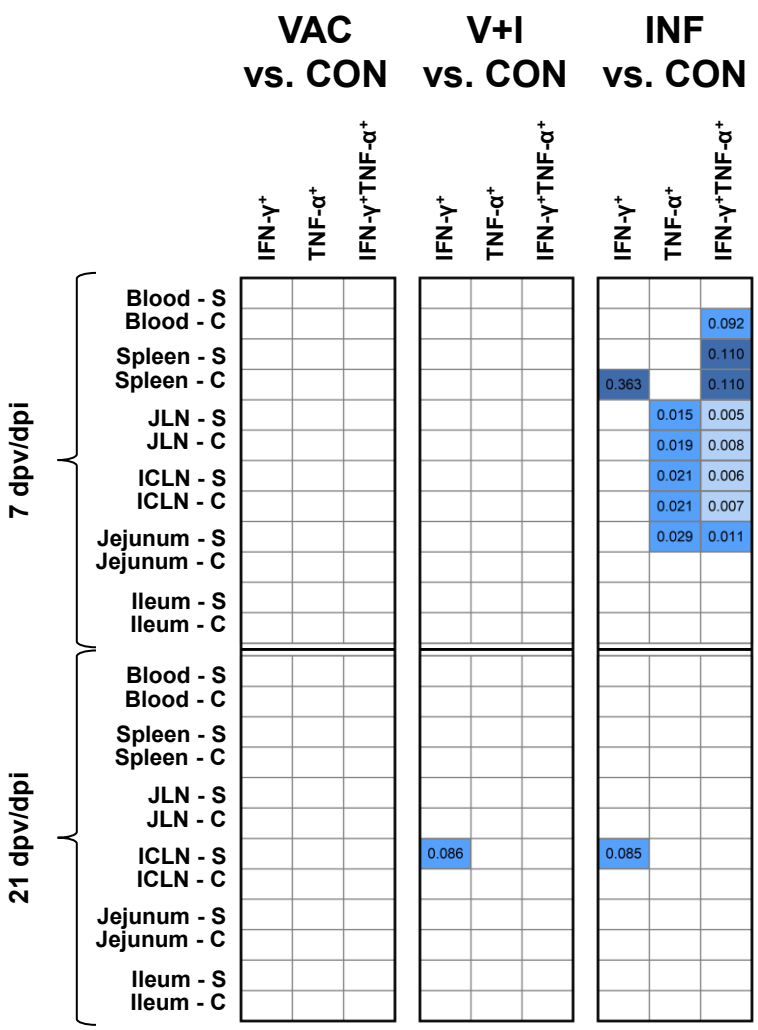


VAC
V+I
INF
CON

S: Salmoporc (vaccine strain)
C: Challenge (infection strain)
M: Medium

day 7: 7 dpv/dpi
day 21: 21 dpv/dpi

Figure S9: CD8⁺ T cells



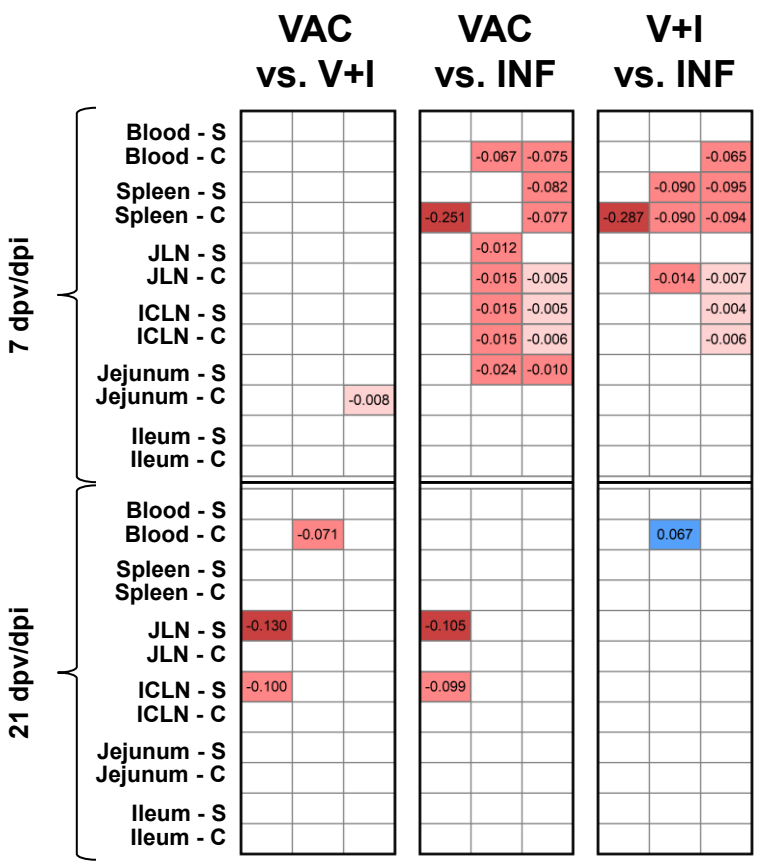
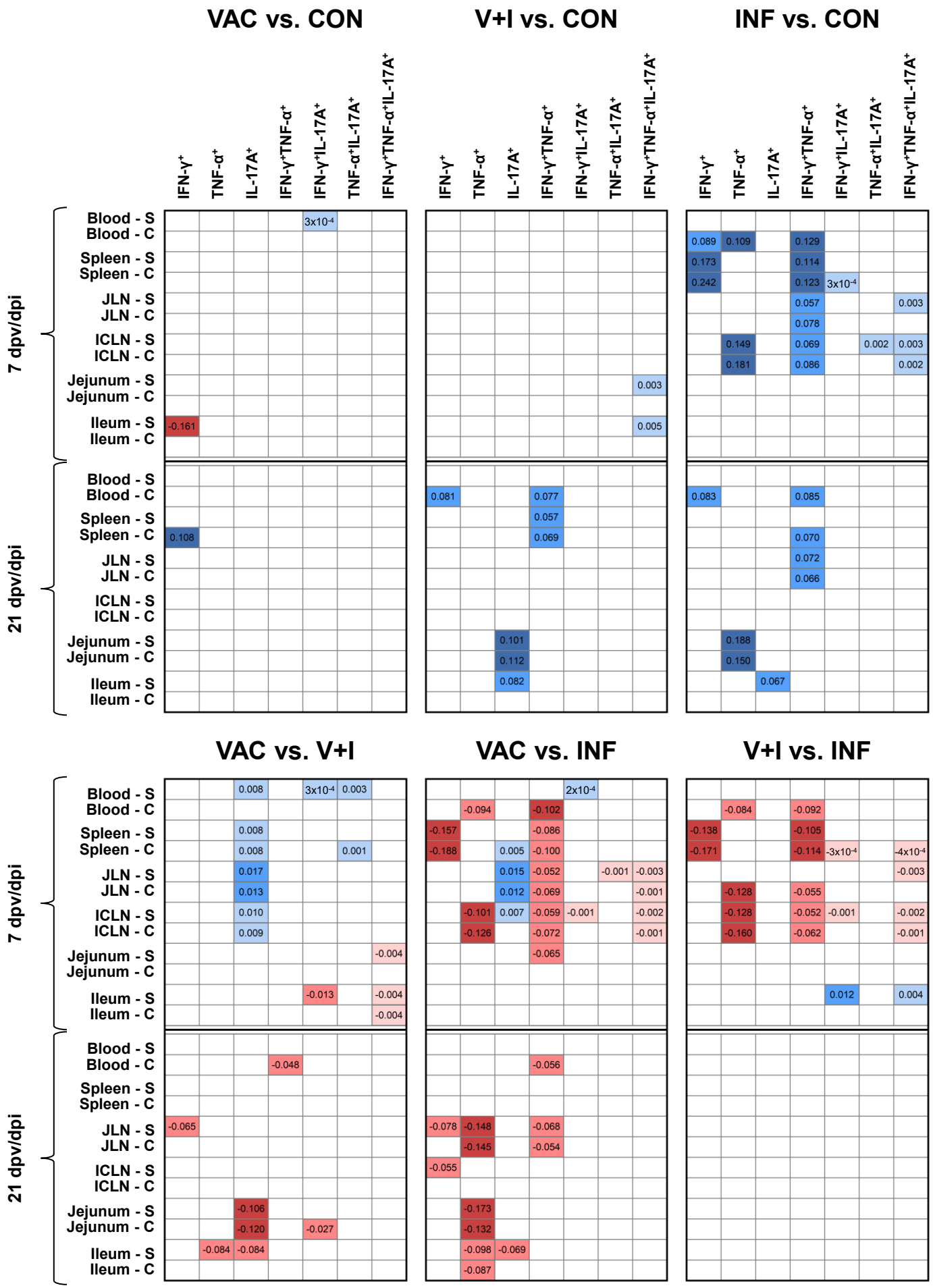


Figure S10: CD4-CD8β- T cells



6 Discussion

Despite regular testing and increased measures for prevention, *Salmonella* Typhimurium (STM) continues to be highly prevalent on swine farms and therefore remains a major food safety hazard for public health. An expansion of the knowledge on the cellular immune mechanisms in response to STM infection as well as those induced by vaccination are therefore crucial to manage the disease on farm level thereby reducing the risk of food contamination at the same time. This PhD project aimed to evaluate the T-cell immune response against STM in the pig. To that end, two animal experiments were conducted where piglets were vaccinated and/or infected with STM followed by analysis of the cellular immune response in local and systemic organs harvested from these pigs. In a pilot trial (Trial 1) with a total of sixteen animals, twelve piglets received two oral immunizations with the Salmoporc vaccine, followed by infection with a virulent STM strain. Four piglets served as a control group and all animals were euthanized 14 days post infection. Findings collected as part of this trial were published in Frontiers in Immunology (Schmidt et al. 2020). In a second trial (Trial 2), forty-four piglets were divided into four treatment groups. Groups 1 (VAC) and 2 (V+I) were vaccinated twice with Salmoporc but only group 2 was subsequently challenged with the virulent STM strain. Group 3 (INF) was challenged but not vaccinated and group 4 served as a control group (CON). Insights gained in Trial 2 were published in *Vaccines* (Schmidt et al. 2021).

6.1 Clinical signs

Clinical signs during the animal experiments were monitored and recorded daily. Typical indications of salmonellosis include lethargy, a rise in temperature and diarrhea, often yellow and watery (Wray and Wray. 2000). Overall, extent and severity of clinical signs observed during both trials were in accordance with data collected in similar studies (Springer et al. 2001; Theuß et al. 2017) and matched our expectations. Prior to any treatment, an increase in rectal temperature and mild diarrhea were observed intermittently at the beginning of the study. This is unsurprising as pigs are highly stress-sensitive animals (Einarsson et al. 2008). Therefore, handling as well as changes in diet and social group formation will be rapidly reflected in raised body temperature and altered consistency of the feces. In Trial 1, the two-time vaccination of piglets with Salmoporc did not result in any notable clinical signs. The same immunization regimen was applied in Trial 2. Here, average daily gain of weight (ADG) was slightly reduced during a one-week period two weeks after the first immunization in VAC and V+I animals. It

is possible that this difference in ADG is due to a lower food intake caused by effects of the vaccination though ADG can also fluctuate naturally and numbers of daily gain varied greatly between individual animals. According to the instruction for use of the vaccine, a transient increase in body temperature and mild diarrhea in piglets are possible side effects after vaccination, however, these were not observed to any notable extent when compared to the non-vaccinated groups in both trials conducted in our experimental facilities. This may be different when the vaccination is applied in a field setting, where pigs are subject to additional environmental impacts.

After STM challenge infection ($5x10^9$ cfu/animal), we observed mild signs of diarrhea in a small number of animals in Trial 1 but individual scores did not exceed values of 1 (1=pasty feces) with rectal temperatures staying within a physiologic range. Since all challenged animals of that study had previously been immunized, no strong negative impact on the health of the pigs was expected and indeed clinical symptoms turned out to be very mild. For Trial 2, the infection dose for both groups that were to be infected was lowered to $1x10^9$ cfu/animal to avoid unnecessary suffering on part of the non-vaccinated INF animals. Consequently, infection led to slightly increased diarrhea scores in both infected groups in comparison to the control group but, similarly to Trial 1, values never exceeded an average group score of 1. In addition, both the V+I and the INF group experienced a comparable increase in rectal temperature as well as decrease in ADG after infection. The lack of difference in the severity of clinical symptoms between V+I and INF animals after infection may be explained by the low infection dose used in this study. Indeed, other studies with a similar setup using infection doses of $5x10^9$ and $1x10^{11}$ cfu/animal, respectively, detected significantly lower diarrhea and clinical scores in vaccinated pigs compared to non-vaccinated control pigs after infection (Roesler et al. 2004; Theuß et al. 2017).

6.2 Humoral immune response

As clinical signs predictably turned out to be very mild, additional methods were employed to ensure that implementation of the vaccination and infection protocol had been successful, one of which was the monitoring of the humoral immune response. Serum samples were collected and analyzed for *Salmonella*-specific antibodies by the commercially available IDEXX Swine *Salmonella* Ab test (Trial 1+2) and an in-house ELISA based on whole-cell lysate of purified STM (Trial 2). While the IDEXX Swine *Salmonella* Ab test is based on LPS antigen and

directed against *Salmonella*-specific IgG, the in-house STM ELISA allowed for the distinction between *Salmonella*-specific IgM, IgA and IgG.

As expected, immunizing pigs twice with the Salmoporc vaccine resulted in a merely moderate rise in serum antibodies in both animal experiments as measured by the IDEXX Swine *Salmonella* Ab test. This is considered to be one of the advantages of the Salmoporc vaccine when it comes to its practical use in the field. In Germany, serum or meat juice samples are routinely taken at the farm or the slaughter house and tested for *Salmonella* antibodies. Based on ELISA results, swine herds will be classified into different categories indicating their risk of contaminating the meat production chain with *Salmonella* (Verordnung zur Verminderung der Salmonellenverbreitung durch Schlachtschweine (Schweine-Salmonellen-Verordnung)). Farms categorized as high-risk have to implement measures to ameliorate the situation. A vaccine that does not significantly or permanently increase serum IgG antibodies and therefore will not negatively impact these monitoring programs which are in place in many EU countries, can be expected to meet with more acceptance from farmers. In fact, when the vaccine is used according to the manufacturer's recommendation, induced antibodies will have subsided by the time the pigs are going to slaughter (Selbitz et al. 2003). However, this also depends on the cut-off values that are applied which vary between countries (Peeters. 2019, see also chapter 3.1.2) and the surveillance cut-off does not always correspond to the test-specific cut-off. Two weeks after challenge infection with the virulent STM strain in Trial 1, S/P ratios according to the IDEXX Swine *Salmonella* Ab test ranged between 2.6 and 4.0. As pigs in Trial 1 were orally infected with the relatively high dose of $5x10^9$ cfu/animal in an experimental setup, S/P ratios were expected to be higher than those measured in field settings. Indeed, most pig herds are subclinically infected with STM and two studies examining the effect of Salmoporc vaccination on subclinically infected herds detected lower mean S/P ratios in the serum of sows and piglets post vaccination (Peeters et al. 2020; Peeters et al. 2019b) than those measured in the study at hand.

Looking at the trajectories of STM-specific IgM, IgG and IgA in Trial 2 over time after vaccination and infection by use of the in-house STM ELISA, emerging patterns varied for the three different immunoglobulin (Ig) classes. While we observed an increase in S/P ratios of both STM-specific IgG and IgA after vaccination, S/P ratios of STM-specific IgM did not seem to be greatly influenced by the immunizations. A lack of distinct differences in levels of IgM between treatment groups after vaccination has also been seen in other studies (Theuß et al. 2017) and may be explained by the presence of natural IgM antibodies (Boes. 2000). After challenge infection, however, STM-specific IgM rose substantially in all infected animals with

highest levels measured in INF animals. Pigs belonging to the INF group, unlike the already vaccinated V+I pigs, had not previously been exposed to STM and the infection therefore induced a strong response of IgM, it typically being the first immunoglobulin to respond after initial antigen exposure. A substantial elevation after infection was also detected for STM-specific IgG with S/P ratios of V+I animals surpassing those of INF animals both at 7 and 21 dpi. Likewise, S/P ratios of STM-specific IgA experienced a strong rise after infection with maximum levels reached at 7 dpi in V+I animals and 21 dpi in INF animals. The half-life of IgA in serum is shorter than that of IgG (Curtis and Bourne. 1973; Leusen. 2015) which may explain its earlier decline in V+I animals at 21 dpi.

Comparing STM-specific IgG levels between the IDEXX Swine *Salmonella* Ab test and the Ig isotype-specific in-house ELISA, kinetics of the IgG response of the respective treatment groups broadly developed in a congruent manner. Similar to the work by Theuß et al. (Theuß et al. 2017), STM-specific IgG levels in INF animals were distinctly below those of V+I animals at 7 dpi when measured with the in-house ELISA while S/P ratios of both groups were closer together at this time point using the IDEXX ELISA. Previous studies examining different ELISA systems for the detection of *Salmonella*-infected pigs found the Ig isotype-specific ELISA (at the time of the studies still commercially available as Salmotype® Pig STM-WCE by Labor Diagnostik Leipzig, Germany) to have the highest sensitivity when compared to three other LPS-based tests, especially for the detection of other *Salmonella* serovars such as *S.* Infantis or *S.* Derby (Roesler et al. 2011; Szabó et al. 2008).

An obvious advantage of the in-house ELISA is its ability to discriminate between STM-specific IgM, IgG and IgA. Especially the induction of an IgA response is desirable when it comes to mucosal pathogens like STM with IgA being the most abundant immunoglobulin class on mucosal surfaces. Intestinal IgA is mainly produced by IgA B cells and plasma cells generated in the gut-associated lymphoid tissues, such as Peyer's patches and isolated lymphoid follicles, and released into the intestinal lumen (Suzuki and Fagarasan. 2009). Bacteria caught by this first line of defense will be prevented from entering the intestinal epithelium thereby reducing bacterial replication and spread. In piglets, the Salmoporc vaccine is administered orally which is in line with the long-standing belief that stimulation of mucosal IgA immunity in the gut can best be achieved when antigens are delivered orally (Lycke. 1998; Pasetti et al. 2011). Since IgA plasma cells induced after oral immunization have also been shown to home to other mucosal sites (Weisz-Carrington et al. 1979), it is tempting to speculate that the elevated levels of STM-specific IgA detected in the sera of Salmoporc-immunized pigs might correlate with secretion of STM-specific IgA in the gut. Indeed, preceding studies in humans

and mice have addressed this very question and found that serum IgA in mice after intragastric immunization with ovalbumin could predict the release of IgA in the gut although a dose-dependent effect was noted (Externest et al. 2000). An investigation of antibody responses in human blood and duodenal biopsies revealed that serum and gut IgA originate from the same B-cell clones but are produced by different plasma cells (Iversen et al. 2017). In contrast to humans, however, where serum IgA is largely produced by long-lived plasma cells in the bone marrow, around 30% of serum IgA in the pig was shown to be synthesized in the intestine and transported to the blood via the mesenteric lymph (Snoeck et al. 2006; Vaerman et al. 1997). That would suggest that at least a percentage of STM-specific serum IgA measured in the vaccinated pigs of Trial 2 originated from intestinal production, supporting the hypothesis that STM-specific IgA plasma cells are generated in the porcine intestine after oral STM immunization. However, this cannot be confirmed until STM-specific secretory IgA or STM-specific IgA plasma cells were to be examined in the gut of vaccinated pigs. While the analysis of porcine STM-specific IgA plasma cells or memory B cells was not possible at the time of this work due to a lack of tools, ongoing work to improve the porcine immunological toolbox should enable the analysis of these cell types in the pig in future studies.

In summary, vaccination and infection with STM was demonstrated to induce a humoral immune response in pigs, that, while reportedly not sufficient to eliminate the pathogen, most likely contributes to local and systemic anti-bacterial defense mechanisms.

6.3 Cellular immune response

While the humoral immune response certainly supports the defense against *Salmonella*, it is the cellular immune response, more specifically the $CD4^+$ T-cell response, that is thought to be the most important agent to promote bacterial clearance. In order to measure this response in the pig, we isolated lymphocytes from different tissue localizations and restimulated them *in vitro* with heat-inactivated STM antigen. Using intracellular cytokine staining, production of IFN-γ, TNF-α and IL-17A by $CD4^+$ T cells (Trial 1+2), $CD8^+$ T cells and $CD4^+CD8\beta b^-$ T cells (Trial 2) were measured by flow cytometry which resulted in seven cytokine-producing phenotypes as identified by Boolean gating: IFN-γ single-producing, TNF-α single-producing, IL-17A single-producing, IFN-γ/TNF-α co-producing, IFN-γ/IL-17A co-producing, TNF-α/IL-17A co-producing and IFN-γ/TNF-α/IL-17A triple-producing cells.

6.3.1 STM-specific CD4$^+$ T-cell responses in systemic versus local tissues

To enable comparisons between systemic and local T-cell responses we chose to sample blood and spleen as representatives of systemic organs in addition to tissues close to or at the site of infection such as jejunal (JLN) and ileocolic lymph nodes (ICLN), jejunum and ileum. In both animal trials, we found the highest frequencies of cytokine-producing STM-specific CD4$^+$ T cells in the lamina propria lymphocytes (LPL) derived from jejunum and ileum when compared to all other organs. While some cytokine-producing phenotypes were present in blood and spleen of Trial 1 pigs 14 days after STM vaccination and infection, the majority was induced in ICLN, jejunum and ileum with five out of seven phenotypes significantly higher in V+I animals when compared to the control group, thus demonstrating a strong induction of the local immune response. This result did not come unexpectedly, since both vaccinations and the challenge infection were delivered orally, mimicking the fecal-oral route of transmission that most frequently occurs in a natural setting. For the most part, STM infection in the pig is restricted to the intestinal tract, rarely spreading beyond this barrier to systemic organs (Boyen et al. 2008). Differently, in the murine model for systemic salmonellosis, STM routinely disseminates to the mesenteric lymph nodes, spleen and liver (Watson and Holden. 2010). Although non-typhoidal strains like STM mostly lead to self-limiting gastroenteritis in humans, more invasive presentations with systemic dissemination and bacteremia have been reported in young African children (MacLennan et al. 2008) and immunocompromised individuals (Gordon. 2008).

Cluster analysis of data obtained from Trial 1 further revealed that cytokine-producing STM-specific CD4$^+$ T cells formed separate clusters for systemic organs, lymphatic organs and gut tissue, respectively, indicating differences not only in abundance of cytokine-producing cells but also in type of cytokine production between these tissue groups. While TNF-α single-producing CD4$^+$ T cells were the most abundant phenotype within lymphatic organs, IFN-γ single and IFN-γ/TNF-α co-producing CD4$^+$ T cells dominated in blood, spleen, jejunum and ileum. In contrast to the systemic sites, however, cytokine phenotypes involving production of IL-17A were almost exclusively induced in gut tissue and mesenteric lymph nodes. These differences between local and systemic sites provide hints that STM-specific CD4$^+$ T-cell responses might, to a certain extent, be tissue-specific and shaped by the local microenvironment. Influencing factors probably include the bacterial load present in the respective tissues as well as the microbiome, which, in case of the gut microbiota, has been shown to be capable of directly modulating the CD4$^+$ T-cell response against *Salmonella* in

mice (Thiemann et al. 2017). Similarly, studies examining mucosal and systemic immune responses in bacterial and viral infections in humans have found immune responses at the site of infection to be distinct from those in the periphery (Booth et al. 2015; Yang et al. 2014). Within the context of *Salmonella* infection, it has been reported that the *S*. Typhi-specific $CD4^+$ T-cell response in humans in LPL isolated from the terminal ileum differed from the one in blood after immunization with the live attenuated *S*. Typhi vaccine Ty21a. Booth et al. showed, among other findings, that ileum-derived $CD4^+$ effector memory T cells after vaccination produced higher amounts of IL-17A, for both multifunctional and IL-17A single-producing phenotypes, than their systemic counterparts (Booth et al. 2019a), matching with observations in our study in the pig. Despite these differences, however, cluster analysis in Trial 1 also showed that IL-$17A^+$ phenotypes tended to cluster apart from IFN-γ single, TNF-α single and IFN-γ/TNF-α coproducing $CD4^+$ T cells across all analyzed organs which may point to differential characteristics of these cytokine-producing phenotypes. Potential roles of IL-17A in STM infection are discussed in more detail in chapter 6.3.3.

The findings obtained in Trial 1 as outlined above were corroborated and complemented by data from Trial 2. Comparisons of V+I animals with either the CON or the VAC group at 7 dpi revealed that significantly higher abundances of cytokine-producing STM-specific $CD4^+$ T cells in the V+I group were limited to the intestine. When contrasting CON and VAC animals with the INF group at 7 dpi, however, cytokine production by STM-specific $CD4^+$ T cells was significantly higher not just in jejunum and ileum but also for most cytokine phenotypes in the mesenteric lymph nodes and the systemic organs reaching highest effect sizes in spleen, jejunum and ileum. A vaccine-specific effect became apparent upon the comparison of V+I and INF pigs. Here, higher abundances of STM-specific cytokine-producing $CD4^+$ T cells were present in the INF group with significantly higher levels in blood, spleen and both of the lymph nodes, but not in the intestinal samples. Correspondingly, significantly lower bacterial loads were detected in ICLN, ileal and cecal mucosa of V+I pigs compared to INF pigs at 7 dpi suggesting that STM-specific effector or memory $CD4^+$ T cells elicited by the vaccine were able to reduce STM colonization in the intestine and inhibit systemic spread of the bacteria to internal organs. Similar mechanisms as shown in mice are conceivable where $CD4^+$ T cells located in the lamina propria increase the bactericidal activity of intestinal macrophages during STM infection (Yu et al. 2018). Additionally, other local mechanisms as mentioned above such as the secretion of STM-specific secretory IgA might have assisted in controlling the bacterial burden in the gut.

6.3.2 Multifunctionality of STM-specific CD4$^+$ T cells

Multifunctional (MF) T cells, defined as T cells with the ability to produce a range of different cytokines, reportedly offer superior and more effective protection than their single cytokine-producing counterparts. In fact, MF CD4$^+$ T cells have already been linked to protection in infections with other intracellular bacteria such as *Chlamydia trachomatis*, *Leishmania major*, *Mycobacterium bovis* and *Mycobacterium tuberculosis* (Darrah et al. 2007; Käser et al. 2017; Lindenstrøm et al. 2009; Maggioli et al. 2016). To investigate the capacity of porcine CD4$^+$ T cells for multifunctionality after STM vaccination and/or infection, we applied Boolean combination gates on STM-specific IFN-γ, TNF-α and/or IL-17A producing CD4$^+$ T cells. In Trial 1, MF STM-specific CD4$^+$ T cells, co-producing IFN-γ/TNF-α, IFN-γ/IL-17A, TNF-α/IL-17A or IFN-γ/TNF-α/IL-17A, were induced after STM vaccination and infection in several tissues. Moreover, significantly higher abundances of cytokine production in V+I animals when compared to control pigs were reached more often for STM-specific MF CD4$^+$ T cells than for those producing just one cytokine. These observations point to a role of MF CD4$^+$ T cells in controlling STM infection in the pig, which fits well with reports of *S.* Typhi-specific MF CD4$^+$ and CD8$^+$ T cells in humans (Wahid et al. 2015; Wahid et al. 2016) that have been demonstrated to be associated with protection (Fresnay et al. 2016).

Of the four MF cytokine phenotypes investigated in Trial 1, STM-specific IFN-γ/TNF-α co-producing CD4$^+$ T cells stood out with significant differences between V+I and control group in all investigated organs with the exception of the tonsil. In addition, as seen in Trial 2, STM-specific IFN-γ/TNF-α co-producing CD4$^+$ T cells were already induced after Salmoporc vaccination in spleen, ICLN, jejunum and ileum, albeit at lower levels than after infection. Indeed, both at 7 and 21 dpi, STM-specific IFN-γ/TNF-α co-producing CD4$^+$ T cells were the cytokine phenotype that reached the largest amounts of significant contrasts between treatment groups. Both IFN-γ and TNF-α are cytokines known as powerful activators of macrophages. During *Salmonella* infection, IFN-γ was shown to activate so-called Guanylate Binding Proteins (GBPs) that are able to lyse the SCV in *Salmonella*-infected macrophages, leading to release of the bacteria into the cytosol with subsequent pro-inflammatory cell death (Ingram et al. 2017). In the mouse model, TNF-α could also influence macrophage development, inhibiting the polarization of M2 macrophages in the context of several intracellular bacterial infections (Li et al. 2020), including STM, where this leads to limited persistence of STM in M2 macrophage granulomas (Pham et al. 2020). In addition, it has been demonstrated that both cytokines can act synergistically and cooperate in the activation of macrophages with TNF-α

inducing enhanced activation of NFκB when IFN-γ is present (Paludan. 2000; Wesemann and Benveniste. 2003). Considering the crucial role of macrophages in pathogen clearance, T cells co-producing both IFN-γ and TNF-α may thus promote a faster and stronger anti-bacterial response. This emphasizes the potential of IFN-γ/TNF-α co-producing $CD4^+$ T cells as a possible correlate of protection, as seen in murine *Salmonella* models (Lehmann et al. 2006; Monack et al. 2004; Ravindran et al. 2005), that merits further investigation.

6.3.3 Multifunctional STM-specific IL-17A producing $CD4^+$ T cells

Apart from co-production of IFN-γ and TNF-α, other MF phenotypes observed in both animal trials involved the production of IL-17A. While $CD4^+$ T cells are considered the classical source of IL-17A, it can also be produced by γδ T cells, innate lymphoid cells type 3, lymphoid tissue inducer cells and NK cells (Abusleme and Moutsopoulos. 2017). Correspondingly, *Salmonella* infection in mice was shown to induce IL-17A production by $CD4^+$ Th17 cells but also by γδ T cells and other $CD4^-$ lymphocytes (Schulz et al. 2008). Our studies in pigs yielded similar results with IL-17A production observed in STM-specific $CD4^+$ T cells and to a lesser extent in $CD4^-CD8\beta^-$ T cells, but not in $CD8^+$ T cells. The latter two T-cell subsets, $CD8^+$ T cells and $CD4^-CD8\beta^-$ T cells, will be discussed in more detail in chapter 6.3.5.

In Trial 1, STM-specific IL-17A-containing MF phenotypes such as IFN-γ/IL-17A co-producing, TNF-α/IL-17A co-producing and IFN-γ/TNF-α/IL-17A triple-producing $CD4^+$ T cells were induced in the pigs after STM vaccination and infection. They featured most prominently in ICLN, jejunum and ileum, however, overall raw frequencies of these phenotypes were considerably lower compared to IFN-γ single and IFN-γ/TNF-α co-producing $CD4^+$ T cells. Interestingly, clustering analysis showed that $IL\text{-}17A^+$ phenotypes were closely related to one another. The observation of MF IL-17A responses to intracellular pathogens is not a novel one. In fact, IFN-γ/IL-17A co-producing T cells have previously been reported in *Listeria monocytogenes* infection in mice (Sheridan et al. 2013) and *Candida albicans* infection in humans (Zielinski et al. 2012). MF IL-17A responses were also observed in the blood and the terminal ileum mucosa sampled from human volunteers after immunization with the *S.* Typhi vaccine Ty21a (Booth et al. 2020; McArthur and Sztein. 2012).

The comparison of different treatment groups possible in Trial 2 revealed that levels of STM-specific MF IL-17A $CD4^+$ T-cell phenotypes after immunization with Salmoporc were very low. Induction of these phenotypes mainly occurred after infection with the virulent STM strain with abundances rising highest in INF animals. Obviously, this leads to the question whether

the IL-17A phenotypes observed after STM infection in swine have a more beneficial or detrimental effect. IL-17A is well known as part of the defense at mucosal barriers where its numerous functions such as the recruitment of neutrophils, the induction of antimicrobial peptides and the regulation of tight junction proteins promote the preservation of epithelial integrity (Abusleme and Moutsopoulos. 2017). Lack of this response in Th17-depleted macaques resulted in increased dissemination of STM from the intestinal mucosa to systemic organs (Raffatellu et al. 2008). Of note, the same IL-17A response that helps contain the spread of the pathogen is also exploited by STM as the intestinal inflammation changes microbiota composition thus favoring its colonization (Liu et al. 2009). In the context of autoimmune diseases, however, IL-17A is described as a driver of chronic inflammation and tissue damage. Blocking of IL-17 signaling in diseases such as rheumatoid arthritis, multiple sclerosis and psoriasis has seen promising results in animal models and clinical trials (Omidian et al. 2019). Unfortunately, this often comes at the expense of higher susceptibility to bacterial and fungal infections. Bearing all that in mind, MF Th17 phenotypes observed in STM-infected pigs most likely supported the anti-bacterial host immune response in a favorable way. Th17 phenotypes in V+I animals were largely limited to the intestine as the local memory response generated by the immunizations seems to have been able to contain the infection. The infection with the virulent STM strain in the non-vaccinated INF pigs presumably promoted the induction of Th17 cells acting as a first line of defense against the pathogen. Had a higher infection dose been chosen, it is conceivable that IL-17A producing phenotypes might have contributed to greater intestinal inflammation leading to higher STM colonization and more pronounced clinical symptoms in INF pigs. Whether Th17 phenotypes have a protective function at systemic sites, once the pathogen has passed the intestinal barrier, remains unclear and requires further study.

Advances in recent years in sequencing technology have given rise to intriguing new insights into the differentiation of $CD4^+$ T-cell phenotypes. Single-cell RNA sequencing on colonic lamina propria-derived $CD4^+$ T cells from *Salmonella*- or *Citrobacter*-infected mice revealed that effector T cells clustered according to the infectious agent and not according to the 'classical' T helper cell lineages as defined by the expression of specific cytokines and transcription factors (Kiner et al. 2021). Interestingly, when looking at different T-cell clonotypes, Kiner et al. found that T cells producing IFN-γ, IL-17A or both could emerge from a single T-cell clone in response to STM infection. This aligns with observations in our study where both IFN-γ single-producing, IL-17A single-producing as well as IFN-γ/IL-17A co-producing $CD4^+$ T cells were elicited in STM-infected pigs. The study by Kiner et al. (Kiner et al. 2021) thus proposes a new model of plasticity wherein T-cell phenotypes do not belong to

different subsets or states but rather exist on a continuum that is shaped depending on the encountered microbes.

6.3.4 Porcine CD4$^+$ T-cell memory phenotypes in response to STM

The generation of memory T cells in response to vaccination or infection with any given pathogen is of utmost importance as their formation will ideally provide a protective immune response upon re-encounter with the antigen. To assess the differentiation status of the CD4$^+$ T cells generated after STM vaccination and infection in Trial 1 pigs, we looked at the expression of CD8α and CD27 within STM-specific cytokine-producing CD4$^+$ T cells. This approach allows the identification of three distinct subsets within porcine CD4$^+$ T cells: naïve cells with a CD8α$^-$CD27$^+$ phenotype as well as a CD8α$^+$CD27$^+$ and CD8α$^+$CD27$^-$ subset that represent central (Tcm) and effector memory (Tem) CD4$^+$ T cells, respectively (Reutner et al. 2013). We found that STM-specific cytokine-producing CD4$^+$ T cells across tissues largely presented with a Tem phenotype. Tem cells circulate between secondary lymphoid organs and blood but also reach non-lymphoid tissue like the gut mucosa where naïve T cells and Tcm are generally absent (Schenkel and Masopust. 2014). Consistent with this fact, almost all STM-specific CD4$^+$ T cells in jejunum and ileum in our study regardless of their cytokine profile were found to display a Tem phenotype. Similarly, Tem cells constituted the dominant CD4$^+$ memory subset in the terminal ileum mucosa of human volunteers vaccinated with the live attenuated *S.* Typhi vaccine Ty21a (Booth et al. 2019a). Apart from re-circulating memory T cells, another memory subset, termed resident memory T cells (Trm), is permanently located in the tissue (Pham and McSorley. 2015a; Schenkel and Masopust. 2014). Due to their ability of remaining in non-lymphoid tissues for a long period of time, they are capable of mounting an immediate local immune response upon pathogen invasion and indeed, resident memory Th1 cells were shown to be essential for protection against STM in mice (Benoun et al. 2018a). While the Ty21a vaccine elicited *S.* Typhi-specific Trm cells in the ileal mucosa of immunized human individuals (Booth et al. 2020), porcine Trm cells, unfortunately, are more difficult to identify due to a lack of available markers for tissue-resident cells such as CD69 and CD103. Works focusing on influenza A virus in swine have determined the numbers of Trm in the lung and other respiratory tissues by infusing pigs i.v. with anti-porcine CD3 mAb prior to euthanasia in order to distinguish intravascular, circulating T cells from Trm cells (Holzer et al. 2018; Martini et al. 2021). This is an interesting approach that could also serve to identify STM-specific Trm in the mesenteric lymph nodes and gut mucosa of STM vaccinated and/or infected pigs in future studies. Until then, however, we can only speculate that a proportion of the STM-specific

cytokine-producing $CD4^+$ T cells in the gut mucosa and herein identified as Tem cells may in fact represent Trm cells. Induced by the vaccination, these cells may have taken up long-term residence in the gut tissue and were potentially re-stimulated by the challenge infection.

6.3.5 Contribution of STM-specific cytokine-producing $CD8^+$ T cells and $CD4^-CD8\beta^-$ T cells

$CD4^+$ T cells are widely recognized as a very important factor in anti-*Salmonella* immunity (Kurtz et al. 2017). Despite putting a strong focus on $CD4^+$ T cells in our studies for this reason, we also aimed to have a look at other T-cell subsets in Trial 2 such as $CD8^+$ T cells and $CD4^-CD8\beta^-$ T cells to assess the potential contributions of these cells in the porcine immune response against STM.

Raw frequencies of cytokine production for the $CD8^+$ T-cell subset were the lowest among all three investigated subsets. Despite that, TNF-α-single and IFN-γ/TNF-α co-producing $CD8^+$ cells at 7 dpi were significantly more abundant in INF pigs than all other treatment groups, most notably for blood, spleen and mesenteric lymph nodes. According to the results coming out of several murine studies, $CD8^+$ T cells seem to be only modestly involved in protection against STM infection (Johanns et al. 2010; Lee et al. 2012a). A study using H-2 congenic strains with an increased resistance to *Salmonella*, however, found an unanticipated $CD8^+$ T-cell mediated protection in these mice (Labuda et al. 2019). In humans, immunization with the *S*. Typhi vaccine Ty21a and challenge infection with *wt S*. Typhi both resulted in potent *S*. Typhi-specific multifunctional $CD8^+$ T cell responses in blood and terminal ileum mucosa (Booth et al. 2019b; Fresnay et al. 2016; Fresnay et al. 2017; Salerno-Goncalves et al. 2002). There are two aspects that have to be taken into consideration when interpreting the results for $CD8^+$ T cells in our study and comparing them with the experiments undertaken in human and mice. Firstly, $CD8^+$ T cells recognize their antigen via presentation by MHC class I molecules which display peptides derived from the cytosol. By using inactivated STM antigen for our T-cell restimulation assays it is unlikely that we achieved antigen presence in the cytoplasm for processing in this manner. In addition, B cells are not able to cross-present STM antigen when it is heat-killed (Wit et al. 2010). For these reasons, studies on the cellular immune response against *S*. Typhi in humans were conducted with *S*. Typhi-infected autologous blasts as antigen presenting cells and target cells (Salerno-Goncalves et al. 2002). Secondly, although STM and *S*. Typhi are both serovars of the *Salmonella enterica* subspecies *enterica*, they possess different virulence factors and parallels between the cellular immune responses they elicit should be

drawn with care. *S.* Typhi is highly host specific and does not cause the strong intestinal inflammation that is associated with STM but instead quickly disseminates to systemic sites (Gal-Mor et al. 2014). Low $CD8^+$ T-cell responses as seen in our study and various murine studies in comparison to studies on *S.* Typhi in humans may therefore simply be the result of serovar-specific differences. This is supported by the fact that *S.* Typhi has been demonstrated to suppress flagellin-expression thereby hindering the $CD4^+$ T-cell response (Atif et al. 2014).

$CD4^-CD8\beta^-$ T cells were identified by gating on $CD3^+CD4^-CD8\beta^-$ cells. While γδ T cells likely represent the majority of cells within this gate, it is important to note that it can also contain other cells types such as NKT cells as well as potentially hitherto uncharacterized porcine MAIT cells. Similar to our findings for the $CD4^+$ T-cell subset, highest abundances of STM-specific cytokine-producing $CD4^-CD8\beta^-$ T cells were located in jejunum and ileum. Overall frequencies of STM-specific cytokine production by $CD4^-CD8\beta^-$ T cells ranked below $CD4^+$ T cells but above $CD8^+$ T cells. In alignment with results for the other two investigated T-cell subsets, relative abundances of STM-specific cytokine-producing $CD4^-CD8\beta^-$ T cells in INF pigs surpassed those of all other treatment groups with significant differences prevailing for IFN-γ/TNF-α co-producing $CD4^-CD8\beta^-$ T cells in blood, spleen and mesenteric lymph nodes at 7 dpi, once again highlighting the potential importance of this phenotype. Looking at significant contrasts across all groups, phenotypes and tissues, however, the response pattern was less distinct than seen for $CD4^+$ and $CD8^+$ T cells. This might be due to the combination of cell types included within the $CD3^+CD4^-CD8\beta^-$ gate, encompassing γδ T cells, NKT cells and possibly MAIT cells.

The few reports on MAIT cells in the context of STM infection in humans suggest that MAIT cells are activated in response to STM though some invasive strains manage to evade this recognition (Preciado-Llanes et al. 2020). Likewise, NKT cells are mobilized in STM-infected mice and produce IFN-γ and TNF-α, though at lower levels than other immune cells (Kirby et al. 2002). Contrary to previous assumptions, they seem to be activated in a TCR-independent manner in STM infection (Holzapfel et al. 2014). More details are available on γδ T cells as they are receiving increasing attention in human and mouse immunology, especially due to their high presence in epithelial tissues and on mucosal surfaces. When it comes to STM infection in mice, studies have suggested a great variety of different roles for γδ T cells with functions ranging from contribution to innate immunosurveillance in the intestine to eradication of infected intestinal epithelial cells and prevention of systemic spread (Dalton et al. 2006; Edelblum et al. 2015; Li et al. 2012). It must be noted, however, that these studies have examined γδ T cells originating from the intestinal epithelium and not LPL-derived

lymphocytes as investigated in our study. Nevertheless, we found significantly higher abundances of STM-specific cytokine-producing $CD4^{-}CD8\beta^{-}$ T cells at 7 dpi in INF pigs when compared to V+I pigs for systemic organs and mesenteric lymph nodes, but not in the intestine, which could indicate that vaccine-induced γδ T cells contribute to local immunity and help hinder systemic dissemination. What is more, γδ T cells have been characterized as an important source of IL-17A in the spleen and gut mucosa in *Salmonella*-infected mice (Godinez et al. 2009; Schulz et al. 2008). In *Listeria monocytogenes* infection in mice, antigen-specific IL-17A producing γδ memory T cells can even be found resident in the mesenteric lymph nodes. Conversely, the expansion of γδ memory T cells in the intestinal mucosa that was detected in response to *Listeria monocytogenes* infection was absent in STM-infected mice (Sheridan et al. 2013). While γδ T cells comprise a small percentage of circulating T cells in mice and humans, pigs, together with cattle, sheep and chickens have high frequencies of blood-circulating γδ T cells. In fact, both avian $CD8\alpha^{+}$high γδ T-cell populations, $CD8\alpha\alpha^{+}$ and $CD8\alpha\beta^{+}$, have been proposed as IFN-γ producers in STM-infected chickens (Pieper et al. 2011). Furthermore, $CD8\alpha\alpha^{+}$ γδ T cells were elevated in the peripheral blood of chickens vaccinated with *S.* Enteritidis (Berndt et al. 2006) and displayed an increased expression of CD25 upon stimulation with a *S.* Enteritidis live vaccine strain *ex vivo* (Braukmann et al. 2015b).

Taken together, our results along with reports of other species suggest that $CD8^{+}$ and γδ T cells assist in the porcine immune defense during STM infection though the extent and exact mechanisms of their support remain unclear.

6.4 Vaccination against STM at the farm level

The data presented in this work demonstrates that two-time immunization of pigs with the live attenuated vaccine Salmoporc reduces the bacterial load in the gut and associated mesenteric lymph nodes upon subsequent infection and impairs dissemination of STM to internal organs. However, one limitation of the project is its exclusive focus on *Salmonella*-free pigs. While this approach is necessary to clearly elucidate the effect of the immunization on the cellular immune response and to make sure no previous antigen-exposure is distorting the data, it does not reflect how the vaccine is usually applied in the field. The implementation of a vaccination regime against STM is not routinely recommended but commonly on farms where STM is already present as a recurring issue. Additionally, vaccination only ever constitutes one of many measures undertaken in affected pig herds to reduce *Salmonella* prevalence and prevent carrier states. Depending on the individual risk factors of the farm, it should be used in conjunction

with other actions such as good hygiene protocols, pest control, waste management and addition of organic acids to the feed (Andres and Davies. 2015). It would therefore be of interest to measure cellular immune responses after immunization in pigs that had previously been exposed to STM and whether this is influenced by other biosecurity measures carried out on the respective farms. The analysis of pig sera in herds subclinically infected with STM after subsequent Salmoporc immunization indicates that the vaccination provides a booster effect on the humoral immune response (Peeters et al. 2020; Peeters et al. 2019b). It therefore seems likely that the vaccination would similarly boost the cellular immune response in pig herds with a primed immune system due to an existing subclinical STM infection. A subsequent oral immunization might lead to the activation of local memory T cells in the intestinal mucosa potentially leading to a stronger stimulation of the $CD4^+$ T-cell response than seen in Trial 2 where frequencies of STM-specific cytokine-producing $CD4^+$ T cells after two-time vaccination, while present, were low compared to those after infection. According to recommendations by the manufacturer of the Salmoporc vaccine, however, sows are immunized subcutaneously, in contrast to the oral application standardly used for piglets, which could provide a more powerful stimulation of the systemic T-cell immune response.

6.5 Conclusions & Outlook

The present PhD project has analyzed the local and systemic T-cell immune response in swine that were vaccinated, vaccinated and challenged, or only infected with STM. It could be shown that STM induces a strong STM-specific $CD4^+$ T-cell response that is multifunctional as characterized by production of the cytokines IFN-γ, TNF-α and/or IL-17A, displays markers consistent with an effector memory phenotype, and dominates in the porcine intestine. While this project has collected some evidence that may suggest an involvement of $CD8^+$ and γδ T cells, further work is required to pinpoint the distinct role and importance of these cell subsets in the porcine immune defense during STM infection. The recent generation of pigs with a knockout of the locus encoding the T-cell receptor delta constant region and consequently lacking γδ T cells (Petersen et al. 2021), will be an essential tool in determining the contribution of γδ T cells to protection against STM as well as many other viral, bacterial and parasitic pathogens in swine. The overall dominance of STM-specific cytokine-producing $CD4^+$ T cells in the lamina propria of the porcine intestine indicates that they inhabit a significant role in the mucosal immune response against STM in swine. Although levels of STM-specific cytokine-producing $CD4^+$ T cells induced after vaccination were low compared to those observed post

infection, results indicate that the immunization significantly reduces bacterial burden and hinders pathogen spread to systemic sites in case of infection with a virulent STM strain. To further elucidate immune mechanisms triggered by STM immunization, investigation of other immune cell subsets such as Tfh cells, plasma cells and memory B cells would be very beneficial. $CD4^+IL\text{-}21^+Bcl\text{-}6^+$ T cells indicative of a Tfh phenotype have previously been identified in cervical lymph nodes and spleen of pigs vaccinated subcutaneously with Salmoporc (Ugolini et al. 2018). The analysis of STM-specific Tfh cells in the porcine intestine and mesenteric lymph nodes in conjunction with resident STM-specific plasma cells in response to oral vaccination with Salmoporc could help clarify their contribution to humoral immunity against STM. As seen in Trial 1, cytokine-producing STM-specific $CD4^+$ T cells largely exhibited an effector memory phenotype, especially in the intestine, and it seems likely that circulating effector $CD4^+$ T cells were recruited from the blood and migrated to the intestinal mucosa upon STM challenge infection. To further investigate T-cell migration patterns as well as the formation of STM-specific Trm cells, analyzing STM-specific $CD4^+$ T cells with regard to their expression of gut homing markers (e.g. CCR9, α4β7) and markers of tissue residency (e.g. CD103, CD69) after STM vaccination and/or infection would be essential. Considering the presence of $IL\text{-}17A^+$ cytokine phenotypes observed at the site of infection, it might also prove useful to determine the expression of CCR6, which has been implicated in regulating the migration of Th17 cells to the gut mucosal lymphoid tissue (Wang et al. 2009). Although the porcine immunological toolbox still lacks antibodies directed against many of these markers, recent advances in sequencing technologies like single-cell RNA sequencing (scRNAseq) allow for their detection and quantification on mRNA level at single-cell resolution. As an added benefit, knowledge gained in swine, in contrast to murine species, is more transferable to humans as the porcine and human immune system are closer related (Dawson. 2011). Additionally, while studies on enteric pathogens in humans are often limited in material to blood samples and small biopsies of intestinal tissue taken from a single time point, less restrictions of that kind apply to studies in the pig, making it an interesting biomedical model for the study of *Salmonella* infections. A deeper understanding of mucosal immunity in the porcine gut would thus provide additional insights for the development and improvement of vaccines against STM and other mucosal pathogens not only in the pig but also in humans.

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