Contribution of innate and adaptive immune cells to the elimination of *Salmonella enterica* serotype Enteritidis infection in young broiler chickens

Short title: Broiler innate and adaptive immune responses to *Salmonella* infection

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Abstract

Salmonella enterica serotype Enteritidis (SE) is a zoonotic pathogen which causes foodborne diseases in humans through contaminated poultry products, as well as severe disease symptoms in young chickens. More insight in innate and adaptive immune responses of chickens to SE infection is needed to understand elimination of SE. Seven-day-old broiler chickens were experimentally challenged with SE and numbers and responsiveness of innate immune cells including natural killer (NK) cells, macrophages and dendritic cells (DCs) were assessed during 21 days post-infection (dpi). In parallel, numbers and function of $\gamma\delta$ T cells, CD8⁺ and CD4⁺ T cells as well as antibody titres were determined. SE was observed in the intestine and spleen of SE-infected chickens at 7 dpi. NK and T cells responded first to SE at 1 and 3 dpi as indicated by increased numbers of intestinal IL-2R α ⁺ and 20E5⁺ NK cells, in addition to enhanced activation of intestinal and splenic NK cells. At 7 dpi in the spleen, the presence of macrophages and the expression of activation markers on DCs was increased. At 21 dpi, an increase in intestinal $\gamma\delta$ and CD8⁺ T cell numbers was observed. Furthermore, SE-specific proliferation of splenic CD4⁺ and CD8⁺ T cells was observed and SE-specific antibodies were detected in all blood samples of SE-infected chickens. In conclusion, SE results in enhanced numbers and activation of innate cells during early stages of infection and it is hypothesized that in concert with subsequent specific T cell and antibody responses, reduction of SE in infected chickens is achieved. A better understanding of innate and adaptive immune responses important in the elimination of SE will aid in developing immune-modulation strategies, which may increase resistance and prevent SE infection and colonization in young broiler chickens and hence increase food safety for humans.

Author summary

Salmonella enterica serotype Enteritidis (SE) causes foodborne zoonotic diseases in humans, as well as a severe disease in young chickens. As a consequence of which health and welfare of humans and chickens are affected, resulting in substantial economic losses. To enable development of immune-mediated prevention strategies in chickens, more insight in the immune responses to SE is needed to understand how the infection is eliminated. For this purpose, we investigated non-specific and specific immune responses upon experimental SE infection in young broiler chickens. In this study, we found SE in the intestine and spleen of SE-infected chickens at 7 days post-infection (dpi). We show that natural killer (NK) cells respond first by enhanced presence and activation, followed by increased presence of macrophages and activation of dendritic cells. These early responses are hypothesized to stimulate the observed subsequent specific T cell and antibody responses. Better understanding of immune responses important in the elimination of SE will aid in developing immune-modulation strategies, which may increase resistance and prevent SE infection and colonization in young chickens and hence reduce SE-related foodborne illness in humans.

Introduction

Salmonella enterica serotype Enteritidis (SE) is one of the leading causes of foodborne diseases in humans, most often due to poultry products as a prominent food source. In chickens infected with faecal *Salmonellae* via oral or respiratory routes, SE colonizes the intestinal tract and disseminate systemically to tissues such as the liver and spleen [1,2]. In young chickens it can lead to severe disease and death, whereas adult chickens are often subclinically infected with SE, carrying the bacteria in their intestines [3]. Prevention of SE infection in poultry is thus important for health and welfare of both young chickens and humans, as well as to avoid substantial economic losses as a consequence. Since therapeutic treatment of SE infection in

chickens with antibiotics is not advised due to limited effectiveness and risk of antibiotic resistance, the use of immune-modulatory strategies to increase the resistance to SE is encouraged [4]. More insight in innate and adaptive immune responses and their interaction in response to SE infection in young broiler chickens will facilitate the design of these strategies. In young chickens, immunity largely depends on maternal antibodies as well activity of the innate immune system, with natural killer (NK) cells and macrophages as key players [5,6]. NK cells are particularly abundant amongst intestinal intraepithelial lymphocytes (IEL) [7,8], which are also rich in $\gamma\delta$ T cells and CD8⁺ T cells expressing the $\alpha\beta$ T cell receptor (TCR) [8,9]. Macrophages, dendritic cells (DCs) and CD4⁺ T cells are located directly underneath the intestinal epithelium [10]. The adaptive immune system is not fully developed yet upon hatch and functional T- and B- cell responses are observed after approximately two to three weeks of life [5,6].

The early response of the innate immune system in chickens within one week post-SE infection is characterised by the upregulation of genes associated with ‘defense/pathogen response’ [11], NK cell-mediated cytotoxicity [12] and production and secretion of the cytokine IFN γ [13]. In chickens, intestinal NK cells comprise a major CD3⁻ IL-2R α ⁺ subset [7,8] and a minor CD3⁻ 20E5⁺ subset [7], both having cytotoxic capacity but to different degrees. In mice and humans, high cytotoxic activity [14,15] and IFN- γ production [15,16] by NK cells have been shown to result in resistance to *Salmonella enterica* serotype Typhimurium. In chickens, the effect of SE infection on the function of NK cells has not been studied so far.

The development of T and B cell responses is initiated with the activation of antigen presenting cells (APCs) such as DCs and macrophages. The presence [17] and activity of intestinal macrophages [18-20], identified by the expression of mannose receptor C-type 1-like B (MRC1LB) [21], and bone marrow-derived DCs [22] increases during SE infection. Furthermore, oral infection of 1-day-old specific-pathogen-free chickens with SE elicits

increased mRNA expression of chemokines and macrophages are attracted to the ileum within 24 hours [23]. In addition, it has been shown that decreased activity of peritoneal macrophage is associated with increased susceptibility for systemic dissemination of SE in chickens [24]. On the other hand, *Salmonella* species have been found to resist killing by macrophages in mammals [25] and chickens [26,27] and even use macrophages as a carrier for systemic dissemination [28]. Despite their involvement in SE infection, a detailed analysis of the effect of SE infection on the function of APCs in chickens has not been performed to date.

Initial T cell responsiveness to SE in chickens has been observed within one week, including increased presence of $\gamma\delta$ T cells in the intestine, blood and spleen after live vaccination [13,29], and enhanced mRNA expression of cytotoxic activity-related genes in the spleen after infection [30], as compared to non-vaccinated and uninfected chickens. After two and three weeks, a second increase in presence of $\gamma\delta$ T cells was observed in the spleen and intestine, respectively, compared to non-vaccinated chickens [29]. CD4⁺ helper T cells and CD8⁺ cytotoxic T cells were also shown to increase in presence within one week post-SE infection and vaccination in the intestine, as compared to uninfected and non-vaccinated chickens [13,17,18]. CD8⁺ cytotoxic T cells kill infected host cells, while CD4⁺ helper T cells release cytokines like IL-2 and IFN γ to further stimulate NK cells, and macrophages and CD8⁺ cytotoxic T cells respectively, and promote the differentiation of B cells into antibody-producing plasma cells. Antibody responses involved in elimination of SE partly depend on maternal antibodies and on the production of IgA in the intestine [31,32], as well as IgM, IgA and IgY antibodies in blood [32,33]. Whereas previous studies have focussed on specific aspects of the immune responses, the present study combines cellular assays to analyze both the innate and adaptive immune responses in intestine and spleen upon SE infection in chickens.

In this study, we investigated how *Salmonella enterica* serotype Enteritidis infection in young broiler chickens affects presence and activation of innate and adaptive immune cells in the

intestine and spleen to obtain more insight in the contribution of the immune system to elimination of the infection. Numbers of SE in ileum and spleen were determined alongside differences in kinetics of presence and activation status of NK cell and APC subsets between uninfected and infected chickens. The subsequent adaptive responses were determined including presence and activation of $\gamma\delta$ T cell, CD4⁺ and CD8⁺ T cell subsets, and serum antibody levels. Hence the present study provides an extensive overview of intestinal and systemic immune responses that are evoked by SE infection in young broiler chickens. Based on the phenotypical and functional data obtained, we will hypothesize on how the various elements of the immune system interact and contribute to elimination of the SE infection, and on potential strengthening of immune responsiveness by immunomodulation strategies, which may prevent SE infection and colonization, and thus increase chicken health and welfare as well as safety of food of chicken origin.

Materials and methods

Animals and husbandry

A total of 30 respectively 35 Ross 308 seventeen- and eighteen-day old embryonated eggs were obtained from the same parent flock of a commercial hatchery (Lagerwey, the Netherlands). Eggs were disinfected with 3% hydrogen peroxide and placed in disinfected hatchers in two different stables (uninfected and SE-infected) at the facilities of the Department of Population Health Sciences, Faculty of Veterinary Medicine, Utrecht University, the Netherlands. Directly upon hatch, chickens were weighed, labelled and housed in floor pens lined with wood shavings (2 kg/m²), and received water and standard *Salmonella*-free commercial starter and grower feeds *ad libitum* (Research Diet Services, the Netherlands). A standard lighting and temperature scheme for Ross broiler chickens was used for both stables.

The animal experiment was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee (registration number AVD1080020174425) of Utrecht University (the Netherlands) and all procedures were done in full compliance with all relevant legislation.

Experimental design

Before the start of the experiment at day 3, five chickens per group (uninfected ($n = 30$) and SE-infected ($n = 35$)) were randomly selected and sacrificed for collection of ileum (± 10 cm distal from Meckel's diverticulum) and spleen to confirm absence of SE before inoculation. At day 7 (0 days post infection (dpi)), five chickens of the SE-infected group only, were randomly selected and sacrificed for collection of ileum and spleen, to determine baseline levels of the various immune parameters as well as absence of SE before infection. Subsequently, chickens of the SE-infected group were challenged at day 7 (0 dpi) by oral inoculation of 0.25 ml brain heart infusion (BHI) medium containing 1.12×10^6 colony-forming units (CFUs) SE, whereas chickens in the other stable (uninfected) were inoculated with 0.25 ml BHI medium. At days 8 (1 dpi), 10 (3 dpi), 14 (7 dpi), 21 (14 dpi) and 28 (21 dpi), five chickens per group were randomly selected and sacrificed for collection of ileum and spleen to determine bacterial CFUs as well as numbers and function of NK cells and T cells. At days 7, 8, 10 and 14 also spleen APCs were assessed. At days 7, 14, 21 and 28 blood (at least 5 ml) was collected in EDTA tubes (VACUETTE® K3E EDTA, Greiner Bio-One, the Netherlands) for determination of SE-specific antibody levels. At day 28, splenic lymphocytes were also used to assess SE-specific T cell reactivity in a proliferation assay. All chickens were weighed prior to post-mortem analyses to determine the growth curve. Ileum segments and spleens were weighed before cell isolation to enable calculation of absolute cell numbers.

SE culture

The *Salmonella enterica* serotype Enteritidis strain (K285/93 Nal^{res}) was kindly provided by Dr. E. Broens, director of the Veterinary Microbiological Diagnostic Center (VMDC) of the Faculty of Veterinary Medicine, Utrecht University, and cultured as described previously [34]. In short, from an overnight culture of the SE strain on blood agar (Oxoid, the Netherlands) a single colony was used to inoculate 45 ml BHI medium (Oxoid), which was incubated aerobically overnight at 200 rpm in a shaking incubator at 37°C. The OD value of a sample of the SE culture diluted 1:10 in PBS was measured using a Ultrospec 2000 (Pharmacia Biotech, Sweden), the SE concentration was calculated from a previously determined growth curve, and SE were diluted in BHI medium to 4×10^6 CFU/ml, to constitute the inoculum. The exact SE concentration of the inoculum, determined by counting the number of CFUs of plated serial dilutions after overnight culture, was 4.49×10^6 CFU/ml.

For the T cell proliferation assay, SE was fixed by resuspending 3.8×10^9 CFUs in 100 µl PBS with 1% formaldehyde (Sigma-Aldrich, the Netherlands) and incubation for 5 min at RT, while the suspension was vortexed shortly every minute. After fixation, the bacteria were washed four times in 1 ml PBS by centrifugation at 12500 rpm to remove the supernatants (Heraeus Pico 17 Centrifuge, Thermo Fisher Scientific). Finally, the bacteria were resuspended in 380 µl X-VIVO 15 cell culture medium (Lonza, the Netherlands) with 50 µg/ml gentamycin (Gibco™, the Netherlands) to create a concentration of 10^7 CFU/ml and stored at 4°C until further use.

Isolation of cells

Ileum segments were washed with PBS to remove the contents, cut into sections of 1 cm² and washed again. Subsequently, the IEL were collected by incubating three times in a shaking incubator at 200 rpm for 15 min at 37°C in EDTA-medium (HBSS 1x (Gibco®) supplemented with 10% heat-inactivated FCS (Lonza) and 1% 0.5M EDTA (Sigma-Aldrich). Supernatants

were collected and centrifuged for 5 min at 1200 rpm at 20°C (Allegra™ X-12R Centrifuge, Beckman Coulter, the Netherlands). Cells were then resuspended in PBS, lymphocytes were isolated using Ficoll-Paque Plus (GE Healthcare, the Netherlands) density gradient centrifugation for 12 min at 1700 rpm at 20°C, washed in PBS by centrifugation for 5 min at 1300 rpm at 4°C and resuspended at 4.0×10^6 cells/ml in complete medium (IMDM 2 mM glutamax I supplemented with 8% heat-inactivated FCS (Lonza), 2% heat-inactivated chicken serum, 100 U/ml penicillin/streptomycin; Gibco®). Spleens were homogenized using a 70 µm cell strainer (Beckton Dickinson (BD) Biosciences, NJ, USA) to obtain a single-cell suspension. Next, lymphocytes were isolated by Ficoll-Paque density gradient centrifugation (20 min, 2200 rpm, 20°C), washed in PBS and resuspended at 4.0×10^6 cells/ml in complete medium as described for ileum.

Whole blood was allowed to coagulate by leaving it undisturbed for 1 hour at room temperature (RT), centrifuged for 10 min at 3000 rpm at 15°C and subsequently, serum was collected and stored at -20°C until further use.

Quantitative bacteriology of IEL and spleen

At -4, 0, 1, 3, 7, 14 and 21 dpi, the numbers of *Salmonella* colonies in IEL and spleen samples were determined by plating 100 µl of the EDTA-isolated IEL and homogenized spleen, 1:10-diluted in PBS before the Ficoll step, on RAPID® *Salmonella* Medium plates (Bio-Rad, the Netherlands). Plates were incubated overnight at 37°C and subsequently, purple colonies were quantified and SE was expressed as CFU per gram tissue.

Phenotypic characterization of lymphocytes by flow cytometry

Presence and activation of NK and T cell subsets were determined among IEL and splenocytes at 0, 1, 3, 7, 14 and 21 dpi as described previously [7,35]. Lymphocyte populations (1×10^6)

were stained with a panel of antibodies specific for surface markers known to be expressed on NK cells, as well as with anti-CD3 to exclude T cells from the analyses. In addition, cells were stained with a panel of antibodies specific for surface markers that distinguishes $\gamma\delta$ T cell, CD4⁺ and CD8⁺ T cell subsets (Table 1). Staining with primary and secondary antibodies was performed in 50 μ l PBS (Lonza) containing 0.5% bovine serum albumin and 0.1% sodium azide (PBA). Cells were incubated for 20 min at 4°C in the dark, washed twice by centrifugation for 5 min at 1300 rpm at 4°C in PBA, after primary staining, and in PBS after secondary staining. Subsequently, to be able to exclude dead cells from analysis, lymphocytes were stained in 100 μ l PBS with a viability dye (Zombie Aqua™ Fixable Viability Kit, Biolegend, the Netherlands) for 15 min at RT in the dark, washed twice in PBA and resuspended in 200 μ l PBA. Of each sample, either 150 μ l or a maximum of 1×10^6 viable lymphocytes were analyzed using a CytoFLEX LX Flow Cytometer (Beckman Coulter), and data was analyzed with FlowJo software (FlowJo LCC, BD Biosciences). The gating strategies used to analyze NK cells, $\gamma\delta$ T cells and cytotoxic CD8⁺ T cells were as described previously [35]. In short, gating included consecutive selection for lymphocytes (FSC-A vs SSC-A), viable cells (Live/Dead marker-negative) followed by selection of the cell subsets and activation markers according to the staining panels. NK cell subsets: CD3⁻ cells expressing either IL-2R α or 20E5. NK activation: CD3⁻CD41/61⁻ cells expressing CD107 or CD3⁻ cells expressing IFN γ . T cell subsets: CD3⁺CD4⁻ cells positive for TCR $\gamma\delta$ ($\gamma\delta$) or negative (CD8⁺ $\alpha\beta$) with both $\gamma\delta$ and cytotoxic $\alpha\beta$ T cells expressing either CD8 $\alpha\alpha$ or CD8 $\alpha\beta$, and only in spleen the CD3⁺ cells expressing CD4. T cell activation: CD3⁺CD41/61⁻CD8 α ⁺ cells expressing CD107 or CD3⁺TCR $\gamma\delta$ ⁺CD8 α ⁺, CD3⁺TCR $\gamma\delta$ ⁻CD8 α ⁻ (CD4⁺) and CD3⁺TCR $\gamma\delta$ ⁻CD8 α ⁺ cells expressing IFN γ .

Table 1. Flow cytometry staining reagents.

Cell population	Primary antibody (mouse-anti-chicken)	Clone / Isotype	Secondary antibody
NK cells	CD45-FITC ¹	LT40 / IgM	-
	CD3-APC ¹	CT3 / IgG1	-
	IL-2R α -UNL ²	28-4 / IgG3	Goat-anti-mouse-IgG3-PE ¹
	20E5-BIOT ³	IgG1	Streptavidin (SA)-PercP ⁶
T cells	CD3-PE ¹	CT3 / IgG1	-
	CD4-APC ¹	CT4 / IgG1	-
	TCR $\gamma\delta$ -FITC ¹	TCR-1 / IgG1	-
	CD8 α -UNL ¹	EP72 / IgG2b	Goat-anti-mouse-IgG2b-APC/Cy7 ¹
	CD8 β -BIOT ¹	EP42 / IgG2a	SA-PercP ⁶
APCs	CD41/61-FITC ⁴	11C3 / IgG1	-
	Bu-1-AF647 ¹	AV20 / IgG1	-
	CD3-FITC ¹	CT3 / IgG1	-
	CD4-PE/Cy7 ¹	CT4 / IgG1	-
	MRC1LB-PE ¹	KUL01 / IgG1	-
	CD11-biotin ³	5C7 / IgG1	SA-Brilliant Violet (BV) 605 ⁷
	MHC-II-UNL ¹	Cia / IgM	Rat-anti-mouse-IgM-BV421 (RMM-1) ⁷
	*CHIR-AB1-UNL ²	8D12 / IgG2a	Rat-anti-mouse-IgG2a-PerCP/Cy5.5 (RMG2a-62) ⁷
	*CD40-UNL ⁵	AV79 / IgG2a	Rat-anti-mouse-IgG2a-PerCP/Cy5.5 (RMG2a-62) ⁷
	*CD80-UNL ⁵	IAH:F864:DC7 / IgG2a	Rat-anti-mouse-IgG2a-PerCP/Cy5.5 (RMG2a-62) ⁷
Assay			
CD107	CD107a-APC ³	LEP-100 I 5G10 / IgG1	-
	CD41/61-FITC ⁴	11C3 / IgG1	-
	CD3-PE ¹	CT3 / IgG1	-
	CD8 α -UNL ¹	EP72 / IgG2b	Goat-anti-mouse-IgG2b-Alexa Fluor (AF) 790 ⁸
	28-4-UNL ²	IgG3	Goat-anti-mouse-IgG3-APC/Cy7 ¹
	20E5-BIOT ³	IgG1	SA-PercP ⁶
IFN γ	CD3-PE ¹	CT3 / IgG1	-
	TCR $\gamma\delta$ -FITC ¹	TCR-1 / IgG1	-
	CD8 α -UNL ¹	EP72 / IgG2b	Goat-anti-mouse-IgG2b-AF790 ⁸
	28-4-UNL ²	IgG3	Goat-anti-mouse-IgG3-APC/Cy7 ¹
	20E5-BIOT ³	IgG1	SA-PercP ⁶
	IFN γ -APC ³	MAb80 / IgG1	-
T cell proliferation	CD3-FITC ¹	CT3 / IgG1	-
	CD4-PE/Cy7 ¹	CT4 / IgG1	-
	CD8 α -APC ¹	CT8 / IgG1	-
	IL-2R α -UNL ²	28-4 / IgG3	Goat-anti-mouse-IgG3-PE ¹

Three APC antibody panels were prepared each containing surface markers plus one out of three antibodies indicated (*). Manufacturer: ¹Southern Biotech, AL, USA, ²Purified supernatant of hybridoma provided by Göbel, T.W., Ludwig Maximilian University, Germany, ³Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA, USA, ⁴Serotec, United Kingdom, ⁵Bio-Rad, ⁶BD Biosciences, ⁷Biolegend, ⁸Jackson ImmunoResearch Laboratories, PA, USA.

245

246 **CD107 assay**

247 Activation of NK cells and cytotoxic CD8⁺ T cells was determined using the CD107 assay,
 248 which measures the increased surface expression of CD107a that results from degranulation of
 249 cytotoxic granules [36]. Briefly, lymphocytes isolated from IEL and spleen were resuspended
 250 in complete medium, and 1 x 10⁶ lymphocytes in 0.5 ml were incubated in the presence of 1
 251 μ l/ml GolgiStop (BD Biosciences) and 0.5 μ l/ml mouse-anti-chicken-CD107a-APC for 4 hours
 252 at 37°C, 5% CO₂. After incubation, lymphocytes were washed in PBA and stained as described

in 2.6 with monoclonal antibodies for NK and T cells, and anti-CD41/61 to exclude thrombocytes from analyses, as mentioned in the CD107 panel (Table 1). Cells were washed in PBS, stained for viability and analyzed by flow cytometry as described in 2.6.

IFN γ assay

Expression of intracellular IFN γ was determined in (subsets of) NK cells, $\gamma\delta$ T cells, CD4⁺ and CD8⁺ T cells, using the assay adapted from Ariaans et al. 2008 [37]. Lymphocytes isolated from IEL and spleen were resuspended in complete medium, and 1 x 10⁶ lymphocytes in 0.5 ml were incubated in the presence of 1 μ l/ml Brefeldin A (Sigma Aldrich) for 4 hours at 41°C, 5% CO₂. After incubation, lymphocytes were washed in PBA and stained as described in 2.6 with surface markers as mentioned in the IFN γ panel (Table 1). Cells were washed in PBS, stained for viability and washed again in PBA. Then, lymphocytes were permeabilized differently by using different solutions as described before. Lymphocytes were incubated in 200 μ l of a mixture of BD FACST[™] Permeabilizing Solution 2 and BD FACST[™] Lysing Solution prepared according to manufacturer's instructions (BD Biosciences) for 8 min at RT, immediately followed by centrifugation for 2 min at 1300 rpm at 4°C. Cells were washed twice in PBA, stained intracellularly with anti-IFN γ -APC in 50 μ l PBA for 20 min at 4°C in the dark, washed in PBA and finally analyzed by flow cytometry as described in 2.6.

Phenotypic characterization of APCs by flow cytometry

Splenocytes isolated at 0, 1, 3 and 7 dpi from infected and uninfected chickens were transferred to a 96 wells V-bottom plate and stained with antibodies of the APC panel to distinguish APC subsets (Table 1). Staining with primary and secondary antibodies (1 x 10⁶) was performed in 50 μ l PBA, incubated for 20 min at 4°C in the dark and washed twice by centrifugation for 3 min at 1300 rpm at 4°C in PBA. Finally, the cells were stained in 50 μ l PBS with ViaKrome

808 viability dye (Beckman Coulter) for 20 min at 4°C. Cells were washed in PBA and analyzed
by flow cytometry as described in 2.6, using 180 µl.

Based on the APC subset staining, a t-distributed Stochastic Neighbor Embedding (t-SNE)
analysis was performed using FlowJo software to identify cell subsets using an unbiased
approach. From each sample of splenocytes at 7 dpi of infected ($n = 5$) and uninfected ($n = 5$)
chickens, 10,000 cells were taken and concatenated into one FCS file that represented all
individual chickens. The t-SNE was performed based on expression levels of CD3, CD41/61,
MRC1LB, CD4, Bu-1, CD11, MHC-II and CHIR-AB1 using published automated optimized
parameters [38]. Based on the t-SNE, three APC subsets were identified based on selection of
MRC1LB and CD11 positive cells, which were negative for CD3, CD4 and CD41/61.
Activation status of the subsets was subsequently evaluated using the expression percentages
of immunoglobulin Y receptor CHIR-AB1, co-stimulatory molecules CD40 and CD80, and the
geometric mean fluorescent intensity (gMFI) of MHC-II. CHIR-AB1 was included as an
activation marker since van den Biggelaar et al. (2020) recently showed increase of its
expression on macrophages after stimulation with LPS [39].

Fluorescence-activated cell sorting of NK cell and APC subsets

Based on marker expression, two NK cell subsets and three APC subsets were separated by
fluorescence-activated cell sorting (FACS) to gain more insight into their functional identity.
To distinguish NK cell subsets, splenocytes were stained with mouse-anti-chicken-CD3-APC,
-Bu-1-FITC, -28-4 and -20E5-biotin. For secondary antibody staining goat-anti-mouse-IgG3-
PE and SA-BV421 were used. To identify APC subsets, splenocytes were stained with mouse-
anti-chicken-MRC1LB-PE and -CD11-biotin. Secondary staining with SA-BV605 was used to
fluorescently label CD11-biotin. To assess viability, the cells were stained with the Zombie
Aqua™ Fixable Viability Kit. Primary and secondary antibody staining of cells used the same

conditions as described in 2.6. Finally, the cells were resuspended in PBA (NK cells) or PBA with 2 mM EDTA (APC subsets), and isolated by FACS using the BD influx™ Cell Sorter and 405-, 488-, 638-, 561- and 640-nm lasers. The cells were gated for viability and subsequently sorted into CD3⁻ Bu-1⁻ 28-4⁺ (IL-2Rα⁺ NK), CD3⁻ Bu-1⁻ 20E5⁺ (20E5⁺ NK), respectively CD11⁺ MRC1LB⁺ (APC subset 1), CD11⁺ MRC1LB⁻ FSC^{low} (APC subset 2a) and CD11⁺ MRC1LB⁻ FSC^{high} (APC subset 2b). The sorted NK cell subsets were collected in 350 µl RLT buffer (Qiagen, the Netherlands) with 1% 2-mercaptoethanol (Sigma Aldrich). The sorted APC subsets, and the original (unsorted) cell population as a control, were centrifuged at 1300 rpm for 5 min and then lysed in 600 µl RLT buffer with 1% 2-mercaptoethanol. Cell lysates of sorted cell subsets and the control cell population were then stored at -20°C until RNA isolation and qPCR analysis.

Gene expression of separated subsets of NK cells and APCs

Target genes (Table 2) were selected based on literature to define functional differences between subsets of NK cells respectively APCs. RNA was isolated from lysates of sorted NK and APC subsets, and control cells, using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, including a DNase treatment using the RNase-Free DNase Set (Qiagen). Next, cDNA was prepared using the reverse transcriptase from the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. RT-qPCRs were performed with primers and either FAM-TAMRA-labeled TaqMan probes combined with TaqMan Universal PCR Master Mix or SYBR Green Master Mix without probes (all from Thermo Fisher Scientific), as indicated in Table 2. Primers were used at 400 nM (SYBR-Green) or 600 nM (Taqman) and probes at 100 nM. RT-qPCRs were performed with a CFX Connect and analyzed with CFX Maestro software (both from Bio-Rad). All RT-qPCRs were evaluated for proper amplification efficiency (95–105%) using serial dilutions of reference cDNA either

from splenocytes that were stimulated with Concanavalin A for 24 hours or from HD11 cells that were stimulated with LPS for 3 hours. RT-qPCRs were performed in triplicate for every sample. For the NK cell subsets, mRNA levels are expressed as 40-Ct and the cycle threshold value (Ct) was corrected for variations in RNA preparation and sampling using the *GAPDH* Ct values, as described elsewhere [40]. Higher gene expression of *NFIL3* and *IL-7 α* is indicative of the cytokine-producing NK cell subset in humans [41-45], whereas higher expression of *PRF1* is indicative of the cytotoxic NK cell subset in humans and chickens [43,46]. Gene expression levels of the APC subsets are shown relative to those of unsorted splenocytes. Furthermore, Ct gene expression values were normalized to housekeeping genes *28S* and *GAPDH*. Changes in gene expression after sorting was expressed as $2^{-\Delta\Delta C_t}$, according to the Livak method [47]. Enrichment of cells expressing *CD14*, *TLR4*, *MERTK* and *MAFB* after sorting of cells was considered indicative for a monocyte/macrophage phenotype, whereas enrichment of cells expressing *ZBTB46*, *XCRI* and *FLT3* after sorting was considered indicative for a DC phenotype, in accordance with previous studies [48,49].

Table 2. Primers and TaqMan probe sequences used for RT-qPCR.

Cell type	Genes	NCBI Reference	TaqMan/SYBR-Green	Type	Sequence (5'-3')
NK cells	<i>NFIL3</i>	XM_017014743.1	SYBR-Green	Forward	TGAATGCCATCAGTTGAGC
				Reverse	GAGAGGCGGAGAATGTGAGT
	<i>IL-7α</i>	NM_001080106.1	SYBR-Green	Forward	ATTCTGGGAAAGCAGGATCAAG
				Reverse	CTTACACAGTCGCTCCAGAGTTATTT
	<i>PRF1</i>	XM_004945690.3	SYBR-Green	Forward	ACCCGCACCAAAAGATGAAG
				Reverse	TAATTTCGCACACCCCTAAACG
	<i>GAPDH</i>	NM_204305.1	SYBR-Green	Forward	GTGGTGCTAAGCGTGTTATC
				Reverse	GCATGGACAGTGGTCATAAG
APCs	<i>CD14</i>	NM_001139478.1	TaqMan	Forward	GGACGACTCCACCATTGACAT
				Reverse	GGAGGACCTCAGGAACCAGAA
				Probe	AATGATCTTCCTGATTTGCAGACTGCCAA
	<i>TLR4</i>	NM_001030693.1	SYBR-Green	Forward	GTCCTGTCTGGCAGGAT
				Reverse	TGTCCTGTGCATCTGAAAGCT
	<i>MERTK</i>	NM_204988.1	SYBR-Green	Forward	TGTGGAAGGATGGCAGGGAG
				Reverse	GCACGGATGCTGAATGTAGAGG
	<i>MAFB</i>	NM_001030852.1	SYBR-Green	Forward	AGGACCGGTTCTCGGATGAC
				Reverse	CCTCGGAGGTGCCTGTTG

	<i>INOS</i>	NM_204961.1	SYBR-Green	Forward	TGGGTGGAAGCCGAAATA
				Reverse	GTACCAGCCGTTGAAAGGAC
	<i>ZBTB46</i>	XM_015296613.2	SYBR-Green	Forward	CTGGACCTGTGGAAGAGGAAAC
				Reverse	CGGTAGTGGGAGGCAATCTC
	<i>XCR1</i>	NM_001024644.2	SYBR-Green	Forward	CCTTCGGGTGGATTTTGGT
				Reverse	CGCTGTAGTAGCCAATGGAGAA
	<i>FLT3</i>	NM_004119.3	SYBR-Green	Forward	CATTCGGACCCAGTACATGTTTAC
				Reverse	TGAGCCGTAGAAGAGCAGGTATAA
	<i>GAPDH</i>	NM_204305.1	SYBR-Green	Forward	GTGGTGCTAAGCGTGTTATC
				Reverse	GCATGGACAGTGGTCATAAG
	<i>28S</i>	XR_00378040.1	TaqMan	Forward	GGCGAAGCCAGAGGAAACT
				Reverse	GACGACCGATTTCACGTC
				Probe	AGGACCGCTACGGACCTCCACCA

All sequences have been described previously except for *NFIL3* and *PRF1* [48-50].

344

345 **T cell proliferation assay**

346 Splenocytes isolated at 21 dpi from uninfected and SE-infected chickens were labelled with
347 CellTrace Violet (CTV, Invitrogen, the Netherlands) to measure proliferation by flow
348 cytometry. The cells were resuspended at 5×10^6 cells/ml in PBS with 5 μ M CTV and incubated
349 for 20 min at RT, while the cell suspension was vortexed every 5 min. Next, the labeling was
350 quenched by the addition of 5 ml complete medium for every ml of CTV staining solution and
351 incubated for 5 min at RT. Cells were centrifuged for 5 min at 1200 rpm at 20°C and
352 resuspended at 2.5×10^6 cells/ml in X-VIVO 15 cell culture medium (Lonza) with 50 U/ml
353 penicillin–streptomycin, 50 μ M 2-mercaptoethanol (Sigma Aldrich) and 50 μ g/ml gentamycin
354 (Gibco™). Aliquots of 200 μ l cell suspension containing 500,000 splenocytes were added to
355 the wells of a 96 wells round-bottom cell culture plate. Fixed SE was added to the splenocytes
356 at 10^4 , 10^5 or 10^6 CFU/well. As a positive control, splenocytes were stimulated with 1 μ g/ml
357 mouse-anti-chicken-CD3, 1 μ g/ml -CD28 and 1:50 diluted conditioned supernatant from COS-
358 7 cells transfected with a pcDNA1 vector (Invitrogen) encoding for recombinant chicken IL-2
359 (a kind gift from prof. Pete Kaiser and dr. Lisa Rothwell), in accordance with a previous
360 publication [51]. Cells were incubated for four days at 41°C and 5% CO₂. After incubation,
361 cells were transferred to a 96 wells V-bottom plate and stained in PBS with ViaKrome 808
362 viability dye (Beckman Coulter). Next, cells were stained with antibodies of the T cell

proliferation panel (Table 1). Primary and secondary staining of cells were conducted in 30 µl PBA and incubated for 20 min at 4°C in the dark. Stained cells were washed twice by centrifugation for 3 min at 1300 rpm at 4°C in PBA and resuspended in 100 µl followed by flow cytometry analysis as described in 2.6, using 80 µl.

SE-specific antibody titers in serum

To detect titers of SE-specific antibodies in the sera collected at 0, 7, 14 and 21 dpi, the commercially available *Salmonella* Enteritidis Antibody Test (IDEXX SE Ab X2 Test) was used according to manufacturer's instructions (IDEXX Europe, the Netherlands). Positive and negative controls were included in the kit, and serum samples were analyzed in duplicate. Endpoint titers were calculated with the following formula:

$$10^{(1.5 * \log_{10}((\text{sample } \mu - \text{negative control } \mu) / (\text{positive } \mu - \text{negative control } \mu)) + 3.47)}.$$

Statistical analysis

First, the data were tested for fitting a normal distribution using the Shapiro-Wilk test. Differences in numbers of leukocytes, NK cell and T cell subsets and percentages of CD107 and IFNγ expression in IEL and spleen between the uninfected and SE-infected groups were analyzed using one-way ANOVA tests. Differences in SE CFUs per gram intestine and spleen as well as SE-specific antibody titers in serum were analyzed using Kruskal-Wallis tests accompanied by Dunn's multiple comparisons tests. Differences in numbers and percentages of the splenic APC subsets 1 and 2a between the uninfected and SE-infected groups were analyzed using one-way ANOVA tests, while subset 2b was analyzed using the Kruskal-Wallis test as the data was not normally distributed. All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, CA, USA). A *p*-value of < 0.05 was considered statistically significant.

Results

Highest presence of SE in ileum and spleen at 7 dpi while intestinal infiltration of leukocytes was observed at 1 and 3 dpi

SE was not observed at -4 and 0 dpi before SE inoculation, in both the IEL fraction of the ileum and the spleen of chickens of both groups (Fig 1A and 1B). After inoculation SE was detected in the IEL fraction of SE-infected chickens only at 7 dpi ($11,100 \pm 4026$ CFU/g, mean $\pm$ SEM, Fig 1A). In the spleen, SE was observed at 7, 14 and 21 dpi with the highest bacterial counts at 7 dpi (1200 ± 566 CFU/g), which subsequently decreased in course of time (Fig 1B). SE was not detected in the IEL fraction and spleen of uninfected chickens at any of the time points (Fig 1A and 1B). One uninfected chicken showed counts of *Proteus* in the spleen at 7 dpi and was therefore excluded from further analyses. Infection with SE did not affect the weight of the chickens, as growth curves were similar between uninfected and SE-infected chickens (Fig 1C). A significant increase in numbers of intestinal leukocytes was found in SE-infected chickens at 1 dpi ($38,509 \pm 3544$) compared to uninfected chickens ($15,401 \pm 1716$, Fig 1D). Numbers tended to be higher at 3 dpi in SE-infected chickens and declined over time to numbers similar to those observed in uninfected chickens (Fig 1D). The numbers of splenic leukocytes were similar between uninfected and SE-infected chickens at all time points (Fig 1E).

Increased presence and enhanced activation of intestinal NK cells upon SE infection

Numbers of intestinal and splenic NK cell subsets were determined to investigate differences between uninfected and SE-infected chickens at several time points post-infection. NK cell subsets were distinguished by membrane expression of IL-2R α or 20E5 (Fig 2A and S1A Fig). In the intestine, numbers of IL-2R α^+ and 20E5 $^+$ NK cells tended to be higher in SE-infected chickens at 1, 3 and 7 dpi compared to uninfected chickens (Fig 2B and 2C). Intestinal 20E5 $^+$

NK cell numbers tended to be lower at 14 dpi and higher at 21 dpi in SE-infected compared to uninfected chickens (Fig 2C). In the spleen, numbers of IL-2R α ⁺ and 20E5⁺ NK cells were similar between uninfected and SE-infected chickens and both increased in course of time (S1B and S1C Fig). To obtain more insight in functional differences between IL-2R α ⁺ and 20E5⁺ NK cells, mRNA levels of genes deemed to be relevant were determined in the spleen. The IL-2R α ⁺ subset showed higher mRNA levels of the NK cell lineage marker NFIL3 and IL-7R α ⁺ as compared to the 20E5⁺ subset, whereas the 20E5⁺ subset showed higher mRNA levels of perforin as compared to the IL-2R α ⁺ subset (PRF1, S1D Fig).

To determine possible changes in NK cell activation upon SE infection, CD107 surface expression and intracellular IFN γ were analyzed in intestinal and splenic NK cells (Fig 3A). Intestinal NK cells showed a significant increase in surface expression of CD107 and IFN γ production in SE-infected chickens at 1 dpi (11.1 $\pm$ 0.1% and 4.6 $\pm$ 0.2% respectively) and 3 dpi (9.0 $\pm$ 0.4%; 4.1 $\pm$ 0.2%) compared to uninfected chickens (Fig 3B and 3C). In the spleen, increased surface expression of CD107 was observed at 1 dpi (8.2 $\pm$ 0.5%) and 3 dpi (8.3 $\pm$ 0.4%) and increased IFN γ production at 1 dpi (8.2 $\pm$ 0.5%), 3 dpi (8.3 $\pm$ 0.4%) and 7 dpi (6.4 $\pm$ 0.1%) in SE-infected compared to uninfected chickens (Fig 3D and 3E).

Increased presence of APCs in the spleens of SE-infected chickens

To investigate whether infection with SE affects the composition of the APC population, amongst splenocytes, these were stained for APC surface markers and analyzed by flow cytometry. A t-SNE analysis was used to determine the differences in APCs between uninfected and SE-infected chickens (Fig 4A-4C). This analysis clustered cells that have high similarity and separated cells that are unrelated based on the APC surface markers that were used, leading to an unbiased visualization of all cell populations. Two populations were found overrepresented in the spleen of SE-infected chickens (Fig 4B). By gating for subset 1 and 2

and assessing their expression of APC markers, subset 1 was identified as CD11⁺ MRC1LB⁺ and subset 2 as CD11⁺ MRC1LB⁻ (Fig 4C, first panel). In addition, subset 2 could be further divided into two subsets, distinguished by FSC-A and SSC-A characteristics, that were further analyzed separately (Fig 4C, second panel).

The APC subsets were sorted, and qPCR was performed to compare the expression levels of macrophage- and DC-specific genes between sorted cells and the unsorted total APC population. High expression levels of the monocyte/macrophage genes CD14, TLR4, MERTK and MAFB (Fig 4D) observed on the CD11⁺ MRC1LB⁺ cells, indicates that this subset 1 includes macrophages (hereafter referred to as macrophages). The CD11⁺ MRC1LB⁻ FSC^{high} subset 2b includes DCs as reflected by high expression of the DC genes ZBTB46, XCR1 and FLT3 (hereafter referred to as FSC^{high} DCs) (Fig 4D). The increase in expression of either macrophage- or DC-specific genes was less clear in the CD11⁺ MRC1LB⁻ FSC^{low} subset 2a, however, DC-specific genes were most abundantly expressed (hereafter referred to as FSC^{low} DCs) (Fig 4D).

Next, the percentages (Fig 4E and S2A-B Fig) and numbers (Fig 4F and S2C-D Fig) of the three APC subsets were followed over time in the spleens of uninfected and SE-infected chickens. Due to limited cell numbers, the analysis of APCs could not be performed for two chickens at 0 dpi. At 7 dpi, the percentage of macrophages was significantly increased in SE-infected compared to uninfected chickens (Fig 4E). The FSC^{low} (S3A and S2C Fig) and FSC^{high} (S3B and S2D Fig) DCs were similar in SE-infected compared to uninfected chickens at all time points, although a slight increase in both the percentages and numbers of FSC^{high} DCs was observed at 7 dpi (S2B and S2D Fig).

APCs become activated in spleens of SE-infected chickens

To assess the activation status of the three APC subsets in response to SE infection, expression levels of immunoglobulin Y receptor CHIR-AB1, co-stimulatory molecules CD40 and CD80, and MHC-II were evaluated by flow cytometry (S3 Fig). The macrophages of SE-infected chickens did not show increased expression of the activation markers compared to uninfected chickens (Fig 5A and 5D and 5G and 5J). Before infection, FSC^{low} DCs of uninfected chickens showed a higher expression of MHC-II (gMFI = $5.0 \times 10^3 \pm 0.2 \times 10^3$ vs $2.4 \times 10^3 \pm 0.1 \times 10^3$, Fig 5K-L respectively) and more cells that were positive for the costimulatory molecules CD40 ($27.8 \pm 6.5\%$ vs $2.0 \pm 0.5\%$, Fig 5E-F respectively) and CD80 ($41.3 \pm 3.8\%$ vs $3.8 \pm 0.9\%$, Fig 5H-I respectively) compared to FSC^{high} DCs. At 7 dpi, FSC^{low} DCs showed significantly increased expression of CHIR-AB1 ($64.1 \pm 5.2\%$ vs $39.9 \pm 4.4\%$, Fig 5B), CD40 ($55.1 \pm 5.5\%$ vs $30.2 \pm 4.3\%$, Fig 5E) and CD80 ($67.7 \pm 2.7\%$ vs $42.1 \pm 3.9\%$, Fig 5H) in SE-infected chickens as compared to uninfected chickens. The FSC^{high} DCs showed at 3 dpi trends towards increased expression of CD40 ($6.6 \pm 1.5\%$ vs $3.7 \pm 0.5\%$, Fig 5F) and CD80 ($7.4 \pm 1.7\%$ vs $4.0 \pm 0.5\%$, Fig 5I) in SE-infected compared to uninfected chickens, and showed at 7 dpi significantly increased expression of CHIR-AB1 ($84.3 \pm 5.0\%$ vs $27.5 \pm 8.8\%$, Fig 5C) and MHC-II (gMFI = $4.9 \times 10^3 \pm 0.2 \times 10^3$ vs $3.3 \times 10^3 \pm 0.5 \times 10^3$, Fig 5L) in SE-infected compared to uninfected chickens.

Increased presence of intestinal $\gamma\delta$ T and cytotoxic T cells at 1 dpi and proliferation of SE-specific splenic T cells ex vivo at 21 dpi

Numbers of $\gamma\delta$ T cells and cytotoxic (CD8⁺) $\alpha\beta$ T cells were determined in course of time in the intestine and spleen of uninfected and SE-infected chickens (Fig 6A). A trend towards increased numbers of intestinal $\gamma\delta$ T cells were found at 1 and 3 dpi, as well as at 21 dpi in SE-infected compared to uninfected chickens (Fig 6B). A significant increase in numbers of intestinal cytotoxic CD8⁺ T cells was observed at 1 dpi (2719 ± 313 , Fig 6C), and at 3 dpi and 21 dpi numbers of intestinal cytotoxic CD8⁺ T cells tended to be higher in SE-infected

compared to uninfected chickens (Fig 6C). In the spleen, numbers of $\gamma\delta$ T cells tended to be decreased at 1 dpi but increased at 3 dpi in SE-infected compared to uninfected chickens (Fig 6D). Numbers of splenic cytotoxic CD8⁺ T cells were similar between uninfected and SE-infected chickens during the course of infection (Fig 6E). Next, $\gamma\delta$ T cells and cytotoxic $\alpha\beta$ T cells were analyzed for their CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ expression (S4A Fig). Numbers of intestinal CD8 $\alpha\alpha$ ⁺ $\gamma\delta$ T cells (S4B Fig) were significantly enhanced at 1 dpi (1084±133) and 21 dpi (694±240), and CD8 $\alpha\beta$ ⁺ $\gamma\delta$ T cell numbers (S4C Fig) tended to increase at those time points in SE-infected compared to uninfected chickens. Similarly, numbers of intestinal cytotoxic CD8 $\alpha\alpha$ ⁺ T cells (S4D Fig) were significantly enhanced at 1 dpi (1550±242) and tended to be higher at 21 dpi. The cytotoxic CD8 $\alpha\beta$ ⁺ T cell numbers (S4E Fig) showed trends to increase at those time points in SE-infected compared to uninfected chickens. In the spleen, CD8 $\alpha\alpha$ ⁺ $\gamma\delta$ T cell numbers (S4F Fig) were higher at 14 dpi (6537±1476), whereas numbers of CD8 $\alpha\beta$ ⁺ $\gamma\delta$ T cells (S4G Fig) were similar in SE-infected versus uninfected chickens. Numbers of splenic cytotoxic CD8 $\alpha\alpha$ ⁺ (S4H Fig) and CD8 $\alpha\beta$ ⁺ (S4I Fig) T cells as well helper CD4⁺ T cells (S5 Fig) were similar between uninfected and SE-infected chickens during the course of infection. Finally, no significant differences were observed in T cell activation, determined by CD107 and IFN γ expression, in the intestine and spleen between uninfected and SE-infected chickens (S6 Fig). Trends to increase were observed at 3 dpi of CD107 expression by intestinal and splenic CD8⁺ T cells (comprising both $\gamma\delta$ and $\alpha\beta$ TCRs, S6A-B Fig respectively), and IFN γ production by splenic CD4⁺ T cells (S6D Fig) in SE-infected compared to uninfected chickens. IFN γ production by T cell subsets in the intestine could not be determined due to too low cell numbers.

SE-specific proliferation of T cells, isolated from spleen at 21 dpi, was determined ex vivo (Fig 7A). Increased proliferation of SE-specific CD4⁺ as well as CD8⁺ T cells isolated from SE-

infected chickens was observed. This proliferation was antigen dose-dependent, whereas T cells from uninfected chickens did not proliferate upon exposure to inactivated SE (Fig 7B-C).

High SE-specific antibody responses were found in all SE-infected chickens after three weeks of infection

The presence of SE-specific antibodies was determined in serum before and after infection in uninfected and SE-infected chickens. In SE-infected chickens, SE-specific antibodies were first detected at 7 dpi, when two out of five chickens showed low antibody titers, that increased in course of time (Fig 8). At 14 dpi SE-specific antibodies were observed in all SE-infected chickens although two chickens showed only low titers. At 21 dpi all SE-infected chickens showed high SE-specific antibody responses with average titers of 455 ± 133 . SE-specific antibodies were not found in sera of the uninfected chickens (Fig 8).

Discussion

In the current study we aimed to provide a detailed analysis of SE-related innate and adaptive immune responses in young broiler chickens up to four weeks of age, to better understand how the immune response contributes to the elimination of infection in course of time. For this purpose, the presence and function of NK cells, various types of APCs and T cells in ileum, as the present infection site and in spleen, as indication of systemic dissemination of SE, were investigated, as well as SE-specific antibody responses in serum, another systemic dissemination indicator. Seven-day-old broiler chickens were successfully infected with SE as was demonstrated by the presence of bacteria in ileum and spleen and the detection of SE-specific T cell proliferation and antibodies. From 1 dpi onwards, increased numbers and activation of innate immune cells as well as numbers of T cells were observed in the intestine and spleen of SE-infected compared to uninfected chickens. From 21 dpi onwards, enhanced T

cell numbers in intestine and antibody titers in serum were observed in SE-infected compared to uninfected chickens, resulting in the reduction of SE.

Although SE was demonstrated in ileum and spleen of infected chickens, the presence of the bacteria did not affect growth nor induced severe disease symptoms similar to previous studies in layer chickens [13,34] and young broiler chickens [32,52]. The absence of severe disease symptoms is related to the SE-dose, which was chosen to avoid welfare issues in the chickens.

Other studies reported the presence of SE for a longer period in the caecum [32,33], suggesting that SE may prefer colonization in the caecum rather than the ileum.

The enhanced activation of intestinal and splenic NK cells upon SE infection, represented by enhanced CD107 expression and IFN γ production, is in agreement with other studies in chickens showing upregulated mRNA levels of intestinal IFN γ [13] and cytotoxicity-related NK cell genes [12] in young chickens. Our observations are also supported by studies in humans and mice, which reported increased cytotoxicity [14], IFN γ production [16] and CD107 expression [15] of intestinal and systemic NK cells after *Salmonella enterica* serotype Typhimurium infection. The enhanced NK cell activation paralleled increased numbers of intestinal IL-2R α^+ and 20E5 $^+$ NK cells. Due to incompatibility of available reagents, we were not able to determine CD107 and IFN γ expression within the IL-2R α^+ and 20E5 $^+$ NK cell subsets. For that reason, we sorted IL-2R α^+ and 20E5 $^+$ NK cells to perform RT-qPCR and both NK cell subsets showed mRNA levels of *NFIL3*, *IL-7R α* and *PRFI* genes albeit to different degrees, suggesting that both may be implicated in cytokine production [41-43] as well as cytotoxic activity [43,46] in response to SE infection. The observation that both NK cell subsets are involved in cytotoxicity is confirmed by previous studies in chickens, in which both NK cell subsets in the intestine and spleen showed CD107 expression [7] and intestinal IL-2R α^+ NK cells exerted cytotoxicity [8]. In humans, the peripheral IL-2R α^+ NK cell population expanded and increased their cytotoxic activity after Toll-like receptor (TLR) stimulation [53].

As SE might activate NK cells directly through TLRs [53-55], more intestinal NK cells as well as enhanced activation, such as cytotoxicity and IFN γ production, may increase the resistance of chickens against SE infection. IFN γ has been reported to activate macrophages resulting in improved clearance of engulfed bacteria [56] and enhanced antigen presentation by APCs inducing T cell responses [57]. Although NK cell activation did not reduce SE numbers in the first week after infection, they may play an important role in the resistance to SE infection by influencing both the innate and adaptive immune cells.

The systemic spread of SE infection as observed, coincided with an increased presence of CD11⁺ MRC1LB⁺ macrophages in the spleen at 7 dpi when bacterial counts were highest. Previous studies have shown that these CD11⁺ MRC1LB⁺ macrophages are largely present in peri-ellipsoid lymphocyte sheaths of the spleen [58], and have a role in clearing blood-borne bacteria in chickens [59], equivalent to that of the marginal zone macrophages in mammals [60]. Therefore, these macrophages are suggested to be involved in clearing the SE from 7 dpi onwards. The FSC^{high} DCs tended to be present in increasing numbers and showed increased expression of CHIR-AB1 and MHC-II at 7 dpi in SE-infected chickens compared to uninfected chickens, indicating a role in antigen presentation. The FSC^{low} DCs did not increase in numbers but showed an increased expression of the activation markers CHIR-AB1, CD40 and CD80 in SE-infected chickens. The two DC subsets were highly similar, and might comprise DCs at different stages of maturation with the FSC^{low} subset being more mature based on the expression of CD40, CD80 and MHCII [22,49,61]. These results suggests that the increased presence of macrophages clear bacteria initially and the increased activation of DC subsets contribute to antigen presentation to stimulate the adaptive immune responses, all together resulting in further reduction of SE in infected chickens.

Whereas the APC subsets are likely to contribute to the clearance of the bacteria, it has also been suggested that they may worsen the impact of infection by acting as a carrier for

Salmonellae [23] and contribute to systemic dissemination [28], since this bacterium is able to survive intracellularly in chicken macrophages [26,27] and DCs [61]. The ability of *Salmonellae* to inhibit activation of APCs might explain why NK cells showed earlier activation than APCs and the high presence of SE found at 7 dpi in our study. Other studies have demonstrated that *Salmonella*-infected APCs secrete IL-12/IL-18 resulting in enhanced expression of the early activation marker IL-2R α^+ on NK cells, thereby inducing their activation by increased cytotoxicity and IFN γ production [15]. This IFN γ production can subsequently stimulate additional macrophages to clear phagocytosed bacteria [62] or impair intracellular survival by direct killing of infected-macrophages [15], which might be involved in the reduction of SE towards and after 7 dpi observed in our study.

T cell presence and SE-specific T cell proliferation and antibodies were addressed as well. All infected chickens in our study had circulating antibodies specific for SE after three weeks, which was in agreement with other studies [32,33]. The observed T cell responses are similar to increased numbers of intestinal and splenic $\gamma\delta$ and cytotoxic $\alpha\beta$ T cells in response to SE early and three weeks after infection [13,17,18], as well as to increased CD8 $\alpha\alpha^+$ $\gamma\delta$ T cell numbers [29,63] in prior studies in chickens. The increase in cytotoxic CD8 $\alpha\alpha^+$ T cell numbers, however, has not been shown before in chickens in response to SE infection. The more innate-like nature of $\gamma\delta$ T cell responses early after infection have been recognized as well as the antigen-specific responses of cytotoxic CD8 $^+$ T cells approximately two weeks after infection, whereas the functional difference between CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ expression is less clear [64-67].

Although expression of CD107 and IFN γ production by intestinal and splenic $\gamma\delta$ T cells, cytotoxic CD8 $^+$ T cells, and splenic helper CD4 $^+$ T cells did not significantly differ between uninfected and SE-infected chickens, proliferation of SE-specific splenic T cells of SE-infected chickens ex vivo was observed three weeks after infection and not in uninfected chickens. The initial increased presence of T cells in our study did not result in reduction of SE, however, the

SE-specific T cells and antibodies in course of infection are suggested to reduce the number of SE.

We propose the following model of SE-induced immune responses. At the start of a SE infection, NK cells become activated either directly through TLRs or indirectly by interaction with activated macrophages that have engulfed SE through contact and cytokine production. Moreover, intestinal $\gamma\delta$ T cells and cytotoxic CD8⁺ T cells are involved in the first response controlling SE infection by cytokine production. Subsequently, NK cells kill infected cells directly by degranulation and indirectly by IFN γ production, which in turn stimulates macrophages to destroy engulfed bacteria. The destruction of bacteria by APCs results in antigens that can be presented to SE-specific CD4⁺ and CD8⁺ T cells, which differentiate into CD4⁺ helper T cells and CD8⁺ cytotoxic T cells, respectively. The CD4⁺ T helper cells stimulate the differentiation of SE-specific B cells into antibody-producing plasma cells and secrete cytokines leading to further stimulation of NK cells, APCs and cytotoxic CD8⁺ T cells, thereby creating a positive feedback loop, resulting in the reduction of SE and thereby elimination of the infection.

In conclusion, this study shows that *Salmonella enterica* serotype Enteritidis infection in young broiler chickens firstly increases local and systemic presence and activation of NK cells as well as local presence of T cells, followed by increased presence of macrophages and activation of DCs. Subsequently, specific T cell and antibody responses are induced, all together resulting in reduction of SE. These insights in understanding the response of innate and adaptive immune cells upon SE infection will aid in developing immune-modulation strategies to stimulate innate cells. The potential strengthening of immune responsiveness by vaccines or feed strategies during early life may increase resistance and may prevent SE infection and colonization in young broiler chickens, eventually increasing the food safety for humans.

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Figure Captions

Fig 1. The bacterial load in course of time after SE infection and its effect on growth and numbers of intestinal and splenic leukocytes. (A) *Salmonella enterica* serotype Enteritidis (CFU/g) in the IEL fraction of the ileum and in the (B) spleen of uninfected (uninf) and SE-infected chickens (SE-inf). (C) Bodyweights (g) of uninfected and SE-infected chickens in the

course of time. (D) Numbers (cells/mg) of leukocytes in IEL and (E) spleen of uninfected and SE-infected chickens in the course of time. Mean + SEM per treatment and time point is shown ($n = 5$) and statistical significance is indicated as ** $p < 0.01$ and **** $p < 0.0001$.

Fig 2. Effect of SE infection on numbers of intestinal NK cells in broiler chickens. (A) Gating strategy for CD3 negative cells expressing either IL-2R α or 20E5. (B) Numbers (cells/mg) of intestinal IL-2R α ⁺ and (C) 20E5⁺ NK cells, in uninfected (uninf) and SE-infected (SE-inf) chickens. Mean + SEM per treatment and time point is shown ($n = 5$).

Fig 3. NK cell activation in intestine and spleen of broiler chickens upon SE infection. (A) Gating strategy for NK cells expressing surface CD107 and intracellular IFN γ in the intestine (first and second panels) and spleen (third and fourth panels). (B) Percentages of intestinal NK cells expressing CD107 and (C) IFN γ in uninfected (uninf) and SE-infected (SE-inf) chickens in the course of time. (D) Percentages of splenic NK cells expressing CD107 and (E) IFN γ in uninfected and SE-infected chickens. Mean + SEM per treatment and time point is shown ($n = 5$), for uninfected chickens at 7 dpi in spleen $n = 4$. Statistical significance is indicated as *** $p < 0.001$ and **** $p < 0.0001$.

Fig 4. Phenotypic characterization of splenic APCs upon SE infection. (A) Splenocytes were gated for size, excluding debris (FSC-A vs SSC-A), singlets (FSC-A vs FSC-H) and viability (Live/Dead marker-negative) consecutively. (B) A t-SNE analysis was performed on spleen samples of 7 dpi uninfected (uninf, blue) and SE-infected (SE-inf, red) chickens combined. Based on the t-SNE analysis, two population (subset 1 and subset 2) were found enriched among the splenocytes of SE-infected chickens. (C) The populations were evaluated for expression of MRC1LB versus CD11. Subset 2 was evaluated for its FSC-A vs SSC-A scatter pattern and further subdivided into subset 2a and subset 2b. (D) Subset 1 (CD11⁺

MRC1LB⁺), subset 2a (CD11⁺ MRC1LB⁻ FSC^{low}) and subset 2b (CD11⁺ MRC1LB⁻ FSC^{high}) were sorted by FACS to assess gene expression of immune markers by RT-qPCR relative to the total splenocyte population. (E) The presence and (F) numbers of macrophages in uninfected and SE-infected chickens were assessed over time. Mean + SEM per treatment and time point is shown ($n = 5$), for uninfected chickens at 0 dpi $n = 3$ and at 7 dpi $n = 4$. Statistical significance is indicated as ** $p < 0.01$.

Fig 5. Activation marker expression by splenic APC subsets upon SE infection. (A-C) Macrophages, FSC^{low} DCs and FSC^{high} DCs were assessed over time for CHIR-AB1, (D-F) CD40, (G-I) CD80 and (J-L) MHC-II expression in uninfected (uninf) and SE-infected (SE-inf) chickens. For CHIR-AB1, CD40 and CD80, the percentage of cells in each APC subset expressing the respective markers is shown, and for MHC-II the geometric mean fluorescent intensity (gMFI) of each subset, in accordance with the gating strategy depicted in S4 Fig. Mean + SEM per treatment and time point is shown ($n = 5$), for uninfected chickens at 0 dpi $n = 3$ and at 7 dpi $n = 4$. Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Fig 6. Numbers of intestinal and splenic T cells in broiler chickens upon SE infection. (A) Gating strategy for $\gamma\delta$ T cells and CD8⁺ $\alpha\beta$ T cells in intestine (first panel) and spleen (second panel). (B) Numbers (cells/mg) of intestinal $\gamma\delta$ T cells and (C) CD8⁺ $\alpha\beta$ T cells in uninfected (uninf) and SE-infected (SE-inf) chickens. (D) Numbers (cells/mg) of splenic $\gamma\delta$ T cells and (E) CD8⁺ $\alpha\beta$ T cells in uninfected and SE-infected chickens. Mean + SEM per treatment and time point is shown ($n = 5$), for uninfected chickens at 7 dpi in spleen $n = 4$. Statistical significance is indicated as ** $p < 0.01$.

Fig 7. Ex vivo proliferation of SE-specific splenic CD4⁺ and CD8⁺ T cells. (A) The gating strategy shows the consecutive selection for viable cells (Live/Dead marker-negative), single cells (FSC-A vs FSC-H), lymphocytes (FSC-A vs SSC-A) and CD3⁺ T cells. T cells were subdivided into CD4⁺ and CD8⁺ T cells. The final gating step selects on T cell subsets which have divided at least once based on dilution of the cell proliferation dye CellTrace Violet (CTV). (B) The percentage of cells that have proliferated is shown for splenic CD4⁺ and (C) CD8⁺ T cells after four days of stimulation with different doses of formaldehyde-inactivated SE expressed in CFU/well, none stimulated controls (-), or after stimulation with anti-CD3, anti-CD28 and recombinant chicken IL-2 (+). All splenocyte samples were stimulated and measured in triplicate for each of the conditions. Mean + SEM is shown; $n = 2$ for uninfected (uninf) and $n = 3$ for SE-infected (SE-inf) chickens.

Fig 8. Serum antibody titers in broiler chickens as a response to SE infection. Titers of SE-specific antibodies in sera of uninfected (uninf) and SE-infected (SE-inf) chickens in course of time. Mean + SEM per treatment and time point is shown ($n = 5$) and statistical significance is indicated as ** $p < 0.01$.

Supporting Information Captions

S1 Fig. Effect of SE infection on numbers of splenic NK cells in broiler chickens. (A) Gating strategy for CD3 negative cells expressing either IL-2R α or 20E5. (B) Numbers (cells/mg) of splenic IL-2R α ⁺ and (C) 20E5⁺ NK cells in uninfected (uninf) and SE-infected (SE-inf) chickens in the course of time. (D) Gene expression levels of NK cell lineage marker (NFIL3), IL-7R α and perforin 1 (PRF1) by RT-qPCR in sorted IL-2R α ⁺ and 20E5⁺ NK cell subsets. Mean + SEM per treatment and time point is shown ($n = 5$), for uninfected chickens at 7 dpi $n = 4$ and for gene expression levels $n = 1$.

S2 Fig. Phenotypic characterization of splenic APCs upon SE infection. (A-B) The presence and (C-D) numbers of FSC^{low} DCs and FSC^{high} DCs in uninfected (uninf) and SE-infected (SE-inf) chickens were assessed over time. Mean + SEM per treatment and time point is shown ($n = 5$), for uninfected chickens at 0 dpi $n = 3$ and at 7 dpi $n = 4$. Statistical significance is indicated as ** $p < 0.01$.

S3 Fig. The gating strategy used to determine the activation status of the APC subsets as depicted in Fig 5. The three identified splenic APC subsets (A) macrophages, (B) FSC^{low} DCs and (C) FSC^{high} DCs were assessed for CHIR-AB1, CD40, CD80 and MHC-II. For CHIR-AB1, CD40 and CD80, the cells expressing the respective markers were selected and expressed as a percentage. The expression of MHC-II by each subset was expressed as the geometric mean fluorescent intensity (gMFI).

S4 Fig. Numbers of intestinal and splenic $\gamma\delta$ T cells and cytotoxic T cells expressing either CD8 $\alpha\alpha$ or CD8 $\alpha\beta$ in broiler chickens upon SE infection. (A) Gating strategy for $\gamma\delta$ T cells and CD8⁺ $\alpha\beta$ T cells expressing either CD8 $\alpha\alpha$ or CD8 $\alpha\beta$ in intestine (first and second panels) and spleen (third and fourth panels). (B) Numbers (cells/mg) of intestinal CD8 $\alpha\alpha$ ⁺ $\gamma\delta$ T cells, (C) CD8 $\alpha\beta$ ⁺ $\gamma\delta$ T cells, (D) cytotoxic CD8 $\alpha\alpha$ ⁺ T cells and (E) CD8 $\alpha\beta$ ⁺ T cells in uninfected (uninf) and SE-infected (SE-inf) chickens in the course of time. (F) Numbers (cells/mg) of splenic CD8 $\alpha\alpha$ ⁺ $\gamma\delta$ T cells, (G) CD8 $\alpha\beta$ ⁺ $\gamma\delta$ T cells, (H) cytotoxic CD8 $\alpha\alpha$ ⁺ T cells and (I) CD8 $\alpha\beta$ ⁺ T cells in uninfected and SE-infected chickens. Mean + SEM per treatment and time point is shown ($n = 5$), for uninfected chickens at 1 dpi in intestine and spleen $n = 4$ due to numbers of events acquired in the gate of interest were < 100 , and at 7 dpi in spleen $n = 4$. Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$. *** $p < 0.001$.

954

955 **S5 Fig. Numbers of CD4⁺ T cells in the spleen of broiler chickens upon SE infection.**

956 Numbers (cells/mg) of splenic CD4⁺ αβ T cells in uninfected (uninf) and SE-infected (SE-inf)
957 chickens in the course of time. Mean + SEM per treatment and time point is shown ($n = 5$), for
958 uninfected chickens at 7 dpi $n = 4$.

959

960 **S6 Fig. T cell activation in the intestine and spleen of broiler chickens upon SE infection.**

961 (A) Percentages of intestinal CD8⁺ T cells expressing CD107 (including both γδ and αβ T cells)
962 in uninfected (uninf) and SE-infected (SE-inf) chickens in the course of time. (B) Percentages
963 of splenic CD8⁺ T cells expressing CD107 (including both γδ and αβ T cells), (C) CD8⁺ γδ T
964 cells expressing IFNγ, (D) CD4⁺ αβ T cells expressing IFNγ and (E) CD8⁺ αβ T cells expressing
965 IFNγ in uninfected (uninf) and SE-infected (SE-inf) chickens over time. Mean + SEM per
966 treatment and time point is shown ($n = 5$), for uninfected chickens at 7 dpi in spleen $n = 4$ and
967 at 1 and 3 dpi in intestine percentages were not determined (n.d.) due to numbers of events
968 acquired in the gate of interest were < 100.

A **B** **C** **D** **E**

Figure

A**Intestine***in CD3⁺ cells***20E5** **IL-2R α** **B****IL-2R α ⁺** **C****20E5⁺** **Figure**

Figure

Figure

Figure

A

B

C

D

E

Figure

A **B** **C**

Figure

SE-specific Abs

Figure