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Circulating γδ T Cells in Response to *Salmonella enterica* Serovar Enteritidis Exposure in Chickens

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γδ T cells are considered crucial to the outcome of various infectious diseases. The present study was undertaken to characterize γδ (T-cell receptor 1$^+$ [TCR1$^+$]) T cells phenotypically and functionally in avian immune response. Day-old chicks were orally immunized with *Salmonella enterica* serovar Enteritidis live vaccine or *S. enterica* serovar Enteritidis wild-type strain and infected using the *S. enterica* serovar Enteritidis wild-type strain on day 44 of life. Between days 3 and 71, peripheral blood was examined flow cytometrically for the occurrence of γδ T-cell subpopulations differentiated by the expression of T-cell antigens. Three different TCR1$^+$ cell populations were found to display considerable variation regarding CD8α antigen expression: (i) CD8α^{+high} TCR1$^+$ cells, (ii) CD8α^{+dim} TCR1$^+$ cells, and (iii) CD8α$^-$ TCR1$^+$ cells. While most of the CD8α^{+high} TCR1$^+$ cells expressed the CD8αβ heterodimeric antigen, the majority of the CD8α^{+dim} TCR1$^+$ cells were found to express the CD8αα homodimeric form. After immunization, a significant increase of CD8αα^{+high} γδ T cells was observed within the CD8α^{+high} TCR1$^+$ cell population. Quantitative reverse transcription-PCR revealed reduced interleukin-7 receptor α (IL-7Rα) and Bcl-x expression and elevated IL-2Rα mRNA expression of the CD8αα^{+high} γδ T cells. Immunohistochemical analysis demonstrated a significant increase of CD8α$^+$ and TCR1$^+$ cells in the cecum and spleen and a decreased percentage of CD8β$^+$ T cells in the spleen after *Salmonella* immunization. After infection of immunized animals, immune reactions were restricted to intestinal tissue. The study showed that *Salmonella* immunization of very young chicks is accompanied by an increase of CD8αα^{+high} γδ T cells in peripheral blood, which are probably activated, and thus represent an important factor for the development of a protective immune response to *Salmonella* organisms in chickens.

Bacteria of the genus *Salmonella* are responsible for a variety of acute and chronic diseases in poultry. Moreover, paratyphoid *Salmonella* organisms are among the most important pathogens inducing food-borne zoonoses throughout the world. Poultry products represent a substantial source of salmonella infection in humans, with *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium being the serovars most frequently associated with human food poisoning. The manifestations and consequences of paratyphoid *Salmonella* infections may vary markedly depending on the age of the birds (5). While infections in young chicks are often characterized by considerable morbidity and mortality, birds aged some weeks and adults are far less susceptible to lethal effects of *Salmonella* exposure and may experience intestinal colonization as well as systemic dissemination without signs of illness.

Vaccination is widely regarded as the most realistic approach to efficiently control the invasive serovars of *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium in the poultry industry. In this context, comprehensive knowledge of immunological defense mechanisms of chickens against *Salmonella* exposure represents a crucial prerequisite for successful vaccine development. The immune-stimulating properties of various *Salmonella* vaccine preparations have already been examined for their potential to enhance antibody production, cell-mediated immunity, and induction of invasion of different cell populations in several organs or peripheral blood (3, 8, 9, 13, 23, 24, 39). Notably, T-cell reactions and cellular immune responses seem to be of central importance for defense against *Salmonella* infections in chickens (8, 9, 23, 24).

T lymphocytes, which are characterized by their expression of special T cell receptors (TCR), have been classified into two subgroups: αβ and γδ T-cell receptor-bearing cells, with the former comprising the classical CD4$^+$ and CD8$^+$ cells. Avian αβ T cells are additionally subdivided into Vβ$_1$ and Vβ$_2$ cells. Compared to the Vβ$_1$ population, the Vβ$_2$ αβ T cells only constitute a small proportion of the total αβ T-cell population of avian peripheral blood. The γδ T-cell subgroup was first described in the middle of the 1980s (4, 11). Meanwhile, a number of interesting distinctions between γδ and αβ T cells have been found, such as tissue distribution, repertoire restrictions, and nonclassical major histocompatibility complex (MHC) restriction (1, 22). In addition, γδ T cells were shown to possess antimicrobial activity in bacterial, parasitic, and viral infections (15, 19, 45) as well as to perform a wide range of functions, such as cytokine production, cytotoxic activity, immunomodulation, granuloma organization, and regulation of inflammation (10, 12, 26, 35, 41). Based on the expression of different Vγ or Vδ elements, tissue localization, TCR diversity, and the expression of some surface antigens, γδ T cells could be divided into various functionally different subpopulations in mice and humans (14), cattle (25, 46), and rats (42).

In mature chickens, T cells bearing the γδ TCR comprise up

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to 50% of the peripheral T-cell pool (2). Recent studies in our lab revealed up-regulation of the percentage of CD8$^+$-γδ-receptor-positive T cells after *S. enterica* serovar Typhimurium infection of chicks in peripheral blood and tissues (8). Functional CD8 is a dimeric protein and exists either as a CD8αβ heterodimer or as a CD8αα homodimer. These two forms are differentially expressed on a variety of lymphocytes and seem to serve distinct functions (21). Although the occurrence of a relatively large proportion of CD8$^+$ γδ T cells expressing only the α-chain of the CD8 could be shown in spleens of embryos and young chicks (44), there is no knowledge of possible functions of these cells in chickens. In mice (28, 29) and humans (27), CD8αα is expressed on αβ T-cell subsets displaying a memory phenotype.

The development of memory CD8$^+$ T cells is a dynamic multistage process that includes an initial T-cell activation with adjacent T-cell expansion, a contraction phase (deletion, activation-induced cell death), and the final memory cell development (40). More recent work has indicated that cytokine receptors, such as interleukin-2 receptor (IL-2R), IL-7R, and IL-15R, all of which include the common γ-chain as an integral signaling component, play an important role in the development, survival, and homeostatic proliferation of lymphocyte populations. This has been most clearly established for CD8$^+$ T cells. Moreover, the expression of IL-2, IL-7, and IL-15 cytokines and their receptors have been shown to be crucial for CD8$^+$ T-cell memory generation and maintenance (40). On the other hand, the accumulation of antiapoptotic factors, such as the Bcl-2 family members Bcl-2 and Bcl-x$_L$, correlate with enhanced lymphocyte survival (40, 36), and Fas-FasL interaction seems to be essential for peripheral T-cell deletion (36).

In the present study, peripheral blood CD8α-positive and -negative γδ T cells were characterized in detail with regard to their expression of several surface antigens (CD4, CD8β, CD28, and MHC class II) and compared with the Vβ1 and Vβ2 αβ T cells of avian peripheral blood. Additionally, morphological and functional aspects of the γδ T-cell response in chickens after immunization and infection with *S. enterica* serovar Enteritidis were examined in blood and tissue. For this purpose, changes of the proportions of γδ T-cell subsets characterized by CD8 and CD28 antigen expression, mRNA expression levels of IL-2Rα and IL-7Rα, and apoptotic markers (Fas, Fas-L, and Bcl-x) were analyzed.

MATERIALS AND METHODS

Chickens. Specific-pathogen-free White Leghorn chickens were hatched at the facilities of the Friedrich-Loeffler-Institute, Jena, Germany, from eggs obtained from Charles River Deutschland GmbH, Extertal, Germany. Experimental and control groups were kept in separate rooms; commercial feed (in powder form without antibiotics or other additives) and drinking water were both available ad libitum. Cleaning and feeding regimens were organized which prevented cross-contamination effectively throughout the course of the experiment.

Experimental design and bacteriology. *Salmonella* vac E (TAD Pharmazeutisches Werk GmbH, Cuxhaven, Germany), a commercial live *Salmonella enterica* serovar Enteritidis (SE-LV) vaccine strain, and a nonattenuated *Salmonella enterica* serovar Enteritidis 147 (SE 147) wild-type strain (33) were used for oral immunization of the chicks on their first day of life. The viable count of the attenuated SE-LV was 1×10^8 to 2×10^8 CFU per bird, and that of the nonattenuated SE 147 was 1×10^7 to 2×10^7 CFU/bird. On day 44 of life, all immunized and nonimmunized chickens were infected orally using a nalidixic acid-resistant variant of the wild-type strain *Salmonella enterica* serovar Enteritidis 147 (SE 147 N) at a dose of 2×10^8 CFU/bird. Oral administration of 0.1

ml bacterial suspension/bird was performed by instillation into the crop using a syringe with an attached flexible tube. Another control group was neither immunized nor infected. In summary, the following animal groups were investigated: group 1 (SE-LV/SE 147 N), immunization with SE-LV on day 1 of life and infection with SE 147 N on day 44 of life; group 2 (SE 147/SE 147 N), immunization with SE 147 on day 1 of life and infection with SE 147 N on day 44 of life; control group 3 (nothing/SE 147 N), no immunization, infection with SE 147 N on day 44 of life; control group 4 (nothing/nothing), no immunization, no infection.

All *Salmonella* strains used in this study were stored in the Microbank system (PRO-LAB Diagnostics, Ontario, Canada) at −20°C. The bacterial suspensions for immunization and infection were cultivated by shaking (18 h at 37°C) in nutrient broth (SIFIN, Berlin, Germany). Doses were estimated by measuring extinction at 600 nm against a calibration graph determined for the strains used and subsequently confirmed by plate counting on nutrient agar (SIFIN).

Bacterial counts in spleens were estimated after infection using a standard plating method as described previously (33). Briefly, homogenized organ samples were diluted in phosphate-buffered saline, plated on deoxycholate-citrate agar (SIFIN) supplemented with sodium nalidixate (50 μg/ml) to detect the challenge organisms (SE 147 N), and incubated at 37°C for 18 to 24 h.

Peripheral blood leukocyte isolation. To study the dynamic of γδ T-cell subsets after *Salmonella* exposure, peripheral blood leukocytes of five animals per day of examination (days 4, 7, 11, 14, 21, 24, 28, 32, 43, 45, 46, 49, 50, 52, 53, 56, 63, and 71 of life) and group were analyzed. For comparison of avian peripheral TCR1$^+$ with TCR2$^+$ and TCR3$^+$ T cells, peripheral blood of chickens was analyzed between days 49 and 56 of life (in total, 15 nontreated animals). For that, the animals were sacrificed and completely bled to death. Lymphocytes of peripheral blood were isolated as described previously (8). Briefly, heparinized blood was mixed with 3% hetastarch (Sigma Immuno Chemicals, St. Louis, Mo.) and centrifuged at $65 \times g$ for 10 min to allow erythrocytes to sediment. The leukocytes of the supernatant were washed twice in phosphate-buffered saline and subsequently used for flow cytometry.

Flow cytometry and cell sorting. For cell sorting, T-cell characterization, and analysis of the dynamic of γδ T-cell subsets, fluorescein isothiocyanate-labeled mouse anti-avian TCR1 (γδ), TCR2 (αβ Vβ$_1$), and TCR3 (αβ Vβ$_2$) antibodies, an R-phycoerythrin-labeled mouse anti-avian CD8α monoclonal antibody, and biotin-conjugated mouse anti-avian CD4, CD8β, CD28 MHC class II antibodies (all from Southern Biotechnology Associates, Eching, Germany) were used. The biotin-conjugated antibodies were detected by Alexa Fluor 633-linked streptavidin (Molecular Probes, Leiden, The Netherlands). All antibody concentrations and dilutions were tested prior to starting the animal experiment. For every test, 2×10^5 leukocytes were incubated in parallel with the appropriate monoclonal antibodies (30 min in the dark). Aliquots of 10,000 to 20,000 cells per sample were analyzed using a FACSCalibur (Becton Dickinson, Heidelberg, Germany) equipped with a 15-mW, 488-nm argon ion laser and a 633 diode laser.

Additionally, γδ T-cell subpopulations (CD8α^{+high} CD8β$^+$ TCR1$^+$, CD8α^{+high} CD8β$^-$ TCR1$^+$, and CD8α$^-$ CD8β$^-$ TCR1$^+$) of three SE 147-infected animals each on days 7, 11, and 14 of life were sorted by means of the FACSVantage SE instrument (Becton Dickinson, Heidelberg, Germany) for quantification of cytokine mRNA expression. For every γδ T-cell subset, at least 50,000 cells were collected and stored at −80°C until use.

Quantitative real-time RT-PCR. Total RNA was extracted from cell samples using the RNeasy mini kit (QIAGEN, Hilden, Germany) by following the manufacturer's instructions, eluted in 50 μl RNase-free water per 50,000 cells, and stored at −80°C. For functional characterization of γδ T-cell subsets, mRNA expression of avian cytokine receptor chains (IL-2Rα and IL-7Rα) and apoptotic related proteins (Fas, Fas ligand, and Bcl-x) was determined by the QuantiTect SYBR green one-step reverse transcription (RT)-PCR kit (QIAGEN) according to the instructions of the manufacturer. Amplification and detection of specific products were performed using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA) using the following cycle profile: one cycle of 50°C for 30 min and 96°C for 15 min, 45 cycles of 94°C for 30 s and appropriate annealing temperature for 30 s, followed by 72°C for 30 s. Primer sequences and annealing temperatures are given in Table 1. To avoid amplification of cellular DNA of each primer pair, at least one primer spans an intron-exon boundary. The threshold method was used for quantification of the mRNA level. ΔC_T (cycle threshold change) values were calculated on the basis of internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results were expressed as 40-ΔC_T values.

Immunohistochemistry. Samples from the cecum and spleen of animals (days 1 to 56 of life) were examined by immunohistochemistry to determine the invasion of different T-cell subsets. Cryostat sections of 7-μm thickness were prepared, and detection of cells was done using unlabeled primary monoclonal

TABLE 1. Primer sequences for real-time RT-PCR

RNA target and primer direction	Sequence (5'–3')	Annealing temp (°C)	Accession no.
IL-2Rα		54	NM_204596
Forward	GCAGATAAATGCCCACGTCTTT		
Reverse	TCAAGGTGTTCCCACTTCGTC		
IL-7Rα		54	XM_423732
Forward	AGCATGCTCAGAATGACACGG		
Reverse	TCATCTCCAAAGGTCCCATCA		
Bcl-x		56	U26645
Forward	GCGAAAGGTCGGATGTGTT		
Reverse	GCTGGACATTTTCACACCTGA		
Fas		54	AF296875
Forward	AAAGCACTCGGTTTGGAGGT		
Reverse	ATGACTCGCAATGTTCACACC		
Fas-L		54	AJ890143
Forward	CACTTAACAGGAAACCCCACAC		
Reverse	TTGATCACAAGGCCCTGGT		
GAPDH		54	K01458
Forward	GTCAGCAATGCATCGTGCA		
Reverse	GGCATGGACAGTGGTCATAAGA		

TABLE 2. Number of challenge organisms of *Salmonella enterica* serovar Enteritidis 147 N in spleens of immunized and control chickens

Day of life	Day after challenge	No. of *S. enterica* serovar Enteritidis 147 N organisms ($\log_{10}$ CFU/g) in group[a]:		
		1	2	3
45	1	0.3	0	0
46	2	0.8[b]	0[b]	2.1
49	5	0.9	0[b]	2.7
50	6	0.3[b]	0[b]	3.5
52	8	0.9	0[b]	1.7
53	9	1.1	0[b]	2.4
56	12	0[b]	0[b]	2.5
63	19	0	0	0.5
70	26	0	0	0.6

[a] On day 1 of life, chickens were immunized as follows: group 1, SE-LV; group 2, SE 147; group 3, nothing (not immunized). Chickens in all three groups were challenged with SE 147 N on day 44 of life. Standard error, ±0.36.

[b] Significantly lower than group 3 result.

antibodies against the TCR1, CD8α, and CD8β antigens (Southern Biotechnology Associates, Eching, Germany) and a commercially available staining kit (PAP, ChemMate Detection kit, peroxidase antiperoxidase, rabbit/mouse; DakoCytomation, Hamburg, Germany). As a negative control, slides were incubated with preimmune mouse serum (dilution, 1:500) instead of the monoclonal antibodies. Sections were counterstained with hematoxylin and mounted with Canada balsam (Riedel de Haen AG, Seelze-Hannover, Germany).

Image analysis. Immunohistological tissue preparations were examined by light-microscopic image analysis (analySIS 3.0; Soft Imaging System GmbH). All measurements were performed at a magnification of ×20. Using the computer software, the regions of interest were drawn interactively as a polygon (cecum) or rectangle (spleen) on the screen. At least 5 regions of interest of each tissue section and antibody were scanned, the percentage of antibody-stained areas was determined, and the mean values were calculated.

Statistical analysis. The data of flow cytometric analyses and image analyses were evaluated statistically. The Student *t* test for comparison of two independent samples was used for statistical evaluation of differences between the groups (each immunized group against the control group). *P* values of ≤0.05 were considered significant.

Viable counts of bacteria were converted into logarithmic form. For the purposes of statistical analysis, a viable $\log_{10}$ count of <1.47 (limit for direct plate detection) obtained from a sample becoming positive only after enrichment was rated a $\log_{10}$ value of 1.0. A sample which yielded no *Salmonella* growth after enrichment was rated a $\log_{10}$ value of 0. Data were evaluated by multifactorial variance analysis. The factors considered were group and time. *P* values of <0.05 were regarded as statistically significant (software from Statgraphics Plus, Inc., Rockville, MD).

RESULTS

Clinical signs after *Salmonella* immunization and infection. In the present study, morphological and functional aspects of the γδ T-cell response in chickens after immunization and infection with *S. enterica* serovar Enteritidis were examined in blood and tissue.

The oral administration of attenuated live SE-LV did not result in morbidity or symptoms of intestinal inflammation. When using a dose of 1×10^7 to 2×10^7 CFU/bird of SE 147 in day-old chicks, strong colonization of the intestine and spread to liver was observed, but severe clinical signs were absent (34). Only a few birds showed signs of intestinal inflammation in the first week after administration. Infection with SE 147 N failed to generate any signs of morbidity or clinical symptoms in immunized and control birds.

Bacteriology. Oral immunization of chickens produced protective effects compared to nonimmunized controls (Table 2). Immunization using the nonattenuated *S. enterica* serovar Enteritidis wild-type strain 147 led to the most efficient protection, and no challenge organisms were detected in the spleen in the course of the experiment.

Birds immunized using an attenuated live *Salmonella enterica* serovar Enteritidis vaccine (SE-LV) revealed a considerably diminished number of the challenge strain in the spleen (reduction of 1.0 to 1.5 $\log_{10}$ units) compared to nonimmunized controls.

Comparison of TCR1+ with TCR2+ and TCR3+ T lymphocytes in nontreated chickens. To compare avian γδ with αβ T lymphocytes in peripheral blood, double- and triple-staining experiments were performed. Flow cytometric analysis proved the presence of three different TCR1+, TCR2+, and TCR3+ T-cell subsets in the blood of nonimmunized animals (Fig. 1). These subpopulations could be discriminated by their variable intensity of CD8α antigen expression as follows: (i) CD8α+high TCR1+, CD8α+high TCR2+, and CD8α+high TCR3+ cells (TCR+ cells expressing high concentrations of CD8α antigen); (ii) CD8α+dim TCR1+, CD8α+dim TCR2+, or CD8α+dim TCR3+ cells (TCR+ cells expressing diminished concentrations of the CD8α antigen); (iii) CD8α− TCR1+, CD8α− TCR2+, or CD8α− TCR3+ cells (TCR+ cells expressing no CD8α antigen).

The majority of γδ T cells were not found to express CD8α antigen on their surface. The CD8α+high TCR1+ and CD8α+dim TCR1+ cell populations occurred in similar proportions. In comparison, most of the TCR2+ and TCR3+ cells displayed diminished CD8α expression. A smaller amount of the TCR2+ and TCR3+ cells either expressed the CD8α antigen at a high concentration or showed no CD8α staining. Compared to TCR1+ and TCR2+ T cells, TCR3+ cells were only found in small quantities in blood (Fig. 1A).

For further characterization of αβ and γδ T cells defined above, the expression of CD4, CD8β, CD28, and MHC class II antigen was examined by flow cytometry (Fig. 1B). Most of the CD8α+high TCR1+, CD8α+high TCR2+, and CD8α+high TCR3+ T cells additionally expressed the CD8β antigen, which
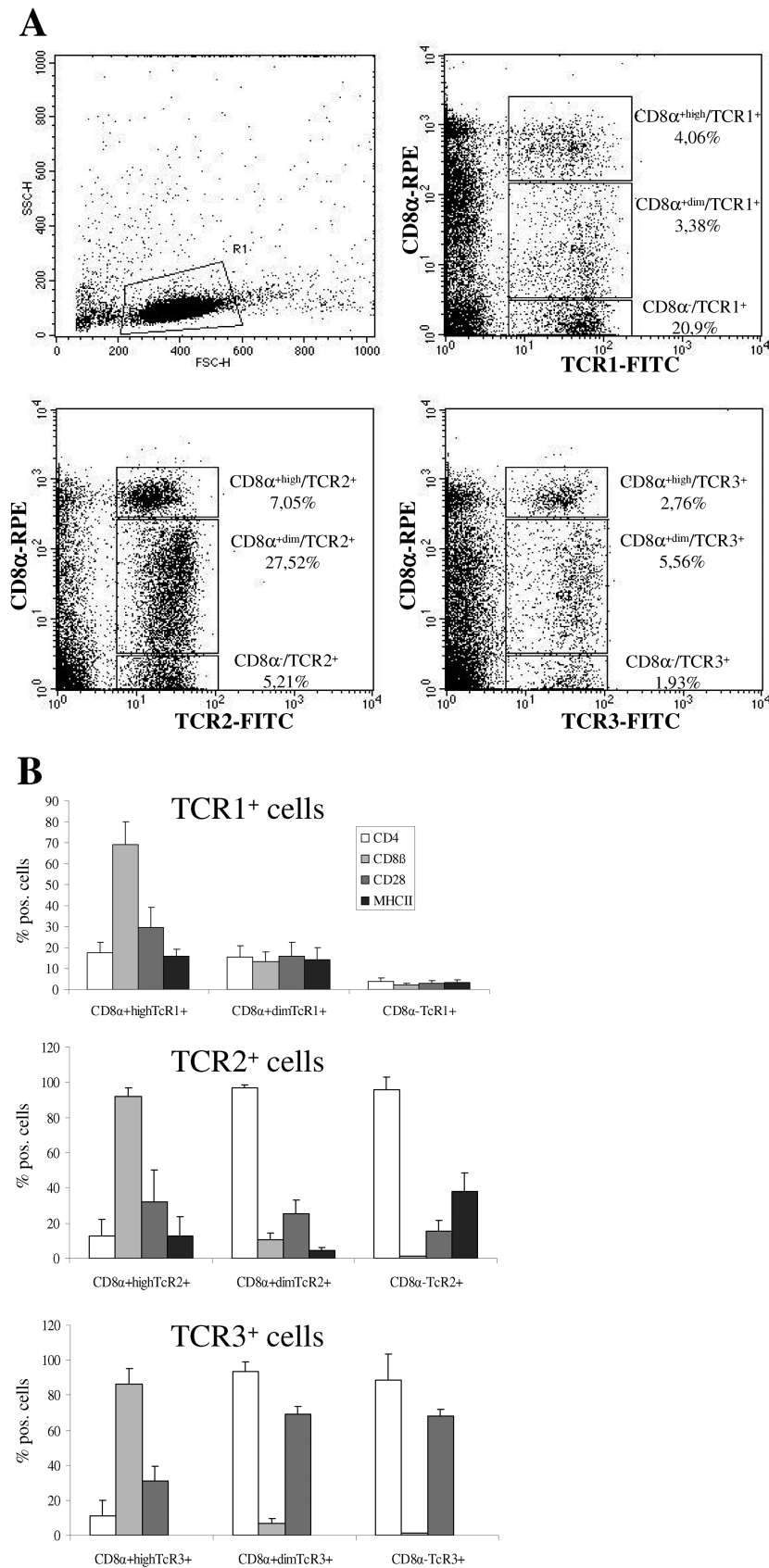
FIG. 1. (A) Flow cytometric analysis of avian peripheral lymphocytes of a representative animal (56 days old). Gated cells shown in the forward scatter/side scatter dot plot diagram represent the lymphocyte population subjected to analysis of T-cell subpopulations (R1). The other dot plot

means that these cells bear the CD8αβ heterodimeric form of the CD8 antigen. In contrast, most of the CD8α^{+dim} TCR1$^+$, CD8α^{+dim} TCR2$^+$, and CD8α^{+dim} TCR3$^+$ T cells showed no CD8β expression, indicating the sole occurrence of the CD8αα homodimeric form of the CD8 antigen on the plasma membrane of these cells.

The CD28 antigen was found on approximately 30% of CD8α^{+high} TCR1$^+$, CD8α^{+high} TCR2$^+$, and CD8α^{+high} TCR3$^+$ T-cell subsets. CD8α^{+dim} TCR1$^+$, CD8α^{+dim} TCR2$^+$, and CD8α$^-$ TCR2$^+$ T cells showed hardly any CD28 antigen expression. A high number (approximately 70%) of CD8α^{+dim} TCR3$^+$ and CD8α$^-$ TCR3$^+$ T cells was CD28 positive.

The vast majority of CD8α^{+dim} TCR2$^+$ and CD8α^{+dim} TCR3$^+$ T cells, as well as CD8α$^-$ TCR2$^+$ and CD8α$^-$ TCR3$^+$ T cells, additionally expressed the CD4 antigen.

Approximately 40% of CD8α$^-$ TCR2$^+$ T cells also showed a distinct MHC class II expression. MHC class II expression of CD8$^+$ TCR3$^+$ cells was not investigated.

Dynamics of TCR1$^+$ cell subsets after *Salmonella* immunization in blood. To examine the possible induction of a γδ T-cell response to *Salmonella* organisms, the percentage of TCR1$^+$ T cells characterized by their CD8α antigen expression was analyzed within the circulating lymphocyte population of immunized chicks compared to nonimmunized control animals. Additionally, the expression of CD8β and CD28 on the defined TCR1$^+$ cell subsets was investigated (Fig. 2).

CD8α^{+high} TCR1$^+$ cells were generally found in very low numbers in the peripheral blood of immunized and nonimmunized chicks on day 4 of life. After immunization with SE-LV or SE 147 wild type, significantly ($P < 0.05$) higher numbers of these γδ T cells were observed (Fig. 2A). The peak in the number of CD8α^{+high} TCR1$^+$ cells was reached on days 7 (SE-LV) and 11 (SE 147) of life. Subsequently, the percentages decreased to the level of nonimmunized animals. The percentages of CD8α^{+dim} TCR1$^+$ cells in peripheral blood were significantly increased ($P < 0.05$) in animals immunized with SE 147 and/or SE-LV compared to nonimmunized controls (data not shown). A peak was seen on days 7 and 11 (SE 147) and on day 14 (SE-LV) of life. Afterwards, the number of these cells dropped to the level of the control birds until day 21 of life. From day 21 onward, a moderate but significant decrease ($P < 0.05$) of these cells was detected in immunized chicks compared to controls (data not shown).

CD8α$^-$ TCR1$^+$ cells were significantly reduced ($P < 0.05$) in immunized animals compared to control birds. This phenomenon was observed from day 21 onward and was most conspicuous in chickens immunized with SE 147. On day 43 of life, the percentages of the CD8α$^-$ TCR1$^+$ T cells within the lymphocyte population of the immunized birds were nearly the same as found in controls (Fig. 2B).

As a consequence of immunization of day-old chicks, the CD8α^{+high} TCR1$^+$-to-CD8α$^-$ TCR1$^+$ cell ratio within the γδ T-cell population of immunized animals shifted significantly in favor of the CD8α^{+high} TCR1$^+$ cell subset between days 7 and 14 of life (data not shown).

In immunized birds, the number of CD8α^{+high} TCR1$^+$ cells expressing the β chain of the CD8 antigen within the CD8α^{+high} TCR1$^+$ cell population was significantly decreased between days 4 and 32 (Fig. 2C). This indicated an enormous increase of CD8αα^{+high} TCR1$^+$ cells in this γδ T-cell subset.

Concerning the expression of the CD28 antigen on TCR1$^+$ cells, an increase of CD28$^+$ cells among the CD8α^{+high} TCR1$^+$ T lymphocytes could be observed in immunized animals between day 4 and day 11 of age (Fig. 2D).

Dynamics of TCR1$^+$ cell subsets after *Salmonella* infection in blood. To evaluate a possible second or memory immune response to *Salmonella* infection on day 44 of life, the percentages of TCR1$^+$ T cells characterized by their CD8α antigen expression were analyzed in the previously immunized and nonimmunized chickens. Furthermore, the expression of CD8β and CD28 antigens on the defined TCR1$^+$ cell subsets was studied after infection between days 44 and 71 (Fig. 2).

In peripheral blood, the nonimmunized but infected group showed a significant ($P < 0.05$) increase of CD8α^{+high} TCR1$^+$ cells 5 and 6 days after infection (days 49 and 50 of life) compared with birds neither immunized nor infected with SE 147 N. In animals immunized on the first day of life, the percentages of the CD8α^{+high} TCR1$^+$ cells remained unchanged after *Salmonella* infection compared to the nonimmunized group. The percentages of the CD8α^{+dim} TCR1$^+$ and CD8$^-$ TCR1$^+$ cells were not significantly changed after infection.

Regarding CD28 antigen expression of the three TCR1$^+$ T cell subsets, no changes were observed after infection of chickens immunized on the first day of life.

Dynamics of TCR1$^+$, CD8α$^+$, and CD8β$^+$ cells after *Salmonella* infection in cecum and spleen. The local immune response after *Salmonella* immunization and infection has been examined by immunohistochemistry using monoclonal antibodies to CD8α, CD8β, and TCR1 antigens in the cecum and spleen.

In the cecum, significantly more CD8α$^+$, CD8β$^+$, and TCR1$^+$ cells were detected in SE-LV- and SE 147-immunized chickens than in the controls between days 4 and 21 of life (Fig. 3). In the ceca of previously immunized chickens, a second significant increase of CD8α$^+$, CD8β$^+$, and at lower degree, TCR1$^+$ cells were detected after challenge on day 44 of life. Whereas the SE 147-immunized animals showed the second increase very rapidly (CD8α, peak on day 45 of life; CD8β, peak on day 46 of life), in the SE-LV-immunized animals, this phenomenon appeared later after challenge (CD8α, peak on day 52 of life; CD8β, peak on day 50 of life). The percentages of cecal TCR1$^+$ cells were also increased after challenge in SE 147-

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diagrams show the two-color fluorescence analyses of lymphocytes for TCR1 and CD8α expression, TCR2 and CD8α expression, and TCR3 and CD8α expression of the gated lymphocytes. FITC, fluorescein isothiocyanate; RPE, R-phycoerythrin. (B) Percentages of cells positively stained for CD4, CD8β, CD28, or MHC class II antigen among the circulating TCR1$^+$, TCR2$^+$, and TCR3$^+$ T-cell subsets defined in dependence on their CD8α antigen expression in nonimmunized animals (49 to 56 days old; in total, 15 animals). MHC class II staining of TCR3$^+$ cells was not done.
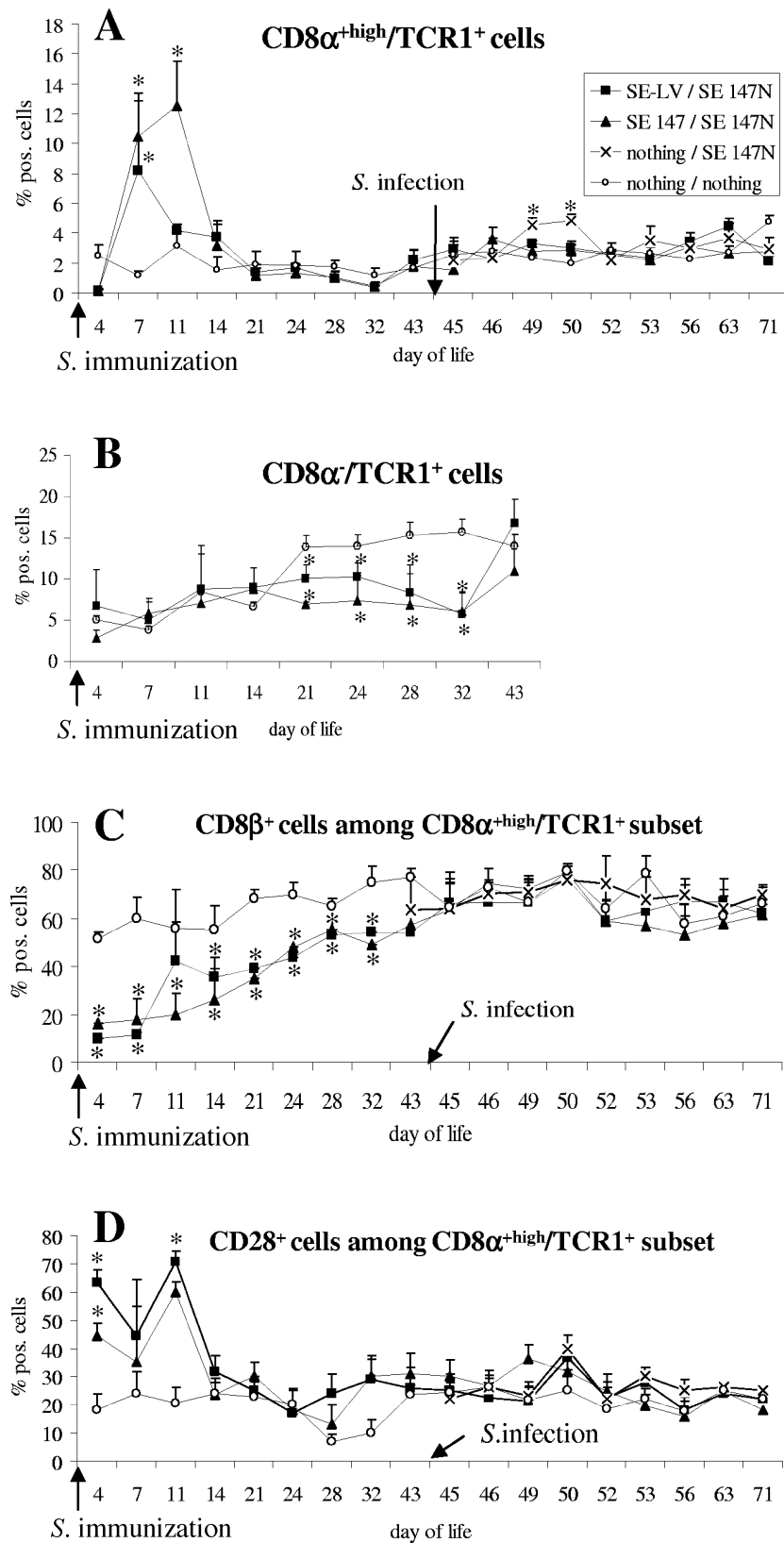
FIG. 2. Dynamic of the percentages of CD8α^{+high} TCR1$^+$ (A) and CD8α^- TCR1$^+$ (B) T-cell subsets in peripheral blood of chicks immunized with *Salmonella enterica* serovar Enteritidis (SE-LV) vaccine strain or nonattenuated *Salmonella enterica* serovar Enteritidis 147 wild-type strain (SE 147) on day 1 of life and infected with *Salmonella enterica* serovar Enteritidis 147 N on day 44 of life compared to nonimmunized controls. Results are shown between days 1 and 71 (A) or 1 and 43 (B) of life. Data represent means ± standard deviations. Asterisks indicate a significant
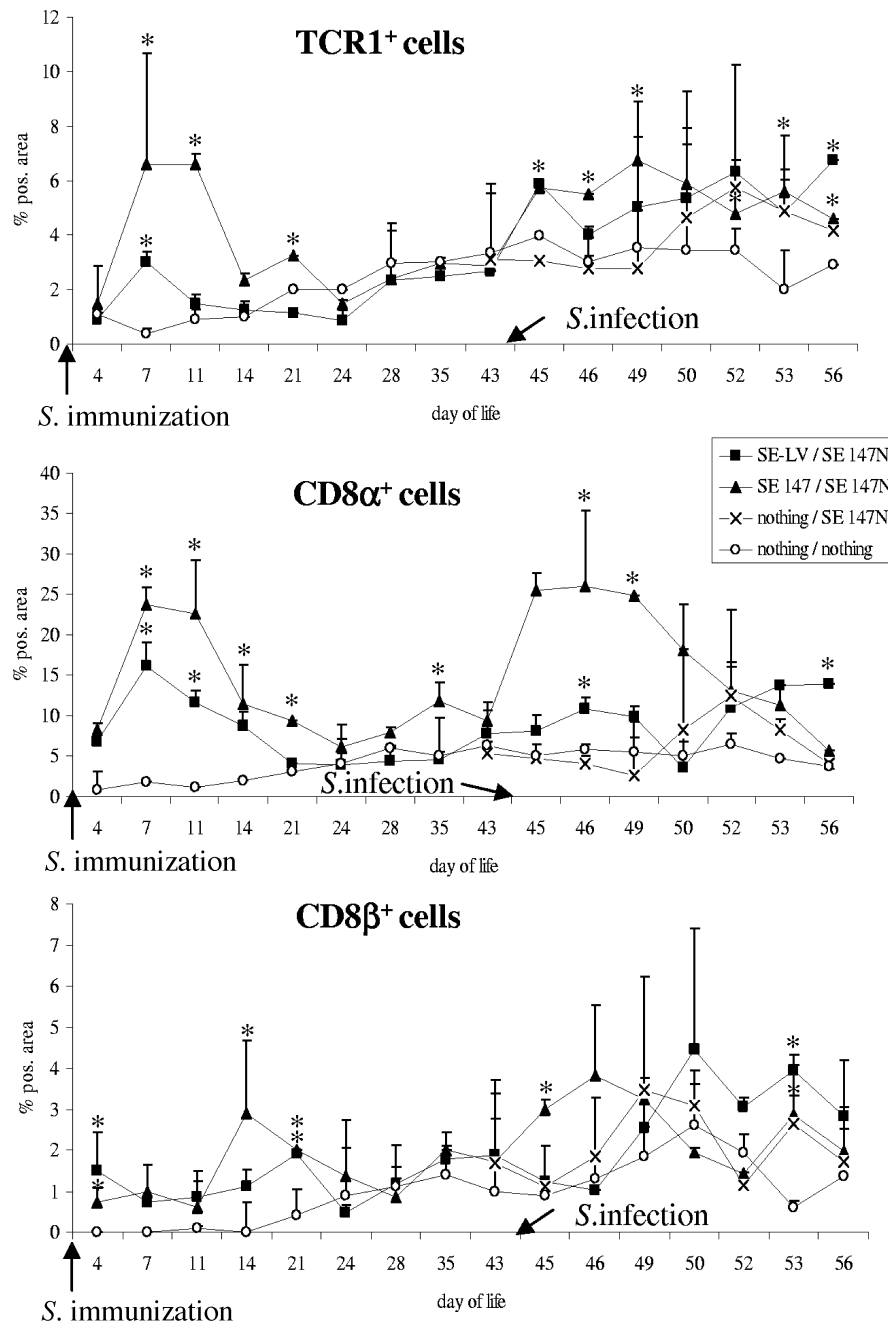
FIG. 3. Percent positive area of TCR1$^+$, CD8α$^+$, and CD8β$^+$ cells in ceca of chickens immunized with the two different *Salmonella enterica* serovar Enteritidis strains (SE-LV or SE 147) on day 1 of life and infected with *Salmonella enterica* serovar Enteritidis 147 N on day 44 of life in comparison to nonimmunized controls. Asterisks indicate a significant difference between the treated and control groups ($P \geq 0.05$).

difference between the treated and control groups ($P \geq 0.05$). (C) Percentages of CD8β$^+$ cells among the CD8α^{+high} TCR1$^+$ and CD8α^{+dim} TCR1$^+$ T-cell subsets in peripheral blood of chickens immunized with the two different *Salmonella enterica* serovar Enteritidis (SE-LV or SE 147) strains on day 1 of life and infected with *Salmonella enterica* serovar Enteritidis 147 N on day 44 of life in comparison to nonimmunized controls. Results are shown between day 1 and day 71 of life. Data represent means ± standard deviations. Asterisks indicate a significant difference between the treated and the control group ($P \geq 0.05$). (D) Percentages of CD28$^+$ cells among the CD8α^{+high} TCR1$^+$ subset in peripheral blood of chickens immunized with the two different *Salmonella enterica* serovar Enteritidis strains (SE-LV or SE 147) on day 1 of life and infected with *Salmonella enterica* serovar Enteritidis 147 N on day 44 of life in comparison to nonimmunized controls. Results are shown between day 1 and day 71 of life. Data represent means ± standard deviations. Asterisks indicate a significant difference between the treated and control groups ($P \geq 0.05$).
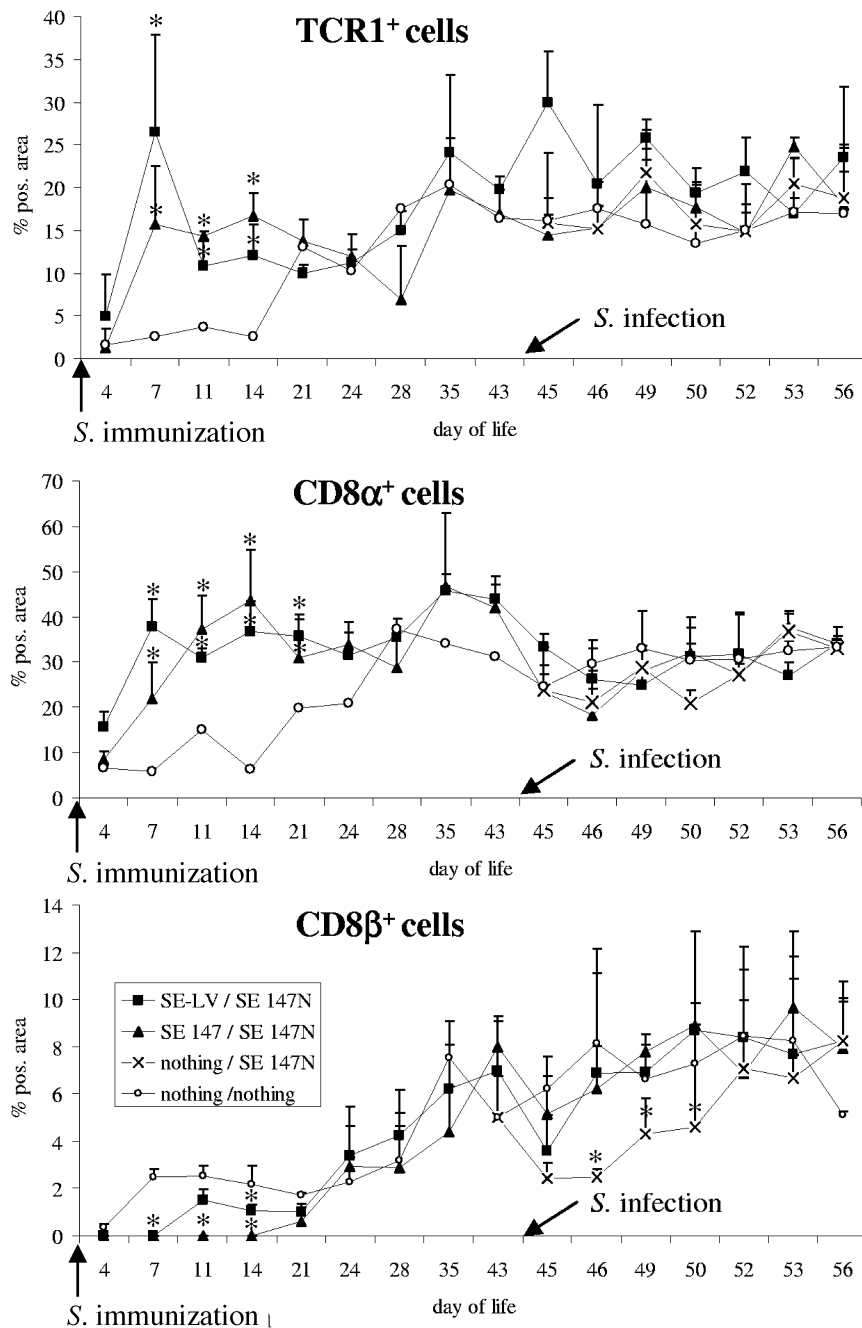
FIG. 4. Percent positive area of TCR1$^+$, CD8α^+, and CD8β^+ cells in spleens of chickens immunized with the two different *Salmonella enterica* serovar Enteritidis strains (SE-LV or SE 147) on day 1 of life and infected with *Salmonella enterica* serovar Enteritidis 147 N on day 44 of life in comparison to nonimmunized controls. Asterisks indicate a significant difference between the treated and control groups ($P \geq$ 0.05).

and SE-LV-immunized birds but showed high standard deviations.

In spleens of SE-LV- and SE 147-immunized animals, the percentages of CD8α^+ and TCR1$^+$ T cells were increased between days 8 and 21 of life. In contrast, the number of CD8β^+ cells was significantly decreased between days 7 and 21 (Fig. 4). After challenge on day 44 of the already immunized animals, no changes concerning the number of the investigated

T-cell populations were detectable. Only the nonimmunized but challenged control animals showed a decrease of CD8β^+ cells, which was observed on days 45 and 46.

Quantification of cytokine mRNA expression of peripheral γδ T-cell subsets. The levels of mRNA expression of the γδ T-cell subpopulation (CD8$\alpha^{+\text{high}}$ CD8β^+ TCR1$^+$, CD8$\alpha^{+\text{high}}$ CD8β^- TCR1$^+$, and CD8α^- CD8β^- TCR1$^+$ cells) after SE 147 immunization are shown in Fig. 5. Higher IL-2Rα mRNA
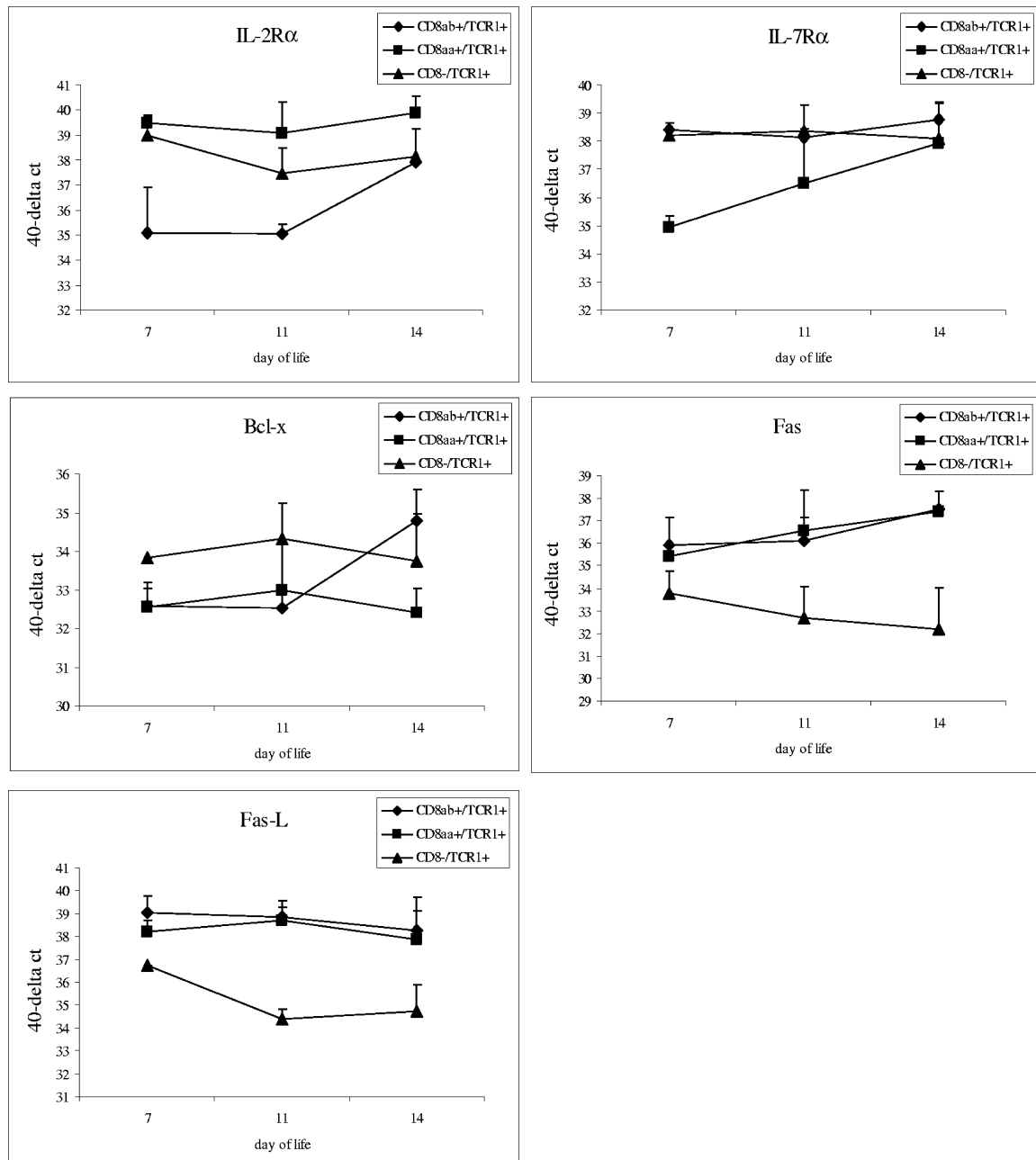
FIG. 5. Expression of cytokine receptor chains (IL-2Rα and IL-7Rα) and apoptotic-related proteins (Fas, Fas-ligand, and Bcl-x) of peripheral γδ T-cell subsets (CD8α^{+high}β$^+$ TCR1$^+$, CD8αα^{+high} TCR1$^+$, and CD8α$^-$ TCR1$^+$ cells) after SE 147 immunization on day 1 of life. Values are given as corrected mean 40-ΔC_T (40-delta ct) values.

expression was evident in CD8α^{+high} CD8β$^-$ TCR1$^+$ (CD8αα^{+high} γδ T cells) cells than in CD8α^{+high} CD8β$^+$ TCR1$^+$ (CD8α^{+high} β$^+$ γδ T cells) and CD8$^-$ TCR1$^+$ (CD8α$^-$ γδ T cells) cells. Additionally, the CD8αα^{+high} γδ T cells showed lower expression of IL-7Rα and Bcl-x mRNA than both CD8α^{+high} β$^+$ γδ cells and CD8α$^-$ γδ T cells. There was an increase of IL-7Rα mRNA expression of the CD8αα^{+high} γδ T cells from days 7 to 14 after immunization.

Hardly any difference of the Fas and Fas-L mRNA expression was found between the CD8αα^{+high} and CD8α^{+high}β$^+$ γδ

T-cell subsets. However, differences between CD8α^{+high} γδ cells and CD8α$^-$ γδ cells were evident. Both CD8α^{+high} γδ T-cell subsets expressed more Fas and Fas-L mRNA than found in CD8α$^-$ γδ T cells.

DISCUSSION

In the present study, the phenotype of avian T cells, particularly CD8α$^+$ γδ T cells, was specified to gain more insight into the role of single γδ T-cell subsets in defense against *Salmo-*

nella organisms of chickens. Based on our previous findings (8), the dynamics of CD8$^+$ and γδ T-cell subsets was investigated after immunization and infection of chickens with attenuated and nonattenuated *Salmonella enterica* serovar Enteritidis strains in peripheral blood and organs.

The phenotypic characterization of avian TCR1$^+$, TCR2$^+$, and TCR3$^+$ cells demonstrated the presence of completely separate cell populations not only concerning the TCR antigen but also because of the expression of further antigens. A closer relationship between the TCR2$^+$ and the TCR3$^+$ subpopulations than with TCR1$^+$ cells was shown by the relatively high frequency of CD4$^+$ antigen expression of both subpopulations. The detected different expression of the CD28 antigen on TCR2$^+$ and TCR3$^+$ cells indicates distinct activation levels of these T-cell populations. It has been already shown that TCR2$^+$ and TCR3$^+$ cells are separate sublineages of avian T cells by observation of a compensatory increase in TCR3$^+$ cell frequency when the development of the TCR2$^+$ subpopulation was inhibited (17). Other authors described the ability of TCR2$^+$ cells, but not of TCR3$^+$ cells, to migrate to the chicken intestine, where they provide help to mucosal B cells for immunoglobulin A production (16).

Each of the three TCR subsets could be further subdivided in three single subsets based on their intensive, weak, or absent CD8α expression. In contrast to CD8α^{+dim} TCR1$^+$ cells, CD8α^{+dim} TCR2$^+$ and CD8α^{+dim} TCR3$^+$ cells of the White Leghorn chickens additionally expressed the CD4 antigen. These cells did not express the CD8β chain, indicating the expression of only the CD8αα homodimeric form of the CD8 antigen. Accordingly, other studies demonstrated that, in some H.B15 chickens, a large subpopulation of peripheral blood CD4$^+$ T cells expresses only CD8α mRNA but not CD8β (30). The same authors have shown that these CD4$^+$ CD8αα T cells can induce an in vivo alloreaction comparable to that of normal CD4$^+$ T cells, which represents direct evidence for peripheral blood CD4$^+$ CD8$^+$ T cells functioning as helper CD4 T cells. It seems that the expression of CD8α does not interfere with the function of peripheral blood CD4$^+$ T cells in chickens.

Based on CD8α antigen expression, we demonstrated for the first time the existence of three γδ T-cell subsets in avian blood and showed that most of circulating CD8^{+high} TCR1$^+$ cells express the αβ heterodimeric form of the CD8 antigen, whereas CD8^{+dim} TCR1$^+$ cells present the αα homodimeric form of the CD8 antigen. It seems that γδ T cells are able to express both forms of the CD8 antigen depending on their localization or species investigated. Straube and Herrmann (42) considered rats to be the only species with frequent expression of the CD8αβ antigen by γδ T cells. In spleens of avian embryos and young chicks, a relatively large proportion of CD8$^+$ γδ T cells expressing only the α-chain of CD8 has been found (44).

The three γδ T-cell subsets defined in dependence on their CD8α antigen expression showed different reactions after *Salmonella* immunization of day-old chicks. This fact indicates not only phenotypic but also functional differences between these subsets. A difference in the overall functional and/or activation status of human circulating CD8$^-$ versus CD8$^+$ γδ T cells has been postulated (25). A comparison of avian CD8α^{+high} and CD8α^{+dim} γδ T cells in our experiment showed that the response of CD8α^{+high} T cells was more evident. In swine,

possible functional differences between CD8^{+dim} and CD8^{+high} αβ T cells have been discussed in connection with different expression of a second α or β chain of the CD8 antigen (38, 43).

The detected decrease of CD8β expression on CD8α^{+high} TCR1$^+$ cells after *Salmonella* immunization indicates an increase in the number of CD8α^{+high} γδ T cells expressing the CD8αα homodimer. Additionally, a decreased number of CD8β$^+$ T cells was seen in the spleens of immunized chickens. Whether this was due to a down-regulation of the CD8β antigen on the single cell or an influx of CD8α$^+$ homodimeric γδ T cells has yet to be examined. However, this is the first demonstration of an increase of CD8αα^{+high} circulating γδ T cells after *Salmonella* exposure of chicks. Similarly, rat splenic T cells showed persistent down-regulation of the expression of CD8β but not of CD8α on γδ T cells after in vitro activation (42). CD8αβ is more effective than CD8αα in facilitating recognition of the same peptide antigen by TCR in vitro (47). Thus, the CD8β down-regulation could presumably be a mechanism for limiting the generation of TCR-mediated signals after *Salmonella* infection in vivo. Moreover, a requirement of transient CD8αα expression for memory T-cell generation has been proven by use of E8$_1$-deficient mice that cannot generate CD8αα homodimers (31). In mice (28, 29) and humans (27), the CD8αα antigen is expressed on distinct T-cell subsets that constitutively display a memory phenotype. That γδ T-cell subsets are also able to differentiate into memory T cells has already been shown in humans (7, 20). However, it is not known whether the rise of CD8αα homodimeric γδ T cells can be considered a suitable marker for memory T-cell generation or memory T cells themselves in chickens.

In this study, avian CD8αα^{+high} homodimeric, CD8α^{+high}β$^+$ heterodimeric, and CD8α$^-$ γδ T-cell subsets were characterized concerning their mRNA expression for interleukin receptor chains involved in the memory generation (IL-2Rα and IL-7Rα) and the apoptosis-related proteins Fas, Fas-ligand, and Bcl-x after *Salmonella* stimulation for the first time. It was demonstrated that all three avian γδ T-cell subsets were able to express mRNA of the investigated proteins. CD8αα^{+high} homodimeric γδ T cells showed relatively reduced levels of IL-7Rα and Bcl-x but an elevated level of IL-2Rα mRNA expression compared to the CD8αβ heterodimeric and CD8α$^-$ γδ T cells. Interestingly, the expression of the IL-7Rα mRNA increased over the time of investigation from 6 to 13 days after *Salmonella* immunization. These results, together with the observed significant increase of the percentage of CD8αα^{+high} γδ T cells and the increased CD28 antigen expression on the CD8α^{+high} γδ T cells after immunization, led to the assumption that the observed CD8αα^{+high} homodimeric γδ T-cell subset represented activated γδ T cells after clonal expansion that underwent the contraction phase of the primary immune response against *Salmonella enterica* serovar Enteritidis. Recent data showed that T-cell stimulation induces considerable modifications of IL-7Rα chain expression (37, 40). While naive T cells are IL-7Rα positive, T-cell activation leads to down-regulation of IL-7Rα and large-scale apoptotic episodes thereafter, resulting in a substantial reduction in the number of antigen-specific T cells. Only a subpopulation of T cells escapes death and remains a stable population of memory T

cells. Progression to memory is accompanied by the reexpression of the IL-7Rα chain (37, 40).

A decrease in the percentage of peripheral CD8⁻ γδ T cells in *Salmonella enterica* serovar Enteritidis-immunized birds was found. This might be due to (i) an up-regulation of CD8 molecule expression or (ii) a fast migration of CD8⁻ γδ T cells to sites of infection, such as the intestine or spleen. It has been shown earlier, as well as in this study, that γδ T cells occur in higher numbers in the gut and spleen after immunization of chickens with *Salmonella enterica* serovar Typhimurium (8) and *Salmonella enterica* serovar Enteritidis, respectively. The peripheral blood may be a major source for γδ T cells of the intestine and vice versa. A proliferation and rapid recirculation of intestinal γδ T cells into the blood was seen in swine (43). Additionally, it has been shown that bovine CD8⁻ γδ T cells of newborn calves express E-selectin ligands, which are responsible for adhesion, and that they are able to migrate to acute and chronic sites of inflammation (18, 32).

After infection, splenic *Salmonella* clearance was more rapid in immunized animals than in nonimmunized controls. Immunization using the nonattenuated *S. enterica* serovar Enteritidis wild-type strain 147 produced the strongest protection: no challenge organisms were detected in the spleen during the course of the experiment. The rapid clearance of the *Salmonella* organisms after infection of *Salmonella*-immunized birds did not correlate with changes of T-cell composition in peripheral blood or spleen after infection. In contrast, our data showed a restriction of the secondary cellular immune response to the intestine after *Salmonella enterica* serovar Enteritidis infection of already immunized birds. A strong increase of CD8⁺ and TCR1⁺ T cells in the intestinal lamina propria could be observed quickly after challenge especially of SE 147-immunized animals. Thus, the *Salmonella* clearance in the spleen correlated well with the observed secondary immune response in the cecum, as higher percentages of CD8⁺ and TCR1⁺ T cells in the cecum mean lower numbers of *Salmonella* organisms in the spleen after challenge. Similarly, Beal et al. (6) found a restriction of the antigen-specific humoral and cellular immune response to the intestine after *Salmonella enterica* serovar Typhimurium infection of immunized chickens.

Generally speaking, immunization of day-old chicks using live attenuated or nonattenuated *Salmonella* strains resulted in variable reactions of γδ T-cell subsets. The emergence of γδ T-cell populations in the blood indicates that they are responding to the pathogen and/or providing a protective immune response. The lower γδ T-cell response after *Salmonella* live vaccine immunization than after *Salmonella* wild-type strain immunization of day-old chicks on the other hand seems to lead to a weaker protection after *Salmonella* infection. Distinct reactions of the defined circulating γδ T-cell subsets after *Salmonella* immunization and infection of chickens indicate that phenotypically characterized γδ T-cell subsets may serve different immunological purposes in chickens. The detected down-regulation of the CD8β antigen on avian circulating CD8α⁺high TCR1⁺ cells and the low IL-7Rα mRNA expression of CD8αα⁺high γδ T cells after *Salmonella* immunization of young chicks in this study might be a consequence of a transient activation or specialization of a single γδ T-cell subset

in vivo that eventually may lead to the generation of specific memory T cells.

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