Characterization of avian gamma/delta T-cell subsets after

*Salmonella* Typhimurium infection of chicks

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Abstract

Avian γδ T lymphocytes are frequently found in blood and organs and assumed to be crucial to the immune defense against Salmonella infections of chicks. To elucidate the so far unknown immunological features of subpopulations of avian γδ T cells in the course of infection, day-old chicks were infected orally with Salmonella Typhimurium. Until 11 days after infection, the occurrence as well as transcription of the CD8 antigen and immunologically relevant protein genes of CD8α⁻ and CD8α⁺high (CD8αα⁺; CD8αβ⁺) γδ cells were analyzed using flow cytometry and quantitative real-time reverse transcription (RT)-PCR in blood, spleen, thymus and cecum.

After infection, an increased percentage of CD8α⁺high γδ T lymphocytes was found in blood and spleen as well as with highest values and most rapidly in cecum. Within the CD8α⁺high subset, a significant rise in the number of CD8α⁺ cells was accompanied by enhanced CD8α antigen expression and reduced gene transcription of the CD8β chain. CD8αα⁺ and CD8αβ⁺ cells showed elevated transcription for Fas, FasL, IL-2Rα and IFNγ. While highest mRNA fold-changes were observed in CD8αβ⁺ cells, the mRNA expression rates of CD8αα⁺ cells have never significantly exceeded that of the CD8αα⁺ cells.

In conclusion, both CD8α⁺high γδ T-cell subpopulations (CD8αα⁺ and CD8αβ⁺) might be a potential source of IFNγ in Salmonella-infected chicks. However, due to their prominent frequency in blood and organs after infection, the avian CD8αα⁺ γδ T cell subset seems to be unique and of importance in the course of the Salmonella Typhimurium infection of very young chicks.
Introduction

The human salmonellosis caused by poultry-derived products is an important food-born disease throughout the world. The most frequently detected *Salmonella* (*S.*) serovars in man are *Salmonella Enteritidis* and *Salmonella Typhimurium*. To reduce the entry of the pathogen into the food chain comprehensive hygiene regimes and immunization programs using live or inactivated *Salmonella Enteritidis* as well as *Salmonella Typhimurium* vaccines are being implemented by the poultry industry in laying hen production and parent flocks. However, the infection with *Salmonella* pathogens often takes place shortly after hatch when the adaptive part of the immune system is not yet fully developed. Therefore, more information about immune cell functions of the fast constituted innate immune response is needed to better protect very young chicks in the future.

The gamma/delta (γδ) T cells are mostly defined as innate-like cells that have been demonstrated to rapidly increase after *Salmonella* infection in humans, cattle, mice as well as chickens (1;22;35). There is no doubt that γδ T cells are involved in the protection against various infectious diseases and able to influence important immunological functions. For example, γδ T cells can stimulate immune defense mechanisms during the early phase of the immune answer and may function as a link between innate and adaptive reactions (7). The cells also act as regulatory cells by limiting immune responses and inflammation (18;18;43). Moreover, γδ T cells produce a wide range of cytokines, present antigens to B cells, are cytolytic and possess memory abilities (21;8;10;21;41;41). Evidence exists that diverse functions are fulfilled by various subtypes of γδ T cells. Accordingly, γδ T lymphocytes have already been subdivided. In primates and mice, T cell receptor chain diversity as well as localization have been used to designate a range of γδ T-cell subsets (12). In other species, the γδ T lymphocytes are subdivided on the basis of the expression of phenotypic markers, as CD2 or CD8 (11;19;24;24;37;37;51;52). A standardized γδ T-cell subtype classification being
valid for all species does not exist, and most of the functional investigations have been
restricted to a small set of exclusive subsets of different species, with the human Vγ2Vδ2
(Vγ9Vδ2) cells of peripheral blood as the best characterized γδ T-lymphocyte population to
date (41).

Some efforts have been undertaken to better characterize γδ T cells of chickens. Based on
their CD8α antigen expression, three main avian γδ T-cell subgroups have been identified:
CD8α-negative (CD8α−), CD8α-diminished (CD8α−dim) and CD8α-highly positive
(CD8α+high) γδ T cells (3). The CD8α+high population additionally consists of the CD8αα
homodimer-bearing and the CD8αβ heterodimer-bearing γδ T-cell subtype (2;3;47). The
subset composition of γδ T cells varies according to their localization as well as the age of
chickens (37). The phenotype of γδ T-cell subsets has been extensively explored in
Salmonella-infected and non-infected chicks (1;4;19), but a more comprehensive
characterization of their potential to produce immune-related mediators and proteins has
solely been conducted in healthy birds. As shown, the defined γδ T-cell subpopulations differ
concerning their gene transcription levels in non-stimulated chickens, and the unique CD8αα+
γδ T-cell subset seems to be a pre-activated as well as a more differentiated population (37).

Preliminary investigations of our laboratory dealt with the role of avian γδ T-cell subtypes of
peripheral blood after Salmonella Enteritidis infection. In this study, an increased number of
peripheral CD8αα+ γδ T cells was accompanied by a temporarily decreased IL-7Rα mRNA
expression level of these cells after infection (3).

The present study was conducted to further characterize γδ T-cell subsets concerning their
composition in blood and organs as well as their capacity to transcribe immune-related genes
after Salmonella Typhimurium infection of chicks.
Material and Methods

Bacteria and culture

Salmonella serovar Typhimurium (strain 9098; phage type DT 177) was stored in the Microbank system (PRO-LAB Diagnostics, Ontario, Canada) at -20 °C. Bacteria were cultivated by shaking (18 h at 37 °C) in nutrient broth (SIFIN, Berlin, Germany) and concentration was estimated by extinction measurement at 600 nm and comparison against calibration graph. Values were confirmed subsequently by plate counting on nutrient agar (SIFIN) (34).

Animals and infection

Eggs of specific pathogen-free White Leghorn chickens were purchased from Charles River GmbH (Extertal, Germany). The chicks were hatched and kept at facilities of the Friedrich-Loeffler-Institut. Experimental and control groups were kept in separate rooms. Cleaning and feeding regimes were organized, which effectively prevented cross-contamination throughout the experiment. There was no transfer of any material, personnel or other things between the rooms of animal groups. Both groups were served separately by two different persons. Commercial feed (in powder form without antibiotics or other additives) and drinking water were both available ad libitum. The animal experiment was performed in accordance with the German Animal Protection Act (registration numbers: 04-01/04 and 03-001/06).

For the study, 145 chicks of group one were infected orally by crop instillation. Each animal received $10^7$ colony forming units of Salmonella serovar Typhimurium at day one of life. As shown in former studies, this infection dose causes high cecum colonization by the strain without severe clinical signs of morbidity (1). A second group of 155 chicks remained non-treated and served as controls.
To analyze \(\gamma\delta\) T-cell subsets, *Salmonella* Typhimurium-infected and control animals were sacrificed at days 2, 4, 7, 9, 11 after infection. At each time point, different organs (thymus, spleen, cecum) and peripheral blood were taken aseptically for further studies. The numbers of infected and control animals used for the single analyses are listed in table 1.

### Cell preparation

For flow cytometric analysis and sorting of \(\gamma\delta\) T-cell subsets, leukocytes were isolated from peripheral blood and organs. Lymphocytes of heparinized blood (PBLs) were separated by centrifugation with hetastarch according to the procedure described previously (1). To isolate splenic and thymic lymphocytes (37), the connective tissue capsule of organs was removed, the subjacent tissue minced and passed through a 23-gauge needle. Resulting cell suspension was mixed with an equal volume of 3 % hetastarch (in 0.9 % sodium chloride solution) and centrifuged at 30 x g for 20 min. The leukocytes of supernatant were washed twice in PBS at 350 x g.

Ceca were opened longitudinally and rinsed several times in PBS to remove cecal content as described (37). Afterwards, cells were abraded and transferred into PBS. After mechanical crushing, extracellular matrix components were digested using collagenase type V (Sigma-Aldrich, Taufkirchen, Germany, 1 mg/ml) for 1 hour at 37 °C. The separated leukocytes were washed twice in PBS.

### Flow cytometric analysis and cell sorting

To investigate the occurrence as well as the CD8α antigen expression intensity of the \(\gamma\delta\) T-cell subsets (CD8α+ and CD8α\(^{\text{high}}\) [CD8αα\(^+\), CD8αβ\(^+\)]) in peripheral blood and tissues (thymus, spleen, cecum) after *Salmonella* Typhimurium infection, \(2 \times 10^5\) isolated lymphocytes were incubated with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or
biotin-conjugated mouse anti-chicken monoclonal antibodies (Southern Biotechnology 
Associates, Eching, Germany) directed against the following chicken surface antigens:
γδ TCR (TCR1-FITC), CD8α (CT-8-PE) and CD8β (EP-42-biotin). To detect the biotinylated 
antibody, Alexa633-coupled streptavidin (SAV) was used. Staining and analysis were carried 
out using the FACSCalibur™ flow cytometer (BD Biosciences, Heidelberg, Germany) as 
previously described (3). The CD8α antigen expression intensity was analyzed by use of the 
geometric mean value of the CD8α^+ high γδ T-cell population.

To study the transcription of different immune-related genes, the CD8-characterised 
subpopulations of γδ T cells were sorted by means of the FACSvantage SE™ device and the 
CellQuest Pro 4.0.2 software (BD Biosciences) from peripheral blood and spleen at days 4, 7, 
9 and 11 after infection. The antibody staining and cell sorting were performed under cooled 
conditions (4 °C) as described (37). In brief, cells were incubated with the appropriate 
monoclonal antibodies for not longer than 20 min and immediately sorted. Before cell 
separating, the instrument settings were adjusted by FACS AccuDrop (BD Biosciences). To 
ensure purity of the single cell populations (> 95 %), test sorts were performed with 
immediate re-analysis of sorted cells. The following subsets were isolated: CD8α^+ high and 
CD8α γδ T cells. Additionally, CD8α^+ high γδ T cells were split into the CD8αα^+ and CD8αβ^+ 
subpopulation. The CD8α^+ dim γδ T cells were not sorted because the number in blood and 
organs was too low for the real-time RT-PCR analysis. The separated cell subpopulations of 
each bird and organ (blood and spleen) were lysed in RLT buffer (Qiagen, Hilden, Germany) 
and stored at -80 °C.

**RNA isolation and real-time RT-PCR**

Using real-time RT-PCR, we determined the transcription levels of different immune-related 
proteins of the sorted γδ T-cell subsets after Salmonella infection (IL-7Rα, IL-15Rα, Bcl-x,
CD8α, CD8β, IL-2, IL-2Rα, IFNγ, XCL1 [lymphotactin, LTCN], Fas, Fas ligand [FasL]). For that reason, RNA samples were prepared. To obtain either 100,000 - 160,000 or at least 25,000 - 70,000 (only spleen 4 dpi) cells per sample, cell lysates of corresponding γδ T-cell subpopulations (CD8α⁺; CD8α⁺^high [CD8αα⁺, CD8αβ⁺⁺]) were pooled. In dependence on the absolute cell number sorted, three to six samples were created per cell subset. After disintegration of cells by means of QIAshredder (Qiagen), RNA was isolated via silica-gel-membrane purification (RNeasy Micro kit; RNeasy Mini kit; Qiagen) and on-column digestion of DNA by RNase-free DNase (Qiagen) according to the manufacturer’s protocol for animal cells. RNAs were eluted with 30 µl (RNeasy Micro kit) or 50 µl (RNeasy Mini kit) RNase-free water. The samples were stored at -80 °C until use. The yield and purity of isolated RNA were controlled by random sampling using the NanoDrop® ND-1000-Spectrophotometer (Peqlab, Erlangen, Germany). The amount of RNA varied from 2 ng to 10 ng per µl. Absorption ratios between 1.8 and 2.4 (A260/A280) were considered to be useable.

Most of the gene-specific primers have already been described and used in previous experiments (4;37). The nucleotide sequences of primers for mRNA detection of the alpha and beta chain of the CD8 antigen are shown in table 2. At least one primer of each pair covers an exon-intron boundary, and different primer pairs display comparable efficiencies in the RT-PCR reaction.

For quantification of gene transcription rates, real-time RT-PCR was performed using the QuantiTect SYBR RT-PCR kit (Qiagen) and primer concentrations of 0.45 µM. For each target mRNA, 2 µl of the isolated RNA sample were used in a total reaction volume of 25 µl. After the reverse transcription of specific mRNA for 30 min at 50 °C, the cDNA was amplified (45 PCR cycles: for 15 s at 95 °C; for 30 s at primer specific annealing temperatures; for 30 s at 72 °C; Table 2) (37). The amplification was controlled and
monitored in real-time by the MX3000P real-time PCR system (Stratagene, La Jolla, CA).

The threshold cycle method (Δct) was used for relative quantification of gene transcription in relation to expression of the internal standard glyceraldehyde-3-phosphate dehydrogenase (3).

Results were expressed as fold-changes of mRNA rates of γδ T cells of infected animals to the controls ($2^{\Delta\text{ct}}$) or as 40-Δct values of infected and control chicks.

Statistical analysis

The data of flow cytometric analyses and real-time RT-PCR were statistically evaluated by Mann-Whitney test to determine significant differences between control and infected animals or between CD8αα$^+$ and CD8αβ$^+$ γδ T cells. Differences were considered significant for p values of ≤ 0.05 (**; a) and tend to be significant for p values of ≤ 0.1(*).
Results

Number of γδ T-cell subsets after Salmonella Typhimurium infection

To determine the importance of avian γδ T-cell subsets after *Salmonella* Typhimurium infection of day-old chicks (2 - 9 dpi), we examined the dynamic occurrence of CD8α- and CD8α+ high γδ T cells by means of flow cytometry in blood, spleen, cecum and thymus.

In comparison to non-infected animals, a significant increase (p ≤ 0.05) of CD8α+ high γδ T cells was observed in cecum, spleen and blood, but not in thymus, after *Salmonella* Typhimurium infection (Fig. 1). Interestingly, changes of CD8α+ high γδ T cells appeared successively during the course of infection. In cecum, a nearly 4-fold increase was detected at 2 dpi and continued to rise by 4 dpi (> 8-fold). In peripheral blood, an almost 4-fold increase was reached at 4 dpi and remained constantly high by the end of the experiment. In spleen, the percentage of CD8α+ high γδ T cells peaked not earlier than 7 dpi (> 5-fold). The number of the CD8α- γδ T-cell subsets remained unchanged (data not shown).

Number of the CD8α+ high γδ T-cell subsets after Salmonella Typhimurium infection

To elucidate the expression of CD8 isoforms on γδ T cells after *Salmonella* Typhimurium infection of young chicks, the percentages of the two CD8α+ high cell subsets expressing either the homodimeric (CD8αα+) or heterodimeric (CD8αβ+) isoform of the CD8 antigen were determined in peripheral blood, spleen and cecum between 2 and 9 dpi.

After infection, a significant increase (p ≤ 0.05) of the percentage of CD8αα+ cells was seen in cecum, peripheral blood and spleen from 4 dpi onwards (Fig. 2). We detected an infection-induced maximum rise of CD8αα+ cells in cecum at 4 dpi (control: 39.5 ± 0.4 %; infected: 76.6 ± 4.9 %) as well as in blood (control: 44.2 ± 7.7 %; infected: 78.2 ± 11.1 %) and spleen (control: 52.9 ± 8.2 %; infected: 90.0 ± 5.4 %) at 7 dpi. Thus, the dynamic changes of the
number of $\text{CD8}^+$ cells corresponded to the overall changes of the $\text{CD8}^{\text{high}}$ $\gamma\delta$ T cells after Salmonella Typhimurium infection.

CD8$\alpha$ antigen expression intensity and CD8$\alpha$ gene transcription of CD8$\alpha^{\text{high}}$ $\gamma\delta$ T cells after Salmonella Typhimurium infection

To answer the question, whether the CD8$\alpha^{\text{high}}$ $\gamma\delta$ T lymphocytes modify their expression levels of the $\alpha$ chain of the CD8 antigen after infection, the geometric mean values of the CD8$\alpha$ expression intensity were examined flow cytometrically (blood, spleen, cecum). In addition, the CD8$\alpha$ gene transcription rates were analyzed by quantitative real-time RT-PCR (blood, spleen).

As shown in table 3, a significant increase ($p \leq 0.05$) of the CD8$\alpha$ chain expression intensity was flow cytometrically evident on the plasma membrane of CD8$\alpha^{\text{high}}$ $\gamma\delta$ T cells of blood (4-9 dpi), spleen (9 dpi) and cecum (4 and 9 dpi) after Salmonella Typhimurium infection. These results were substantiated by quantification of the CD8$\alpha$ gene transcription rates of the CD8$\alpha^{\text{high}}$ $\gamma\delta$ T-lymphocyte subsets. Notably, a significantly increased ($p \leq 0.05$) transcription level of the CD8$\alpha$ chain was observed in CD8$\alpha\alpha$ as well as CD8$\alpha\beta$ cells of blood and spleen (Fig. 3) at day 9 after Salmonella Typhimurium infection. In CD8$\alpha$ $\gamma\delta$ T cells, CD8$\alpha$ mRNA expression has never been detected (data not shown).

CD8$\beta$ gene transcription of CD8$\alpha^{\text{high}}$ $\gamma\delta$ T-cell subsets after Salmonella Typhimurium infection

In order to show a possible down regulation of the CD8$\beta$ gene transcription, being potentially responsible for the enhanced CD8$\alpha$ chain expression intensity on the plasma membrane of...
CD8α<sup>high</sup> γδ T lymphocytes after infection, the transcription rate of the β chain of the CD8 antigen was examined in CD8αα<sup>+</sup> and CD8αβ<sup>+</sup> γδ T cells of blood and spleen.

The results are presented in figure 4. Interestingly, both the CD8αβ<sup>+</sup> and the CD8αα<sup>+</sup> cells exhibited gene transcription of the CD8β chain. However, in CD8αα<sup>+</sup> cells, the CD8β chain transcription level was generally low and significantly lower than in the CD8αβ<sup>+</sup> γδ T lymphocytes. After Salmonella infection, the transcription rates of the β chain dropped additionally (p < 0.05) in CD8αα<sup>+</sup>, but not in CD8αβ<sup>+</sup> γδ cells.

**Transcription of immune-related protein genes of γδ T-cell subsets after Salmonella Typhimurium infection**

For further characterization of γδ T-cell subsets of peripheral blood and spleen, changes of immune gene transcription were analyzed by quantitative real-time RT-PCR between 4 and 11 dpi after Salmonella infection. The analyses included apoptosis-related genes (Fas, FasL, Bcl-x), genes of mediators (IL-2, CXL1 [LTCN], IFNγ) and genes of the α-chain of growth factor receptors (IL-2Rα, IL-7Rα, IL-15Rα).

In comparison to CD8α<sup>+</sup> γδ T cells (data not shown), the CD8α<sup>high</sup> γδ T cells showed significant changes of transcription levels of some analyzed genes in blood and spleen after infection (Fig. 5, 6; Tab. 4, 5).

Concerning the mRNA levels of IL-2, Bcl-x, IL-7Rα, IL-15Rα and lymphotactin, CD8α<sup>high</sup> cells of blood and spleen showed no significant changes compared to the control animals after Salmonella infection (Fig. 5), and differences between the CD8αβ<sup>+</sup> and CD8αα<sup>+</sup> lymphocyte subset were not detected (data not shown). In contrast, the transcription of Fas, FasL, IL-2Rα and IFNγ changed significantly in CD8αα<sup>+</sup> and CD8αβ<sup>+</sup> γδ T cells (Fig. 6) of blood and spleen. Generally, the mRNA fold-changes of the proteins Fas (only blood), FasL, IL-
2Rα (blood and spleen) and IFNγ (with the exception of 7 and 9 dpi in blood) were higher in
CD8αβ+ γδ T cells than in CD8αα+ γδ T cells after infection (Table 4, 5). However, the
transcription levels (40-Δct values) of the CD8αβ+ cells have never significantly exceeded
that of CD8αα+ γδ T cells (Fig. 6).

Significant increases were seen regarding the IFNγ gene transcription levels (40-Δct values)
of the CD8αα+high γδ T-cell subsets in blood and spleen after Salmonella infection of day-old
chicks (p ≤ 0.05; Fig. 6). In blood, the IFNγ mRNA rates rose continuously in CD8αβ+ and
CD8αα+ γδ T cells up to 11 dpi, however, the CD8αα+ cells started on a higher level
compared to the CD8αβ+ γδ T lymphocytes. In both of the CD8ααhigh γδ T-cell subsets of
spleen, the INFγ transcription rates (40-Δct values) remained on a very high level between 7
and 11 dpi (p ≤ 0.05). However, CD8αα+ cells showed always highest mRNA rates.
The present study was undertaken to characterize different \( \gamma\delta \) T-cell subsets, distinguished according to their CD8 expression pattern, in blood and organs after *Salmonella Typhimurium* infection of day-old chicks. Beside the analysis of the occurrence of \( \gamma\delta \) T cells at selected sites of the body, the present work focused on the detection of immune-related gene transcription of avian \( \gamma\delta \) T-cell subpopulations after infection. We proved an enhanced CD8\( \alpha \) chain expression of avian CD8\( \alpha^{\text{high}} \) \( \gamma\delta \) T cells and an increased frequency of CD8\( \alpha\alpha^{+} \) \( \gamma\delta \) T cells after *Salmonella* Typhimurium infection. Our findings suggest that the CD8\( \alpha\beta^{+} \) and CD8\( \alpha^{+} \) \( \gamma\delta \) T cells, but not the CD8\( ^{-} \) cells of blood and spleen, were infection-dependently stimulated to transcribe activation- and Th1-related proteins, as Fas, FasL, IL-2R\( \alpha \) and IFN\( \gamma \).

As shown earlier (4), an increase of \( \gamma\delta \) T cells and especially of CD8\( \alpha^{\alpha+} \gamma\delta \) T lymphocytes is evident in peripheral blood after *Salmonella Enteritidis* infection of chicks. In the present study, we found a significant rise in the number of CD8\( \alpha^{\alpha+} \gamma\delta \) T cells also after *Salmonella Typhimurium* infection of day-old chicks. Moreover, this increase was seen not only in blood but also in cecum and spleen. A successive emergence of elevated numbers of CD8\( \alpha^{\text{high}} \) cells was observed, with the cecum being the first organ showing increased \( \gamma\delta \) T-cell fractions followed by blood and spleen. It remains unclear whether avian CD8\( \alpha^{\text{high}} \gamma\delta \) T cells are able to proliferate in the cecal mucosa or other organs. However, a cellular migration via blood seems likely. An expression of chemokine receptors after *in vitro* stimulation of \( \gamma\delta \) T cells with *Salmonella* organisms as well as a migration of \( \alpha\beta \) and \( \gamma\delta \) T lymphocytes to special sites of the body have already been described for mice and humans (16;17;30). Studies in pigs additionally suggest a re-circulation of intestinal intraepithelial \( \gamma\delta \) T lymphocytes that lead to an overall \( \gamma\delta \) T cell pool in peripheral blood (46).
In the present study, the increase of the number of CD8αα+ γδ T cells after *Salmonella* Typhimurium infection was accompanied by an enhancement of the CD8α gene transcription in these cells and an increase of the CD8α chain expression intensity on the plasma membrane of the CD8ααhigh γδ T lymphocytes after infection. These results correspond well to those from studies in rats and swine showing a stimulation-induced expression of CD8αα antigens on γδ T cells (42;42;45). There seems to be a general relation between antigen load and emergence of the CD8α chain on T cells. Accordingly, a CD8αα antigen expression has already been demonstrated on both activated γδ and αβ as well as on memory precursor T cells (32;33). Nevertheless, our results indicate for the first time an infection-triggered up-regulation of the CD8α chain expression on a γδ T-cell subset in chickens. The principle function of the α chain of the CD8 receptor has not been fully understood yet. Earlier reports showed that the CD8αβ is more effective than the CD8αα antigen to facilitate the recognition of the same peptide antigen by TCR and better interacts with MHC tetramers (9;36;38;49).

This may be caused by the property of the β-chain to enhance the kinase activity of the CD8α-chain-associated lck (27;31). Moreover, the stalk region of the CD8β antigen is capable of fine tuning the co-receptor function of CD8 proteins due to a distinct protein structure, smaller physical size and the unique glycan adducts associated with this region (50).

Interestingly, a low rate of CD8β gene transcription was generally demonstrated in avian CD8αα+ γδ T cells of our study. This low amount of CD8β gene transcripts additionally decreased in CD8αα+ γδ T cells after *Salmonella* infection. In contrast, the comparably high CD8β mRNA level of the CD8αβ+ γδ T cells has never changed after infection. Straube and Herrmann (45) proved a down regulation of the CD8β mRNA level in CD8αβ γδ T cells of rats and the generation of CD8αα+ lymphocytes after *in vitro* stimulation. The authors
suggest that the CD8β modulation provides a mechanism to escape possible over-stimulation by the antigen.

Of special interest is the relation between single phenotypically defined γδ T-cell subsets and their specific functional role. Frequently, a cellular function is associated with the secretion of a characteristic cytokine pattern. For human, murine and bovine γδ T-cell subpopulations, the ability to produce different cytokines has already been demonstrated (6;24;29). Especially in infectious processes, however, the cytokine transcription rates or the characteristic cytokine panel may change and, in this way, define the specific functional capacity of a unique cell subset. Consequently, the cytokine or chemokine secretion level has been utilized to characterize γδ T-cell subsets after stimulation (23;23;25;25;26;29). For example, human Vδ1+ T cells were triggered to produce IFNγ after activation with Gram(-) bacterial products (14). Similar, the avian CD8αα+ as well as CD8αβ+ γδ T-cell subsets of our study were able to express elevated IFNγ mRNA levels after Salmonella Typhimurium infection of chicks. Though, a secretion of this important mediator by avian γδ T cells has to be proven yet. IFNγ is a unique cytokine with a wide range of functions. On the one hand, it plays a critical role to enhance bactericidal functions of macrophages. On the other hand, IFNγ is the hallmark for the development of a T helper-type-1-dependent cell-mediated immune response (Th1; also called type-1). Nowadays, it is accepted that not only CD4+ but also CD8+ lymphocytes are able to promote the cell-mediated immune answer by augmented IFNγ secretion after stimulation. Like αβ T cells, murine γδ T cells have been subdivided into Th1-like and Th2-like T cells, depending on whether they produce IL-2 and IFNγ, or IL-4, IL-5 and IL-10 (48). Thus, the CD8αα+ and CD8αβ+ γδ T cells of our study might potentially be cell subsets that contribute to the development of a Th1-like cell-mediated immune response after Salmonella Typhimurium infection of young chicks. However, we merely showed increased transcription
rates of the immune mediator. Further studies are needed to demonstrate the actual capability of these cells to secrete INF\(_\gamma\) or other mediators after stimulation with \textit{Salmonella} organisms.

After \textit{Salmonella} infection, we observed increased mRNA levels of Fas and FasL in CD8\(\alpha\alpha^+\) as well as CD8\(\alpha\beta^+\) \(\gamma\delta\) T cells. In bovine, human and murine \(\gamma\delta\) T-cell subsets, FasL expression has been shown (5;13;20). FasL appears on activated T cells and regulates T cell homeostasis as well as T cell cytotoxicity (28). Especially the latter one seems to be of great interest mainly after infection with intracellular surviving bacteria as salmonellae. In fact, human V\(\gamma\)2V\(\delta\)2 (V\(\gamma\)9V\(\delta\)2) T lymphocytes are able to detect and kill infected cells through Fas-FasL interaction (15;22;44).

The IL-2R is generally considered as an activation receptor of T cells. It is able to bind IL-2 mostly produced by the same cell that also exhibits the receptor. Of note, the CD8\(\alpha\alpha^+\) \(\gamma\delta\) T cells of healthy and \textit{Salmonella}-infected chickens expressed comparably high mRNA levels of this receptor. The CD8\(\alpha\beta^+\) \(\gamma\delta\) T cells increased their IL-2R\(\alpha\) transcription but never reached the high rates seen in the CD8\(\alpha\alpha^+\) cells after infection. The group of CD8\(\alpha\alpha^+\) \(\gamma\delta\) T cells, therefore, seems to be the most activated \(\gamma\delta\) T-cell population after \textit{Salmonella} infection of chickens. The IL-2R function contributes not only to activation of cells, which results in altered cytokine production pattern and secretion intensity, but also to proliferation and survival of lymphocytes. Together with the receptors for IL-7 and IL-15, the IL-2R is responsible for fine tuning of the T cell immune reaction after infection (39;40). Additionally, it regulates the later fate of T cells, as apoptosis or memory development (39;40). A high IL-2R\(\alpha\) transcription together with an enhanced CD8\(\alpha\) chain expression on CD8\(^+\) \(\gamma\delta\) T cells might be an indication for a longer survival and a development of these cells towards memory cells (16). Other authors postulated the expression of the CD8\(\alpha\alpha\) antigen as a marker for a differentiation into long-lived mature memory T cells (33). Whether the CD8\(\alpha\alpha^+\) \(\gamma\delta\) T-cell
population of our animal experiment is able to generate memory cells remains to be elucidated.

In summary, our study demonstrated for the first time a changed transcription pattern of different avian $\gamma\delta$ T-cell subsets after *Salmonella* infection. While the CD8$\alpha\alpha^+$ and CD8$\alpha\beta^+$ $\gamma\delta$ T cells showed enhanced transcription of a range of immune-related proteins, the CD8$^+$ $\gamma\delta$ T cells hardly responded to the infection. Notably, the demonstrated ability of the avian CD8$\alpha\alpha^+$ $\gamma\delta$ T-cell subset to emerge at inflammatory sites of the body combined with an enhanced property to transcribe IFN$\gamma$ and other immune-relevant proteins represents a unique feature of these cells after *Salmonella* Typhimurium infection that deserves further investigation.

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Figures

Figure 1
Change of the proportion of CD8α<sup>+</sup>γδ T cells in peripheral blood and different organs after *Salmonella* Typhimurium infection of day-old chicks. Percentages of CD8α<sup>++</sup>γδ T cells of infected chicks were related to the percentages of non-infected animals. The percentages of the CD8α<sup>++</sup>γδ T cells of non-infected animals were normalized to 1. Asterisks indicate significant differences between the infected and control group (** p ≤ 0.05).

Figure 2
Percentages of CD8α<sup>+</sup> and CD8αβ<sup>+</sup> cells within the CD8α<sup>++</sup>γδ T-cell subset of control (-infection) and *Salmonella* Typhimurium-infected animals (+infection). Standard errors are shown as vertical bars. Asterisks indicate significant differences between the infected and control group (** p ≤ 0.05).

Figure 3
Quantification of CD8α mRNA transcription rates of CD8αα<sup>+</sup> and CD8αβ<sup>+</sup> γδ T cells in spleen and blood after *Salmonella* Typhimurium infection of day-old chicks. Data are given as n-fold changes of mRNA levels for infected birds relative to those for non-infected control animals (fold change = 1). Standard errors are shown as vertical bars. Asterisks indicate a significant difference between the treated and control group (** p ≤ 0.05 and * p ≤ 0.1).

Figure 4
Transcription levels of the CD8β gene in blood and splenic CD8αα<sup>+</sup> and CD8αβ<sup>+</sup> γδ T cells of non-infected and *Salmonella* Typhimurium-infected animals (shown as 40-ΔΔct values).
Standard errors are shown as vertical bars. Significant transcription differences between infected and non-infected chickens were indicated by asterisks (**p ≤ 0.05). Significant differences between the CD8αα⁺ and CD8αβ⁺ γδ T cells of the control animals were indicated by the letter “a” (p ≤ 0.05).

Figure 5
Fold-changes of transcription rates of different immune-related genes in the flow cytometrically separated CD8αα⁺high γδ T cell population of blood and spleen after Salmonella Typhimurium infection of day-old chicks. Standard errors are shown as vertical bars. Significant differences of the expression levels of CD8αα⁺high γδ T cells between infected and control animals were indicated by asterisks (** p ≤ 0.05).

Figure 6
Transcription levels (shown as 40-Δct values) of different immune-related genes in blood and splenic CD8αα⁺ and CD8αβ⁺ γδ T cells of non-infected and Salmonella Typhimurium-infected chicks. Standard errors are shown as vertical bars. Significant differences between control and infected animals were indicated by asterisks (** p ≤ 0.05). Significant differences between the CD8αα⁺ and CD8αβ⁺ γδ T cells after infection were indicated by the letter “a” (p ≤ 0.05).
1 Table 1
2 Numbers of infected and control animals used for the different tests

<table>
<thead>
<tr>
<th>Day of life</th>
<th>3</th>
<th>5</th>
<th>8</th>
<th>10</th>
<th>12</th>
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<tbody>
<tr>
<td>dpi</td>
<td>2</td>
<td>4</td>
<td>7</td>
<td>9</td>
<td>11</td>
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<tr>
<td>Group</td>
<td>Con</td>
<td>ST</td>
<td>Con</td>
<td>ST</td>
<td>Con</td>
</tr>
<tr>
<td>Total number of animals</td>
<td>15</td>
<td>10</td>
<td>30</td>
<td>30</td>
<td>40</td>
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<tr>
<td>Peripheral blood</td>
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</tr>
<tr>
<td>Subset analysis</td>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
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<tr>
<td>Cell cycle</td>
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<td>-</td>
<td>6</td>
<td>5</td>
<td>6</td>
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<tr>
<td>Sort for qPCR</td>
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<td>-</td>
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<td>8</td>
<td>16</td>
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<tr>
<td>Spleen</td>
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<td></td>
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<tr>
<td>Subset analysis</td>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
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<tr>
<td>Cell cycle</td>
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<td>9</td>
<td>9</td>
<td>9</td>
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<tr>
<td>Sort for qPCR</td>
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<td>-</td>
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<td>8</td>
<td>18</td>
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<tr>
<td>Cecum</td>
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</tr>
<tr>
<td>Subset analysis</td>
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<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Thymus</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Subset analysis</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

dpi: days after infection; Con: control group; ST: Salmonella Typhimurium-infected group
### Table 2
Primer sequences and annealing temperatures ($T_A$) for RT-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>(accession no.)</th>
<th>Primers (5’-3’)</th>
<th>$T_A$ (°C)</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8α</td>
<td>NM_205235</td>
<td>F: TCTTCCAGCCACAACACAG  R: ACTGCTTGTCCTGGCTCTGA</td>
<td>54</td>
<td>110</td>
</tr>
<tr>
<td>CD8β</td>
<td>NM_205247</td>
<td>F: AGCCAGGAGAAGTTTCAGCAGATCC  R: GCAAAACATCGACCACGTCGA</td>
<td>54</td>
<td>166</td>
</tr>
</tbody>
</table>

F = forward primer; R = reverse primer

### Table 3
Intensity (geometric mean) of the CD8α antigen expression on CD8α$^{^{\text{high}}}$ γδ T cells

<table>
<thead>
<tr>
<th>Infection</th>
<th>Peripheral blood</th>
<th>Spleen</th>
<th>Cecum</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>505 ± 53</td>
<td>1005 ± 154</td>
<td>396 ± 62</td>
</tr>
<tr>
<td>+</td>
<td>610 ± 27</td>
<td>1501 ± 351</td>
<td>424 ± 18</td>
</tr>
<tr>
<td>2 dpi</td>
<td>486 ± 92</td>
<td>1803 ± 215</td>
<td>357 ± 22</td>
</tr>
<tr>
<td>4 dpi</td>
<td>560 ± 35</td>
<td>1643 ± 238</td>
<td>457 ± 14</td>
</tr>
<tr>
<td>7 dpi</td>
<td>560 ± 92</td>
<td>1643 ± 238</td>
<td>457 ± 14</td>
</tr>
<tr>
<td>9 dpi</td>
<td>560 ± 35</td>
<td>1643 ± 238</td>
<td>457 ± 14</td>
</tr>
</tbody>
</table>

*significant differences between the control and the infected group (p ≤ 0.05)

- : non-infected control animals
+ : *Salmonella* Typhimurium-infected animals
Table 4

Fold-changes of transcription rates of different immune-related proteins detected in CD8αα+ and CD8αβ+ γδ T lymphocytes of peripheral blood after *Salmonella Typhimurium* infection of day-old chicks

<table>
<thead>
<tr>
<th>Protein</th>
<th>CD8αα+</th>
<th>CD8αα+</th>
<th>CD8αβ+</th>
<th>CD8αα+</th>
<th>CD8αβ+</th>
<th>CD8αα+</th>
<th>CD8αβ+</th>
<th>CD8αα+</th>
<th>CD8αβ+</th>
</tr>
</thead>
<tbody>
<tr>
<td>FasL</td>
<td>2.1 ± 0.8</td>
<td>3.9 ± 1.3*</td>
<td>1.5 ± 0.5</td>
<td>3.5 ± 0.1*</td>
<td>1.9 ± 0.8</td>
<td>3.3 ± 1.4</td>
<td>5.9 ± 0.4*</td>
<td>1.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Fas</td>
<td>2.5 ± 0.6</td>
<td>2.7 ± 1.4</td>
<td>1.8 ± 0.8</td>
<td>2.4 ± 1.6</td>
<td>1.6 ± 0.5</td>
<td>5.2 ± 3.1*</td>
<td>6.6 ± 1.9*</td>
<td>2.0 ± 0.8</td>
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</tr>
<tr>
<td>IL-2Rα</td>
<td>1.6 ± 0.5</td>
<td>5.6 ± 1.9*</td>
<td>1.7 ± 0.4</td>
<td>4.2 ± 0.1*</td>
<td>1.6 ± 0.5</td>
<td>28.5 ± 2.2*</td>
<td>16.6 ± 12.5</td>
<td>29.4 ± 7.7</td>
<td></td>
</tr>
</tbody>
</table>

*significant differences between CD8αα+ and CD8αβ+ γδ T lymphocytes (p ≤ 0.05)

Table 5

Fold-changes of transcription rates for different immune-related proteins detected in CD8αα+ and CD8αβ+ γδ T lymphocytes of spleen after *Salmonella Typhimurium* infection of day-old chicks

<table>
<thead>
<tr>
<th>Protein</th>
<th>CD8αα+</th>
<th>CD8αα+</th>
<th>CD8αβ+</th>
<th>CD8αα+</th>
<th>CD8αβ+</th>
<th>CD8αα+</th>
<th>CD8αβ+</th>
<th>CD8αα+</th>
<th>CD8αβ+</th>
</tr>
</thead>
<tbody>
<tr>
<td>FasL</td>
<td>2.0 ± 1.1</td>
<td>4.2 ± 2.6</td>
<td>3.2 ± 1.4</td>
<td>2.9 ± 0.9</td>
<td>0.8 ± 0.2</td>
<td>14.9 ± 7.7*</td>
<td>3.4 ± 1.2</td>
<td>23.7 ± 9.3*</td>
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<tr>
<td>Fas</td>
<td>2.8 ± 1.5</td>
<td>2.4 ± 1.4</td>
<td>6.8 ± 3.7*</td>
<td>1.8 ± 0.5</td>
<td>0.7 ± 0.2</td>
<td>1.8 ± 0.7*</td>
<td>9.4 ± 3.3</td>
<td>10.5 ± 3.4</td>
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<tr>
<td>IL-2Rα</td>
<td>3.1 ± 1.7</td>
<td>9.4 ± 6.0*</td>
<td>7.5 ± 3.6</td>
<td>8.4 ± 2.5</td>
<td>0.6 ± 0.3</td>
<td>3.5 ± 0.7*</td>
<td>4.1 ± 1.3</td>
<td>28.4 ± 9.5*</td>
<td></td>
</tr>
</tbody>
</table>

*significant differences between CD8αα+ and CD8αβ+ γδ T lymphocytes (p ≤ 0.05)
References

25. Huber, S. A., W. Born, and R. O’Brien. 2005. Dual functions of murine gammadelta cells in inflammation and autoimmunity in coxsackievirus B3-induced myocarditis: role of Vgamma1+ and...


Figure 1

Dr. J. Pieper

Fold change of CD8α^high γδ T cells

control level

dpi
Figure 2
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Figure 3
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Figure 4
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Figure 5
Dr. J. Pieper
Figure 6
Dr. J. Pieper