

## Research Article

**Loss of  $\alpha\beta$  but not  $\gamma\delta$  T cells in chickens causes a severe phenotype**

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The availability of genetically modified mice has facilitated the study of mammalian T cells. No model has yet been developed to study these cells in chickens, an important livestock species with a high availability of  $\gamma\delta$  T cells. To investigate the role of  $\gamma\delta$  and  $\alpha\beta$  T cell populations in birds, we generated chickens lacking these T cell populations. This was achieved by genomic deletion of the constant region of the T cell receptor  $\gamma$  or  $\beta$  chain, leading to a complete loss of either  $\gamma\delta$  or  $\alpha\beta$  T cells. Our results show that a deletion of  $\alpha\beta$  T cells but not  $\gamma\delta$  T cells resulted in a severe phenotype in KO chickens. The  $\alpha\beta$  T cell KO chickens exhibited granulomas associated with inflammation of the spleen and the proventriculus. Immunophenotyping of  $\alpha\beta$  T cell KO chickens revealed a significant increase in monocytes and expectedly the absence of CD4<sup>+</sup> T cells including FoxP3<sup>+</sup> regulatory T cells. Surprisingly there was no increase of  $\gamma\delta$  T cells. In addition, we observed a significant decrease in immunoglobulins, B lymphocytes, and changes in the bursa morphology. Our data reveal the consequences of T cell knockouts in chickens and provide new insights into their function in vertebrates.

**Keywords:** Adaptive immune system · Chicken · CRISPR/Cas9 · Genetically modified animals · T cells



Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Introduction**

T lymphocytes can recognize a variety of peptides to facilitate the humoral and cytotoxic immune responses and are greatly involved in variable processes including inflammatory and autoimmune diseases. They are characterized by their heterodimeric T cell receptor (TCR), which consists of a constant and a variable region with either  $\alpha\beta$  or  $\gamma\delta$  chains. Therefore, T lymphocytes are divided into two major subgroups, the  $\alpha\beta$  and  $\gamma\delta$  T cells

[1]. The role of these cells has been largely studied in human and mammalian animal models including mice, which harbor a comparatively low percentage of peripheral blood  $\gamma\delta$  T cells [2]. In contrast, chickens harbor a high percentage of  $\gamma\delta$  T cells, which makes them an intriguing research model.

$\gamma\delta$  T cells in chickens are known for their cytotoxic activity [3], and high availability in the peripheral epithelial tissue [4]. The function of  $\alpha\beta$  T cells is defined by the two co-receptors CD4 and CD8. The CD4<sup>+</sup>  $\alpha\beta$  T cells are activated through antigens bound

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to MHC II molecules, leading to a humoral immune response against pathogens [5]. Regulatory T cells ( $T_{\text{regs}}$ ), typically expressing  $CD4^+CD25^+$  and the transcription factor  $FoxP3^+$  [6], play an important role in maintaining immune tolerance [7].  $CD8^+ \alpha\beta$  T cells recognize peptides presented by MHC I molecules [8] and show cytotoxic activity [5]. Similar to other species, chicken  $CD8^+$  T cells have two isoforms, composed of a  $CD8\alpha\alpha$  homodimer or a  $CD8\alpha\beta$  heterodimer. The  $TCR1^+$  cells can further be divided into  $CD8^{\text{high}}$ ,  $CD8^{\text{dim}}$ , and  $CD8^{\text{neg}}$  populations, which were shown to respond differently to pathogens such as salmonella [9].

The establishment of a reverse genetic system in chickens allowed the generation of a knockout (KO) of the B cell receptor (BCR) using the genetic modification of primordial germ cells (PGCs) [10, 11]. Not only the use of PGCs but also the availability of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 technology has facilitated the generation of genetically edited chickens and simplified the gene editing process [12]. To define specific T cell populations' functions, such as  $\alpha\beta$  and  $\gamma\delta$  T cells, genetically modified chicken lines lacking these populations are pivotal.

Due to the unavailability of genetically modified avian models, early research was based on thymectomy combined with monoclonal antibody injections to investigate chicken T cell biology, which can be associated with significant alterations of the immune system [13]. Using this method, it was not possible to define T cell functions since the frequency of epithelium-associated  $\gamma\delta$  T cells (TCR1) as well as  $V\beta1^+ \alpha\beta$  T cells (TCR2) cells were not affected [13]. More recently, the generation of RAG1 KO chickens led to the loss of both B and T cell populations [14] which helped in understanding the role of this gene in chicken lymphocyte development, but it did not allow a separate study of distinct immune cell populations.

In this study, we successfully generated both  $TCR C\gamma^{-/-}$  and  $TCR C\beta^{-/-}$  chickens, lacking  $\gamma\delta$  or  $\alpha\beta$  T cells, respectively.  $TCR C\beta^{-/-}$  chickens exhibited a severe phenotype that resulted in inflammation in the spleen, proventriculus, and skin associated with structural deterioration of the thymus and bursa of Fabricius as early as 14 days after hatch, while no adverse phenotype was observed in  $TCR C\gamma^{-/-}$  chickens.

## Results

### Deletion of the T cell receptor $\beta$ and $\gamma$ constant chains

Chickens lacking either  $\gamma\delta$  or  $\alpha\beta$  T cells were generated by targeting the  $\gamma$  or respectively  $\beta$  constant region of the corresponding TCR. CRISPR/Cas9 mediated homology-directed repair was used in chicken PGCs to replace the constant region with a selectable marker cassette (Fig. 1A,B). Precise targeting was confirmed by an allele-specific KO PCR and correctly targeted clonal PGC lines were used to generate  $TCR C\gamma^{+/-}$  (germline transmission rate = 5.3%) and  $TCR C\beta^{+/-}$  (germline transmission rate = 1.5%) animals (Supporting information Fig. S1A). To remove the selectable marker cassette, PGCs were re-derived from these lines and elec-

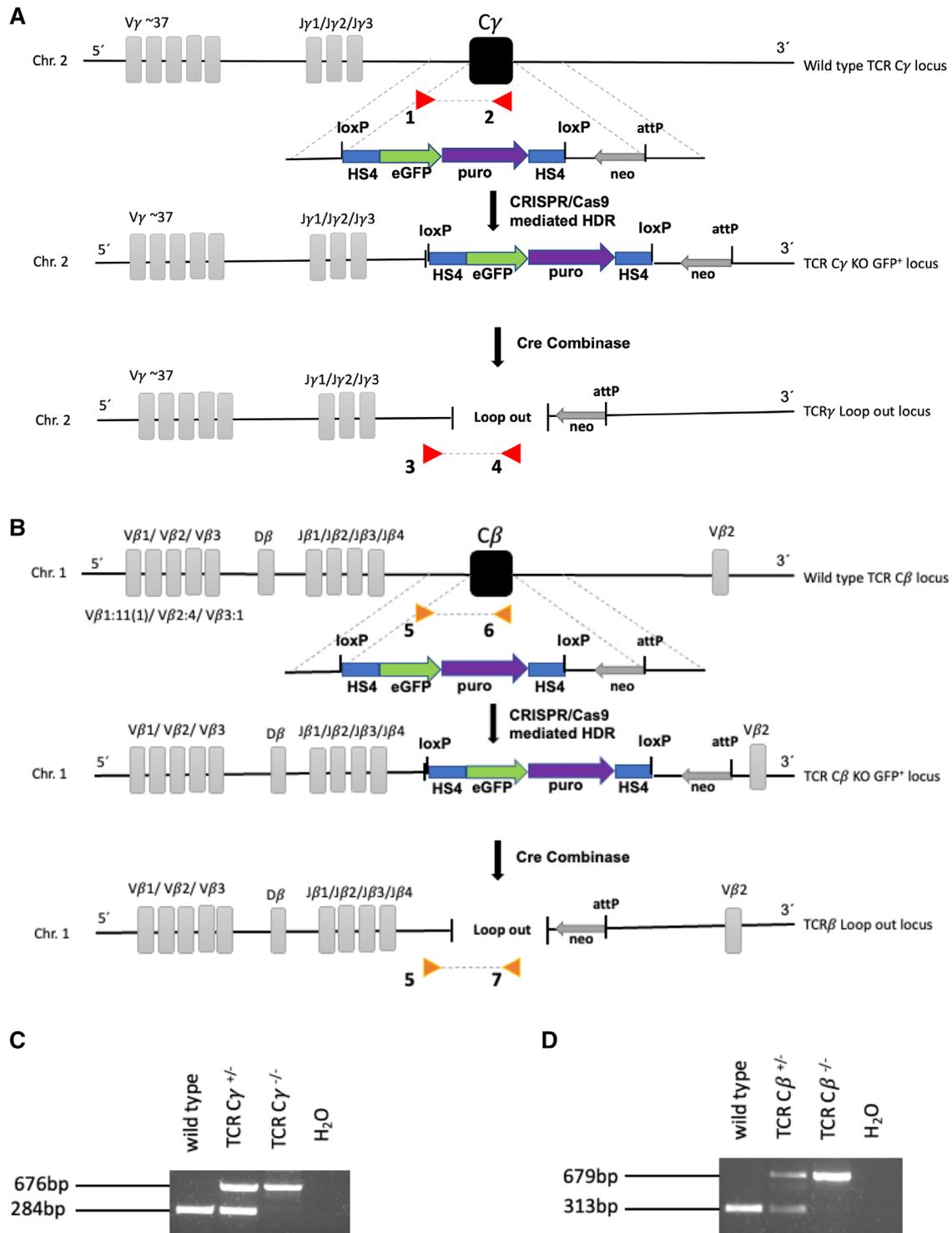
troporated with Cre recombinase to remove the selectable marker cassette. eGFP<sup>neg</sup> PGCs were sorted by FACS and injected into embryos to finally generate  $TCR C\gamma^{+/-}$  (germline transmission rate = 9%) and  $TCR C\beta^{+/-}$  (germline transmission rate = 5.9%) chickens that do not express eGFP (Fig. 1A,B; Supporting information Fig. S1A). Knockout and WT allele-specific PCRs were established to detect  $TCR C\gamma^{-/-}$  and  $TCR C\beta^{-/-}$  chickens (Fig. 1C,D).

### TCR $C\gamma^{-/-}$ and TCR $C\beta^{-/-}$ chickens lack either $\gamma\delta$ or $\alpha\beta$ T cells

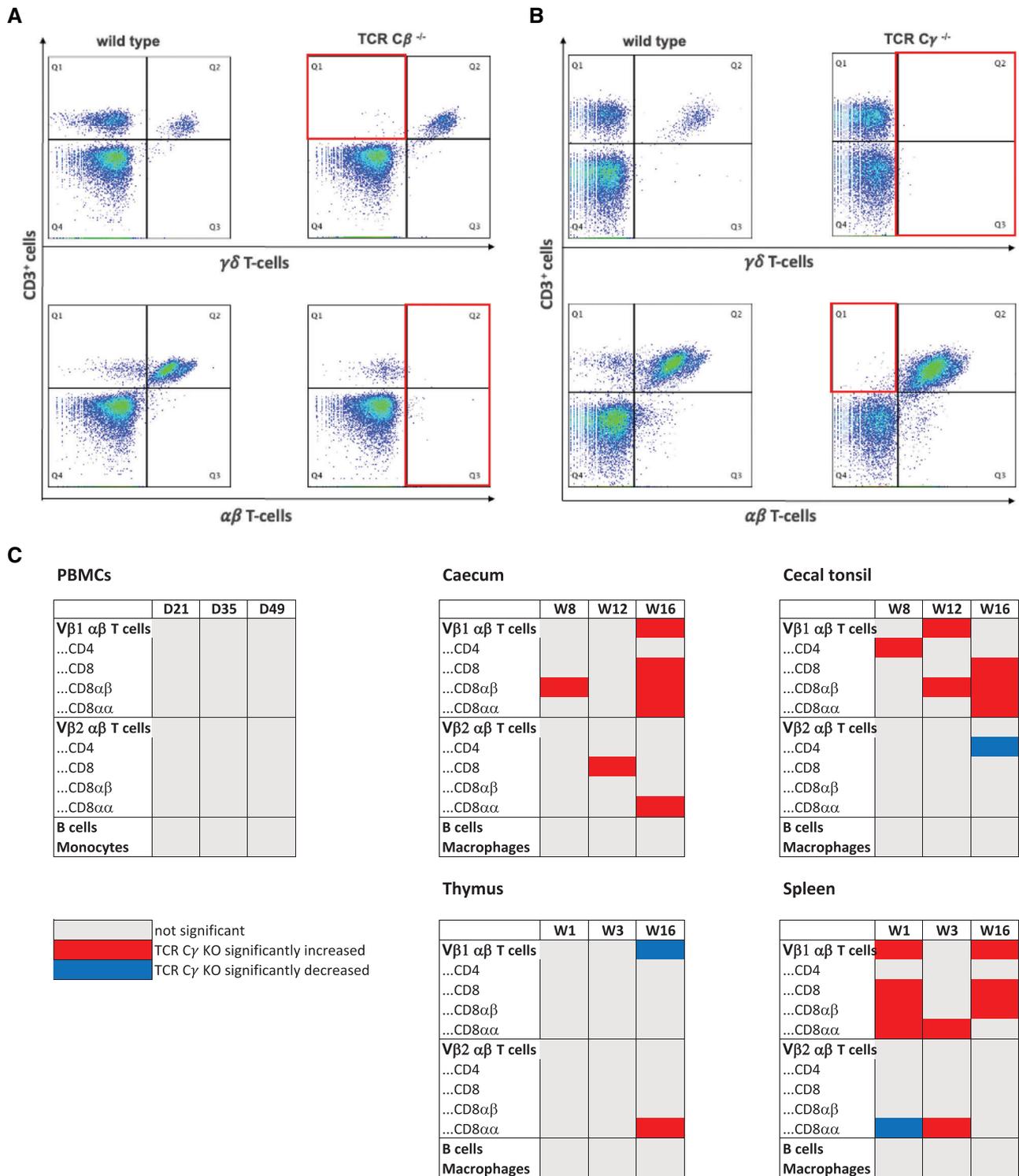
The immunophenotype of the generated T cell KO chicken was analyzed via flow cytometry.  $TCR C\gamma^{-/-}$  chickens showed a complete depletion of  $\gamma\delta$  T cells and  $TCR C\beta^{-/-}$  chickens a complete depletion of  $\alpha\beta$  T cells. Simultaneous staining for the pan T cell antigen CD3 confirmed the successful KO and revealed that no uncharacterized T cell population exists in chickens (Fig. 2A,B). While  $TCR C\gamma^{-/-}$  chickens are phenotypically similar to WT animals,  $TCR C\beta^{-/-}$  chickens develop a severe phenotype as early as two weeks post-hatch. Macroscopic lesions like epithelial granulomas at the comb, beak, and legs associated with inflammation in the mucosal surface of the proventriculus and the spleen (Supporting information Fig. S2; Fig. 4A,B) were seen. Biopsy samples from the spleen of 2-week-old  $TCR C\beta^{-/-}$  animals and WT animals were cultivated for bacterial detection. No bacterial growth was observed, confirming that the phenotype was not associated with culturable bacterial invasion (data not shown). Neither  $TCR C\gamma^{-/-}$  nor  $TCR C\beta^{-/-}$  chickens showed impaired weights compared with their WT siblings (Supporting information Fig. S1B).

### The absence of $\gamma\delta$ T cells is compensated through $CD8^+ \alpha\beta$ T cells in the gut

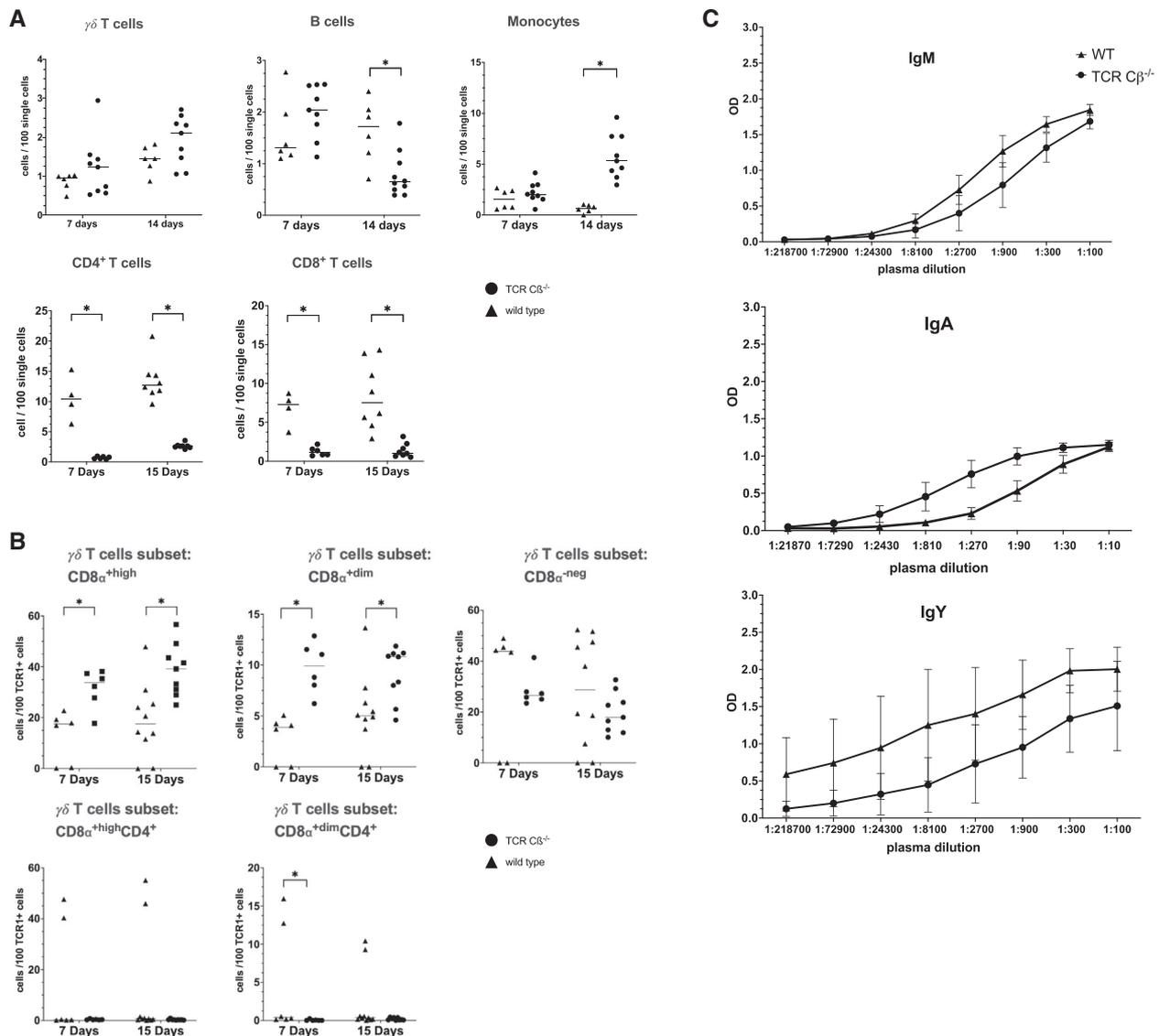
Analysis of peripheral blood mononuclear cells (PBMCs) in  $TCR C\gamma^{-/-}$  chickens showed, that the loss of  $\gamma\delta$  T cells does not influence other blood circulating T cell subpopulations, B cells, or monocytes between 21- and 49 days posthatch. However, a significantly higher number of  $V\beta1^+ \alpha\beta$  T cells was found in the spleen after one and 16 weeks ( $p < 0.05$ ). Within these, the  $CD8^+$  T cells in both isotypes  $CD8\alpha\beta^+$  and  $CD8\alpha\alpha^+$  were increased. In the caecum, a significantly higher number of  $CD8\alpha\beta^+ V\beta2^+ \alpha\beta$  T cells was found eight weeks posthatch, and a significantly higher number of  $CD8^+ V\beta1^+ \alpha\beta$  T cells was found after 16 weeks ( $p < 0.05$ ). In the cecal tonsils, significantly more  $CD4^+ V\beta1^+ \alpha\beta$  T cells were detected ( $p < 0.05$ ) after eight weeks. However, after 12 weeks the  $CD8\alpha\beta^+ V\beta1^+ \alpha\beta$  T cells were increased ( $p < 0.05$ ), and after 16 weeks both isotypes  $CD8\alpha\alpha^+$  and  $CD8\alpha\beta^+$  from  $V\beta1^+ \alpha\beta$  T cells were increased ( $p < 0.05$ ). In the thymus, the  $V\beta1^+ \alpha\beta$  T cells were decreased ( $p < 0.05$ ), and the  $CD8\alpha\alpha^+ V\beta2^+ \alpha\beta$  T cells were increased ( $p < 0.05$ ) after 16 weeks (Fig. 2B, Supporting information Fig. SF6). The intestinal integrity was examined by morphometric analysis of histological sections including the length of the tunica muscularis, crypts, and villi in the duodenum, jejunum,



**Figure 1.** Generation of the TCR  $C\beta^{-/-}$  and TCR  $C\gamma^{-/-}$  KO animals. (A) The establishment of a  $\gamma\delta$  T cell-deficient chicken line was achieved by targeting the constant region of the TCR  $\gamma$  chain on chromosome 2. The targeting vector contained a selectable marker cassette (eGFP + puromycin resistance gene) to allow the selection of successfully targeted cells. Cre-mediated recombination resulted in the removal of the selectable markers. (B) The establishment of a  $\alpha\beta$  T cell-deficient chicken line was achieved by targeting the constant region of the TCR  $\beta$  chain on chromosome 1. The same targeting strategy as shown for the TCR  $\gamma$  chain was used. (C) Genotyping of TCR  $C\gamma^{-/-}$  chicken by multiplex PCR. WT allele-specific primers (#1 and #2) with an amplicon length of 284bp and TCR  $C\gamma$  KO-specific primers (#3 and #4) with an amplicon length of 676bp were used. (D) Genotyping of TCR  $C\beta^{-/-}$  chicken by multiplex PCR. WT allele-specific primers (#5 and #6) with an amplicon length of 313bp and TCR  $C\beta$  KO-specific primers (#5 and #7) with an amplicon length of 679bp were used.



**Figure 2.** Immunophenotyping TCR C $\beta^{-/-}$  and TCR C $\gamma^{-/-}$  KO animals (A) One representative flow cytometry dot plot of WT against TCR C $\beta^{-/-}$  (day 14;  $n = 10$ ) and (B) WT against TCR C $\gamma^{-/-}$  KO chickens (day 24;  $n = 3$ ) is shown. PBMCs were stained for the T cell marker CD3 and  $\gamma\delta$  T cells (TCR1) or  $\alpha\beta$  T cells (TCR2 + TCR3). (C) Analysis of TCR2 subsets, TCR3 subsets, B cells, and macrophages at day (D) 7, 21, 35, and 49 from PBMCs and mononuclear cells isolated from thymus, and spleen, at week (W) 1, 3, and 16 and from caecum, and cecal tonsils at week (W) 8, 12, 16 of TCR C $\gamma^{-/-}$  KO chickens ( $n = 4$ ). \* $p < 0.05$ .



**Figure 3.** Immunophenotyping TCR C $\beta$ <sup>-/-</sup> and WT animals (A) Flow cytometric analysis of PBMCs of TCR C $\beta$ <sup>-/-</sup> ( $n \geq 6$ ) and WT ( $n \geq 4$ ) chickens at seven and 14 days of age. (B) CD8<sup>+</sup> cells within the  $\gamma\delta$  T cell (TCR1) subset were analyzed based on their level of CD8 expression and co-expression of CD4 from TCR C $\beta$ <sup>-/-</sup> ( $n \geq 6$ ) and WT ( $n \geq 4$ ) chickens at seven and 15 days of age. (C) Total plasma IgM, IgY, and IgA immunoglobulin levels of TCR C $\beta$ <sup>-/-</sup> ( $n = 10$ ) and WT ( $n = 12$ ) chickens at 14 days of age were measured by ELISA. The mean and SD of  $n \geq 4$  are shown. \* $p < 0.05$ .

ileum, and caecum of TCR C $\gamma$ <sup>-/-</sup> animals compared with WT birds. No significant differences were found between both groups ( $p > 0.05$ ; Supporting information Fig. S5).

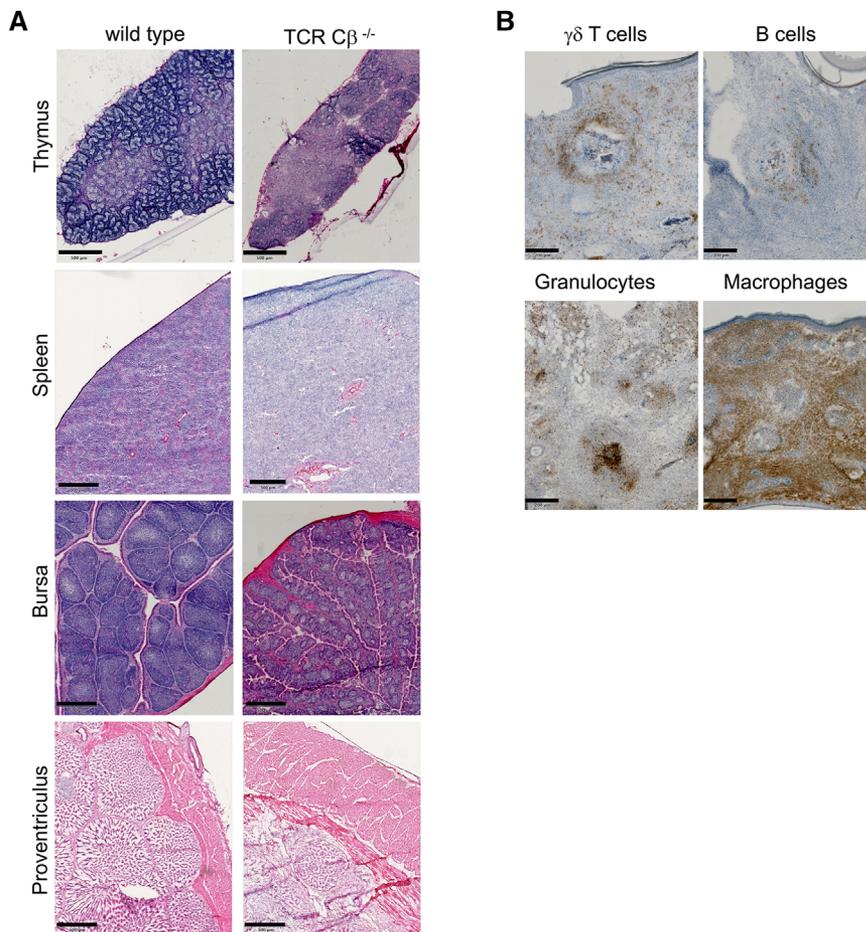
### TCR C $\beta$ <sup>-/-</sup> chickens show an increase in monocytes and a decrease in B cells

In the TCR C $\beta$ <sup>-/-</sup> chicken no significant increase in the  $\gamma\delta$  T cells was detected. 14 days after hatch significantly fewer B cells, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells were detected ( $p < 0.05$ ) along with significantly increased numbers of monocytes of TCR C $\beta$ <sup>-/-</sup> chicken ( $p < 0.05$ ) (Fig. 3A). The number of blood circulating  $\gamma\delta$  T cells was significantly lower in double positive CD8<sup>dim</sup> CD4<sup>+</sup> cells,

1 week after hatch ( $p < 0.05$ ). On the other side, levels of both CD8<sup>high</sup> and CD8<sup>dim</sup> cells within the  $\gamma\delta$  T cells were significantly higher after seven- and 15-days post-hatch (Fig. 3B). Additionally, immunoglobulin levels in the plasma of 14-day-old TCR C $\beta$ <sup>-/-</sup> chickens were decreased compared with WT chickens while no differences between TCR C $\gamma$ <sup>-/-</sup> and WT chickens were observed (Fig. 3C, Supporting information Fig. S1C).

### TCR C $\beta$ <sup>-/-</sup> KO impacts the development of lymphatic organs

The thymus in WT chickens showed a clear separation in the cortex and medulla. No such separation was found in TCR C $\beta$ <sup>-/-</sup>



**Figure 4.** Histological analysis of TCR C $\beta^{-/-}$  chicken (A) Sections of the thymus, spleen, bursa, and proventriculus of 14-day-old WT and TCR C $\beta^{-/-}$  chicken have been prepared and stained with H&E. (B) Skin granulomas of 14-day-old TCR C $\beta^{-/-}$  chicken have been dissected and stained for  $\gamma\delta$  T cells (TCR1), B cells (BU1/AV20), granulocytes (GRL1) and macrophages (Kul01). Always one representative picture of at least three different animals per genotype is shown. Scale bar (A) thymus, spleen, bursa = 500  $\mu\text{m}$ ; proventriculus = 100  $\mu\text{m}$  (B) = 200  $\mu\text{m}$ .

chickens (Fig. 4A). The spleen and proventriculus of TCR C $\beta^{-/-}$  chickens showed inflammatory lesions (Fig. 4A). Surprisingly, a strong phenotype was observed in the bursa of Fabricius in TCR C $\beta^{-/-}$  chickens, where B cell follicles appeared underdeveloped in comparison to WT siblings (Fig. 4A). Immunohistochemistry of the epithelial granulomas 14 days after hatch showed infiltration of the surrounding tissue by macrophages and a central granulocyte accumulation, surrounded with B cells and  $\gamma\delta$  T cells (Fig. 4B). Splenic immunofluorescence staining of B cells, CD4<sup>+</sup>, CD8<sup>+</sup>,  $\gamma\delta$  T cells revealed the absence of germinal center formations and CD4<sup>+</sup> T cells in TCR C $\beta^{-/-}$  animals, which was not the case in WT birds (Supporting information Fig. S3A). In addition, TCR C $\beta^{-/-}$  chickens exhibited a random splenic distribution of B cells and a few CD8<sup>+</sup> T cells, while typical white pulp formation of B cells surrounded by CD8<sup>+</sup> T cells was found in WT animals (Supporting information Fig. S3B).  $\gamma\delta$  T cells did not colocalize with the B cells in the spleen of TCR C $\beta^{-/-}$  or WT chickens (Supporting information Fig. S3C).

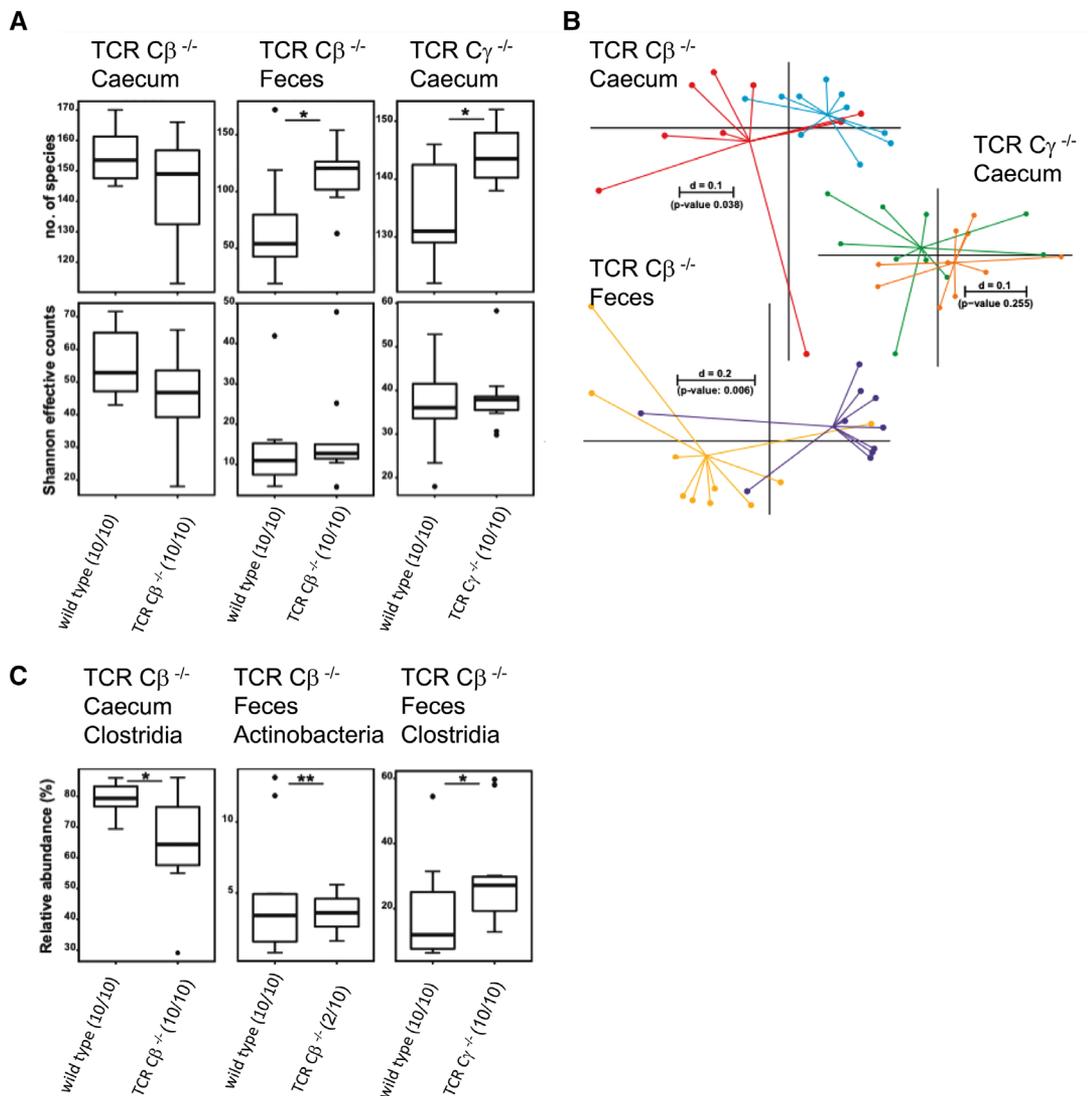
### The gut microbiome of TCR C $\beta^{-/-}$ chickens show a significantly different beta diversity

To analyze the influence of T cells on the chicken gut microbiome, 16S RNA sequencing of feces and caecum content was performed.

Significant differences in the number of species were found in the feces of 14-day-old TCR C $\beta^{-/-}$  chickens as well as in the caecum of 35-day-old TCR C $\gamma^{-/-}$  chickens ( $p < 0.05$ ). There were no differences in the Shannon effective counts ( $p > 0.05$ ). Analyzing the beta diversity of both fecal and cecal samples of TCR C $\beta^{-/-}$  chickens demonstrated significantly different populations compared with WT chickens ( $p < 0.05$ ). In the caecum content of 14-day-old TCR C $\beta^{-/-}$  chickens, significantly fewer bacteria of the class Clostridia were found, while in the feces, significantly fewer Actinobacteria and significantly more Clostridia were detected at the class level ( $p < 0.05$ ; Fig. 5).

### FoxP3<sup>+</sup> expression of T<sub>reg</sub> cells is significantly lower in TCR C $\beta^{-/-}$ KO chicken

The expression of several immune-related genes was compared between WT, TCR C $\beta^{-/-}$  and TCR C $\gamma^{-/-}$  chickens, 14 days after hatch. Significantly lower TGF $\beta$  and IL-5 expression was found in the spleen ( $p < 0.05$ ), while significantly higher IL-6 levels were detected in the thymus of TCR C $\beta^{-/-}$  animals ( $p < 0.05$ ). In addition, a significantly increased IL-1 $\beta$  expression was detected in PBMCs of TCR C $\beta^{-/-}$  animals ( $p < 0.05$ ). IL-22 expression was significantly lower in the spleen of TCR C $\beta^{-/-}$  chickens, whereas it was significantly higher in the spleen of TCR C $\gamma^{-/-}$  chickens ( $p$



**Figure 5.** The gut microbiome of TCR Cβ<sup>-/-</sup> and TCR Cγ<sup>-/-</sup> chickens were compared with WT chickens by 16S rRNA gene amplicon sequencing. Caecum and feces samples were taken 14 days posthatch from TCR Cβ<sup>-/-</sup> animals and caecum samples were taken from TCR Cγ<sup>-/-</sup> and WT animals 35 days posthatch. (A) The number of species and the Shannon effective count are displayed as well as (B) the β-diversity based on generalized UniFrac distances and (C) significant differences in relative abundance for Clostridia (class) in caecum and feces of TCR Cβ<sup>-/-</sup> and for Actinobacteria (class) in feces of TCR Cβ<sup>-/-</sup>. For statistical analysis of group separation (B), PERMANOVA was used. Bar plots (A+C) were analyzed with the Wilcoxon rank sum pairwise test. Mean and SD are shown. \**p* < 0.05.

< 0.05). Following the significantly decreased CD4<sup>+</sup> T cell population FoxP3 expression was significantly lower in the spleen, thymus, and PBMCs in TCR Cβ<sup>-/-</sup> animals (*p* < 0.05; Supporting information Fig. S4).

## Discussion

T cells play a crucial role in regulating various physiological functions and providing defense against pathogens in humans and other mammals [1]. However, the role of each subpopulation in chickens remains unclear due to the lack of investigative tools such as genetically engineered chickens in the past. This study was carried out to characterize the functions of different T cell subpopulations

by generating chickens that lack γδ T cells (TCR Cγ<sup>-/-</sup>) and those that lack αβ T cells (TCR Cβ<sup>-/-</sup>). The successful generation and detailed characterization of these lines showed that the loss of γδ T cells in TCR Cγ<sup>-/-</sup> chickens were asymptomatic, while the TCR Cβ<sup>-/-</sup> chickens exhibited a severe phenotype.

It is widely believed that γδ T cells play a central role in mediating various functions of the immune system in chickens since they harbor a high number of γδ T cells with up to 50% in blood [2]. Surprisingly, the TCR Cγ<sup>-/-</sup> chickens did not display major phenotypic differences compared with WT chickens. In mice, a significant population of γδ T cells is present in the intestine and plays an important role in maintaining intestinal integrity [15]. Fujihashi et al. [16] showed that γδ T cells, as a major population of intraepithelial lymphocytes, might influence epithelial cell growth

and function. Contrary to expectations no differences were recorded in the morphometry of the intestine in the TCR  $C\gamma^{-/-}$  compared with WT chickens. It is well established that the majority of  $\gamma\delta$  T cells in chickens are  $CD8^+$  T cells [17]. In TCR  $C\gamma^{-/-}$  chickens, the  $CD8^+$   $\alpha\beta$  T cell population is significantly increased in the caecum, spleen, and cecal tonsils. Concluding that this  $CD8^+$   $\alpha\beta$  T cell population compensates for the absence of the  $\gamma\delta$  T cells in the gut and therefore, maintains intestinal integrity in TCR  $C\gamma^{-/-}$  chickens. This phenomenon is also supported by the findings of Sandrock et al. [18], who reported that  $\gamma\delta$  T cell-deficient mice show mild phenotypes and that other lymphocytes take over the functions of  $\gamma\delta$  T cells in the absence of these cells.

The TCR  $C\beta^{-/-}$  chicken developed a pathological phenotype around two weeks of age. To determine if the observed phenotype is caused by a microbial infection, the spleens of 14-day-old TCR  $C\beta^{-/-}$  and WT chickens were cultured to check for bacterial growth, but no bacteria were detected after a week of cultivation. Even though the chickens are kept under SPF conditions, it is still open to investigate if the set-off causing the severe phenotype appears through an infectious agent. Our findings of this study indicate that the severe phenotype of the TCR  $C\beta^{-/-}$  results in an imbalance of humoral and cytotoxic responses through the loss of  $CD4^+$  T cells and therewith  $T_{reg}$  cells. This phenotype was not in agreement with Cihak et al. [13] who reported depletion of T cell subsets after thymectomy and antibody injections without a pronounced phenotype. The method employed by Cihak et al. [13] was deemed inefficient, as the combination of TCR2 antibody treatment and thymectomy led to an increase of TCR3<sup>+</sup> cells, thus demonstrating the inability to effectively eliminate T cells. Later research showed, that mice with TCR $\alpha^{-/-}$  KO also develop an inflammation of the stomach [17]. Likewise, SCID mice [19] and RAG1 deficient chicken show similar phenotypes to the TCR  $C\beta^{-/-}$  chickens. Even though Rag-1 deficient chickens it is suggested that the severe phenotype appears through the loss of both B and T cells [14], in SCID mice it is suggested that TCR  $\beta$  gene rearrangement is obligatory for T cell development [20]. However, we showed in this study, that the phenotype in TCR  $C\beta^{-/-}$  chickens appear only because of the loss of  $\alpha\beta$  T cells. Also, chickens lacking B cells [10] and TCR  $C\gamma^{-/-}$  chickens do not show this phenotype, underlining that only the missing  $\alpha\beta$  T cells are leading to the severe phenotype in TCR  $C\beta^{-/-}$  chicken.

The significant decrease in  $CD4^+$  T cells in the PBMCs of TCR  $C\beta^{-/-}$  chicken is one of the key findings to explain the severe pathology of this phenotype.  $CD4^+$  T cells secrete anti-inflammatory cytokines to coordinate the functions of other immune cells, primarily B cells, macrophages, and cytotoxic T cells [8, 21]. This helps to prevent an overactive immune response that can result in autoimmune diseases [22].

Indeed, the TCR  $C\beta^{-/-}$  chickens showed a reduction in the expression of IL-4, and IL-5, a significant decrease of B cells, and a concurrent reduction of IgY, IgM, and IgA compared with WT chickens. These observations may be attributed to the absence of  $CD4^+$  T cells, as the secretion of IL-4 and IL-5 controls the activation and proliferation of B cells and therefore the production of immunoglobulins [23].

The lower IL-22 mRNA expression especially in the spleen of TCR  $C\beta^{-/-}$  chickens compared with WT chickens can be explained by the fact that  $CD4^+$  T cells are a major source of IL-22 in chickens [24]. IL-22 plays a critical role in enhancing the innate immunity of tissues and facilitating repair and healing mechanisms during inflammation, which is essential for restoring tissue homeostasis and preventing autoimmune diseases [25]. This could also contribute to the phenotype observed in TCR  $C\beta^{-/-}$  chickens.

Moreover, the significantly lower expression of FoxP3 and TGF $\beta$  in TCR  $C\beta^{-/-}$  chickens matches the absence of  $CD4^+$  T cells including the loss of  $T_{reg}$  cells. However, since these results are based on expression data, they need to be further proofed by additional experiments such as adoptive transfer of  $CD4^+$  ab T cells in the TCR  $C\beta^{-/-}$  chicken. IL-10 and TGF $\beta$  secreted by  $T_{reg}$  cells have anti-inflammatory and immunosuppressive effects, inhibiting the activation and function of  $CD8^+$  T cells and monocytes, which are involved in the cytotoxic response [26]. In the TCR  $C\beta^{-/-}$  chicken we see a high cytotoxic activity of  $\gamma\delta$  T cells and macrophages.

In total fewer  $CD8^+$  T cells were found in the PBMCs of the TCR  $C\beta^{-/-}$  chickens. However, within the TCR1 subsets, both  $CD8^{+high}$  and  $CD8^{+dim}$  T cells were significantly increased. Whereas after infection with *Salmonella enterica*, the  $CD8^{+high}$  TCR1<sup>+</sup> population increases while the  $CD8^{+dim}$  TCR1<sup>+</sup> subset decreases [9]. In TCR  $C\beta^{-/-}$  chicken the majority of TCR1<sup>+</sup> T cells are  $CD8^+$  [26], leading to a disbalance of the cytotoxic and humoral response and therefore higher levels of both  $CD8^{+high}$  and  $CD8^{+dim}$  T cells. Interestingly, even though it has been described, that double-positive  $CD8^+CD4^+$  T cells are increasing in autoimmune diseases, for example, thyroiditis [27], in the TCR  $C\beta^{-/-}$  chickens fewer double-positive T cells were seen. It is still open to investigate whether this is related to the loss of  $CD4^+$  T cells in general. The increase in the monocyte population and the higher expression of T cell-related proinflammatory cytokines such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$  might be explained by the lower expression of TGF $\beta$  and FoxP3 in TCR  $C\beta^{-/-}$  chickens. The higher levels of monocytes in TCR  $C\beta^{-/-}$  chickens are associated with inflammation in the spleen and gut, and the infiltration of macrophages into epithelial tissue, leading to the formation of granulomas which occur when the immune system is unable to effectively eliminate persistent antigens [21].

In the TCR  $C\beta^{-/-}$  chicken line there is no division between the cortex and medulla of the thymus visible. During maturation, T cells migrate from the cortex into the medulla [28]. Whereas  $\alpha\beta$  T cells need several days to migrate,  $\gamma\delta$  T cells migrate much faster. This is why Bucy et al. [29] suggest that  $\gamma\delta$  T cells do not undergo the same selection process as  $\alpha\beta$  T cells. This underlines our findings indicating that the  $\gamma\delta$  T cells do not need the cortex for the maturation process. Also, we found that the bursa development is impacted by the KO because smaller B cell follicles are found in the TCR  $C\beta^{-/-}$  chickens. Whether this effect is caused by the loss of  $CD4^+$  T cells, needs to be further investigated. The avian spleen serves as the primary site for interaction between  $CD4^+$  T cells and B cells, where after antigenic stimulation, B cells give rise to germinal centers [26]. The presence of  $\alpha\beta$  T cells is necessary for this process, although reports of spontaneous

germinal center formation in TCR $\alpha^{-/-}$  mice have been documented [30]. Conversely, in TCR $\beta^{-/-}$  mice germinal center formation was absent [30], a phenomenon also observed in the spleens of TCR C $\beta^{-/-}$  chickens in the current study. However, after a repeated parasitic infection, germinal center formation of B cells and  $\gamma\delta$  T cells and increased immunoglobulin levels were found in TCR $\beta^{-/-}$  mice [31]. For chickens, no comparable study has been performed yet.

The gut-associated lymphatic tissue is an important part of the chicken immune system. It has been shown that leukocytes can be regulated by the microbiome of the chicken [32]. Herewith it was shown that also the missing  $\alpha\beta$  T lymphocytes can influence the microbiome, as we found significant differences in the microbiome of TCR C $\beta^{-/-}$  chicken compared with their WT siblings. In TCR $\alpha^{-/-}$  mice it was shown, that only specific pathogen-free housed KO mice develop colitis, but not germ-free mice or TCR  $\beta^{-/-}$  mice, concluding that a microbial agent activates the immune system to cause a spontaneous autoimmune reaction [17]. This can also explain the inflammation in the stomach of the TCR C $\beta^{-/-}$  chicken.

The results showed that the knockout of  $\gamma\delta$  T cells does not result in a pronounced phenotype, whereas the knockout of  $\alpha\beta$  T cells leads to a severe phenotype including granulomas on the comb, leg, and beak, inflammations of the spleen and the proventriculus, and impaired B cell function and immunoglobulin production due to the loss of CD4<sup>+</sup> T cells including T<sub>reg</sub> cells. These findings highlight the crucial role of  $\alpha\beta$  T cells in regulating the immune response and demonstrate their importance in the chicken immune system. These genetically modified chickens will serve as a tool to study the nature and function of T cell subpopulations in detail by performing various infection experiments. Understanding the distinct functions of  $\gamma\delta$  and  $\alpha\beta$  T cells in chickens will help to improve our knowledge of the chicken's immune system and to develop new strategies for controlling diseases in chickens by targeting specific components of the immune system. Additionally, these genetically modified chicken lines provide a model for understanding the functions of T cells in other species, including humans, which can deepen our understanding of the evolution of the immune system and its role in protecting against pathogens.

## Materials and methods

### Animals

White Leghorn (Lohmann selected White Leghorn, Lohmann-Tierzucht GmbH) chickens were used. Animal experiments were approved by the government of Upper Bavaria, Germany (ROB-55.2-2532.Vet\_02-17-101 & 55.2-1-54-2532-104-2015). Experiments were performed according to the German Welfare Act and the European Union Normative for Care and Use of Experimental Animals. All animals received a commercial standard diet and water ad libitum. Genetically modified animals were generated as

previously described [10]. Shortly, PGCs, with the desired genetic modification, were injected into the vasculature of 65-year-old embryos transferred into a turkey surrogate eggshell, and incubated until the hatch of chimeric roosters. Upon sexual maturity, sperm was collected for DNA isolation and genotyping. The germline-positive roosters were bred with WT hens, first to obtain heterozygous animals, and then siblings were bred together to obtain homozygous animals. All animals were housed under SPF conditions. In this study, only homozygous and WT animals were included since heterozygous animals showed no difference compared with WT siblings.

### Genotyping assays

For the TCR C $\beta^{-/-}$  chickens' primers were designed to detect the TCR C $\beta^{-/-}$  KO: forward: 5' GGTTGCGAAATGACCGACCAAGC 3'; reverse: 5' GGCTTGACACTCAGCTCTATAG 3'. A second primer pair was used to detect the TCR C $\beta$  WT allele: forward: 5' GGTTGCGAAATGACCGACCAAGC 3'; reverse: 5' CACACCATTACCTTCCAGAC 3'. FIREPol Multiplex DNA Polymerase Mastermix (Solis Biodyne) was used according to the manufacturer's instructions with an annealing temperature of T<sub>m</sub> 58°C.

For the TCR C $\gamma^{-/-}$  chickens' primers were designed to detect the TCR C $\gamma^{-/-}$  KO: forward: 5' GCCATTCCTATTCCTATCCTAAGT 3'; reverse: 5' GGTTGCGAAATGACCGACCAAGC 3'. A second primer pair was used to detect the WT constant region of the TCR  $\gamma$  chain: forward: 5' GAGCTCCAGCCATGAAACCATAG 3'; reverse: 5' GTTGTCAGTGTCACTGGCTG 3'. FIREPol Multiplex DNA Polymerase Mastermix (Solis Biodyne) was used according to the manufacturer's instructions with an annealing temperature of T<sub>m</sub> 60°C.

### Flow cytometry

PBMCs were isolated using histopaque density gradient centrifugation (Sigma).  $1 \times 10^6$  cells were used per sample and washed with 1% BSA in PBS + 0.01% Na<sub>3</sub>N. Cells were first washed and then stained with primary antibodies (Supporting information Table S1) for 20 min in the dark on ice. Subsequently, cells were washed and incubated with secondary antibodies (Supporting information Table S1) for 20 min in the dark on ice. Thereafter cells were again washed and analyzed using an AttuneNXT flow cytometer (LifeTechnologies). Data were analyzed with FlowJo 10.8.1 software (FlowJo). For the FACS analysis of organs, the organ was strained through a 100  $\mu$ m cell strainer into a falcon holding 5 mL PBS. The single-cell solution was then further processed as described above.

### Enzyme linked immunosorbant assay

ELISA was performed as described before [11]. Used antibodies are listed in Supporting information Table S4. OD was

measured at 450 nm with FluoStar Omega (Version 5.70 R2 BMG LABTECH).

## Histology

The tissue was frozen at  $-80^{\circ}\text{C}$  in O.C.T. Tissue Tek Compound (Thermo Fisher Scientific). Before sectioning the tissue was stored overnight at  $-20^{\circ}\text{C}$ . About 7–8  $\mu\text{m}$  sections were prepared. For H&E Histology sections were stained with Mayer's hematoxyline (Medité) followed by Eosin (Medité). For mounting Vecta-Mount Express Mounting Medium (Biozol) was used. Slides were scanned using a Precipoint Microscope. (Version 1.0.0.9628 PreciPoint)

For immunohistology, sections were stained with the antibodies listed in Supporting information Table S2. Antibodies were detected using the Vectastain ABC Peroxidase Kit (Biozol) followed by the Vector DAB Kit (Biozol). Sections were counterstained with Mayer's hematoxyline (Medité). For mounting Vecta-Mount Express Mounting Medium (Biozol) was used. Slides were scanned using Precipoint Microscope. (Version 1.0.0.9628). For fluorescence histology, sections were stained with the following antibodies shown in Supporting information Table S3. Cell nuclei were stained with 0,01% DAPI (Applichem). Vectashield Mounting Medium (Biozol) was used to mount the stained sections. Images were taken using an ECHO Revolve Microscope. (Revolve Software Version 4.0.5 Discover Echo Inc.)

## RNA extraction

Bursa, spleen, and thymus were collected 14 days posthatch. Samples were frozen in RNAlater (Sigma) at  $-20^{\circ}$  until further processing. After defrosting samples were rinsed with PBS and homogenized using a SpeedMill Homogenisator (Analytik Jena) for  $6 \times 60$  s. Thereafter the samples were processed according to the manufacturer's protocol for ReliaPrep RNA Tissue Miniprep System (Promega). PBMCs were isolated using histopaque density gradient centrifugation (Sigma). Further processing was according to the Manufacturer Protocol using ReliaPrep RNA Cell Miniprep System (Promega). RNA integrity was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Only RNA with a RIN  $\geq 7.5$  was used for downstream analysis.

## cDNA Synthesis

Using Go Script cDNA Kit (Promega) RNA samples were processed according to the manufacturer's protocol.

## Quantitative RT-PCR

Primers (Supporting information Table S5) were adapted from literature or designed using Benchling. Promega GoTaq qRT-PCR kit (Promega) was used according to the manufacturer's instructions.

Expression was measured using QuantStudio5 (QuantStudio™ Design&analysis Software v1.5.2, Thermo Fisher Scientific). Samples were measured in duplicates.

## Morphometry

Sections from the duodenum, jejunum, ileum, and caecum were taken from TCR  $\text{C}\gamma^{-/-}$  ( $n \geq 3$ ) and WT chickens ( $n \geq 5$ ) 35 days after hatch and frozen in O.C.T. Tissue Tek Compound (Thermo Fisher) at  $-80^{\circ}\text{C}$ . Before cutting, the tissue was stored overnight at  $-20^{\circ}\text{C}$ . About 7–8  $\mu\text{m}$  sections were prepared. H&E Stain was performed as described above. Measurements were done with the Precipoint Microscope (Version 1.0.0.9628 PreciPoint). From each slide, the longest villus and the corresponding crypt were measured, and for the Tunica muscularis four sites were measured. At least three sections per organ and animal were prepared.

## Microbiome analysis

Feces were taken from 14-day-old TCR  $\text{C}\beta^{-/-}$  chicken and WT chickens. The feces were collected using a sterile spoon attached to a fecal tube. Feces samples were stored in 1 mL of Stool Stabilizer (Invitex). Caecum content was taken from TCR  $\text{C}\beta^{-/-}$  animals after 14 days and from TCR  $\text{C}\gamma^{-/-}$  animals after 35 days both compared with WT. Caecum content was flushed into a tube holding 1 mL Stool Stabilizer (Invitex). From all samples, DNA was extracted and processed as previously described [33].

## Bacterial cultivation of spleen tissue

Spleen tissue was transferred and streaked on Columbia Blood Agar (44g/L Columbia Blood Agar Base, 5% defibrinated sheep blood after autoclaving), Trypticase Soy Broth Agar (30g/L Trypticase Soy Broth, 15g/L Agar) and Gifu Anaerobic Medium modified (41.7 g/L GAM Broth, 15 g/L Agar), and incubated at  $37^{\circ}\text{C}$  for 72 h under anaerobic (DonWhitley Anaerobic workstation A95 with 10%  $\text{CO}_2$ , 4.5%  $\text{H}_2$ , and 85.5%  $\text{N}_2$ ) microaerophilic (Biomerieux AnaeroPack™ in and anaerobic jar) and aerobic conditions. After 72 h, agar plates were examined for the occurrence of bacterial colonies.

## Statistical analysis

Statistical analyses were performed using SPSS statistics software (version 28.0.1.1. IBM). Normally distributed data (Shapiro-Wilk test  $p < 0.05$ ) were analyzed by student's *T*-test and Mann-Whitney *U* test was applied for not normally distributed data. All *p*-values  $< 0.05$  were marked as significant. Graphs were designed with GraphPad Prism (Version 9.3.1. Dotmatics). Statistics of the microbiome analysis were performed using the Wilcoxon rank-sum pairwise test and PERMANOVA.

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**Author contributions:** Theresa von Heyl performed and analyzed the experiments and wrote the paper. Benjamin Schusser supervised the work and wrote the paper. Hicham Sid and Christian Zenner performed experiments and wrote the paper. Mohammed Alhussien planned experiments and wrote the paper. Romina Klinger, Dorothea Aumann, Antonina Schlickerrieder, Kamila Lengyel, Hanna-Kaisa Vikkula, and Teresa Mittermair performed experiments. All authors approved the submitted version.

**Ethics approval statement:** All animal work was conducted according to relevant national and international guidelines for the humane use of animals. Animal experiments were approved by the government of Upper Bavaria, Germany (ROB-55.2-2532.Vet\_02-17-101 & 55.2-1-54-2532-104-2015).

**Data availability statement:** The data that supports the findings of this study are available from the corresponding author upon reasonable request. Raw data for the microbiome analysis are available on SRA (accession number: PRJNA934268).

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**Abbreviations:** **bp**: basepairs · **BSA**: albumin bovine serum · **CD**: cluster of differentiation · **CRISPR**: Clustered Regularly Interspaced Short Palindromic Repeats · **DAB**: diaminobenzidine · **DNA**: deoxyribonucleic acid · **GC**: germinal center · **IgA**: immunoglobulin A · **IgM**: immunoglobulin M · **IgY**: immunoglobulin Y · **HRP**: horseradish peroxidase · **mAb**: monoclonal antibody · **MHC**: histocompatibility Complex · **PBMC**: peripheral blood mononuclear cells · **PGC**: primordial germ cell · **Scid**: Severe combined immunodeficiency · **TCR**: T cell receptor · **TMB**: tetramethylbenzidine · **T<sub>regs</sub>**: regulatory T cells

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