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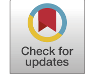

Characterization of a novel chicken γδ TCR-specific marker

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ABSTRACT

Chickens are a species with a high number of γδ T cells in various tissues. Despite their abundance, γδ T cells are poorly characterized in chickens, partially due to a lack of specific reagents to characterize these cells. Up until now, the TCR1 clone has been the only γδ T cell-specific monoclonal antibody (mAb) in chickens and additional reagents for γδ T cell subsets are needed. In order to address this issue, new mAb were generated in our laboratory by immunizing mice with *in vitro* cultured γδ T cells. In an initial flow cytometric screen a new mAb, clone "8D2", displayed an interesting staining pattern that mirrored γδ TCR up- and down-regulation in the γδ T cell line D4 over time, prompting us to characterize this antibody further. We compared the expression of the unknown 8D2 epitope in combination with TCR1 staining across various primary cells. In splenocytes, peripheral blood lymphocytes and intestinal epithelial cells, 8D2 consistently labeled a subset of $TCR1^+$ cells. To determine, whether specific γδ T cell receptors were recognized by 8D2, we sorted γδ T cells according to their 8D2 and TCR1 expression and analyzed their TCR V(D)J gene usage by TCR profiling. Strikingly, sorted $8D2^+$ cells preferentially expressed Vγ3 genes, whereas the TCR Vγ genes used by $TCR1^+$ $8D2^-$ cells were more variable. γδ TCR in $8D2^+$ cells were most frequently comprised of gamma chain VJ genes TRGV3-8 and TRGJ3, and delta chain VDJ genes TRDV1-2, TRDD2, TRDJ1. To confirm binding of 8D2 to specific γδ TCR, the preferentially utilized combination of TRG and TRD was expressed in HEK293 cells in combination with CD3, demonstrating surface binding of the 8D2 mAb to this Vγ3 γδ TCR-expressing cell line. Conversely, HEK293 cells expressing either Vγ1 or Vγ2 TCR did not react with 8D2. In conclusion, 8D2 is a novel tool for identifying specific Vγ3 bearing γδ T cells.

1. Introduction

T lymphocytes are an essential part of the adaptive immune system in both mammals and birds. They can be divided into αβ T cells and γδ T cells based on the expression of the heterodimeric membrane-bound T cell receptor (TCR) that is comprised of either an alpha (TCRα) and a beta (TCRβ) chain, or a gamma (TCRγ) and a delta (TCRδ) chain, respectively (Davis and Bjorkman, 1988; Chen et al., 1996). Each TCR chain consists of a constant and a variable region, with the highly diverse variable regions mainly formed by somatic DNA recombination of the V (variable), D (diversity; only for TCRβ and TCRδ) and J (joining) genes in combination with additional mechanisms that further increase junctional and combinatorial diversity (Tonegawa, 1983; Schatz and Swanson, 2011). In chickens, the TCRγ VJ genes are located on chromosome 2 (Six et al., 1996; Liu et al., 2020) and the TCRβ cluster of VDJ genes on chromosome 1 (Liu et al., 2020; Zhang et al., 2020). The TCRα/δ V(D)J genes form a hybrid cluster on chromosome 27, with the TCRδ genes located between the V and J genes of the alpha chain. In this locus, gene rearrangement can occur between Vα and Dδ and Jδ genes, leading to increased combinatorial diversity (Göbel et al., 1994; Kubota et al., 1999; Liu et al., 2020). Furthermore, in chickens, a second TCRδ locus was identified on chromosome 10 that only consists of a single set of VDJC genes (Parra et al., 2012).

The tissue distribution and phenotypes of various T cell subsets have been well characterized in humans and mice (Kumar et al., 2018). Additionally, there is considerable knowledge about chicken T cells (Chen et al., 1994). The number of γδ T cells varies significantly between different species. While human, mouse and rat are among the species with comparatively few γδ T cells in the peripheral blood (Hayday, 2000; Shekhar et al., 2012), chickens (Sowder et al., 1988), along with goats (Caro et al., 1998), cattle, sheep (Mackay and Hein, 1989; McClure et al., 1989) and pigs (Piriou-Guzylack and Salmon, 2008), are classified as γδ high species, characterized by a γδ T cell frequency of more than 10% in peripheral blood. In chickens, the frequency of γδ T cells can reach up to 50% of all T cells in the peripheral blood (Cihak et al., 1993). Both age and sex appear to have an impact on the frequency of γδ T cells in chickens. For example, an androgen-induced increase in the number of γδ T cells in the peripheral blood was observed in male chickens between 3 and 6 months of age (Arstila and Lassila, 1993). The distribution of T cell subsets in peripheral tissues also varies in chickens. While αβ T cells predominantly occur in the splenic periarteriolar sheath and the lamina propria of the intestine, γδ T cells are primarily located in the splenic sinusoids and the intestinal epithelium (Bucy et al., 1988).

The antigen-discriminating function of αβ T cells is mediated by the

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TCR, which specifically recognizes cognate peptide bound to MHC molecules on antigen-presenting cells. While $CD4^{+}$ T helper αβ T cells bind peptides presented on MHC class II molecules, $CD8^{+}$ cytotoxic αβ T cells recognize peptide-MHC class I complexes. Both CD4 and CD8 coreceptors also exist in chickens (Chan et al., 1988) and they seem to be very similar to their mammalian counterparts (Chen et al., 1990; Cooper et al., 1991).

Compared to αβ T cells, the function of γδ T cells is less well characterized. γδ T cells have characteristics of innate and adaptive immune cells, therefore they likely contribute to both responses (Chien et al., 2014). In contrast to αβ T cells, γδ T cells are not restricted to antigens presented on MHC molecules. Instead, they often recognize specific non-peptide molecules. Human Vγ9Vδ2 T cells, for example, recognize phosphoantigens (Holoshitz et al., 1993; Constant et al., 1994; Tanaka et al., 1995; Sireci et al., 2001), but overall few γδ T cell ligands have been identified. In chickens, there is evidence that γδ T cells exhibit cytotoxic activity in an MHC-independent manner (Fenzl et al., 2017). γδ T cells also contributed to the immune responses against Marek's disease virus (MDV) by producing cytokines such as IFN-γ, IL-2, and IL-17, and exhibiting cytotoxic activity (Laursen et al., 2018; Matsuyama-Kato et al., 2022, 2023). In chickens infected with *Salmonella typhimurium* an expansion of CD8αα-positive γδ T cell subsets in different organs was observed (Berndt et al., 2006). In addition, the frequency of γδ T cells was increased in the peripheral blood after immunization against *Salmonella* (Berndt et al., 2006; Braukmann et al., 2015). Likewise, activated γδ T cells were detected in the peripheral blood of chickens immunized against Newcastle disease virus (NDV) (Dalgaard et al., 2010; Andersen et al., 2017). A large proportion of chicken splenic γδ T cells express CD8, while only a few γδ T cells in the blood are $CD8^{+}$ (Chen et al., 1988; Kasahara et al., 1993). CD8-expressing γδ T cells, similar to those found in chickens, have also been identified in various mammalian tissues. Approximately 50% of γδ T cells in the human spleen express CD8 on their surface, with up to 30% of γδ T cells in human blood also exhibiting CD8 expression (Bucy et al., 1989). Similarly, porcine γδ T cells are classified into $CD8^{+}$ and $CD8^{-}$ cells (Kim et al., 2021). Furthermore, $CD8^{+}$ γδ T cells have been detected in the murine intestinal epithelium (Goodman and Lefrançois, 1988).

In chickens, only few antibodies exist against cell surface antigens that are exclusively expressed on T cells. As in mammals, CD3 is found on all T cells (Chen et al., 1986), since it is mandatory for the surface expression of the TCR. In addition, three monoclonal antibodies (mAb) specific for the chicken TCR have been generated until now: Clone TCR1 is a specific marker for γδ T cells (Sowder et al., 1988) and clones TCR2 and TCR3 for αβ T cells (Chen et al., 1988, 1989; Cihak et al., 1988), with TCR2 specifically recognizing Vβ1 family TCR and TCR3 recognizing Vβ2 family TCR (Lahti et al., 1991; Chen et al., 1996).

The different subtypes and functions of γδ T cells in chickens and other species are poorly understood, and new tools to better characterize these cells are needed. Next-Generation Sequencing of T cell receptors has recently been adapted to chickens, allowing to characterize the clonal distribution of T cells in various tissues (Dixon et al., 2021; Zhang et al., 2021; Früh et al., 2024; Linti et al., 2024). Annotation of expressed TCR is based on the germline V(D)J genes. Our group recently published an updated annotation of the chicken TCR loci (Früh et al., 2024): The TCR γ locus contains 4 TRGV families (TRGV1-4) comprising 8, 27, 10 and 8 members, respectively. This locus also includes 3 TRGJ genes and 1 TRGC gene. The TCR δ locus contains 5 TRDV families (TRDV1-5) comprising 41, 3, 1, 9 and 3 genes, respectively, and 2 TRDD genes, 2 TRDJ genes and 1 TRDC gene.

In recent years, several chicken T cell receptor repertoire analyses have been performed and revealed new information about the expression of the different TRG and TRD families in various tissues (Six et al., 1996; Parra and Miller, 2012; Liu et al., 2020; Dixon et al., 2021; Zhang et al., 2021; Huang et al., 2023; Früh et al., 2024). A characteristic distribution of V(D)J gene usage was found for all chains, yet a tissue-specific expression bias of specific Vγ genes was not observed in chickens. This was in sharp contrast to human tissues, where distinct biases for specific γδ T cells were described in various tissues. In human peripheral blood, for example, the majority of γδ T cells expressed $V\gamma9V\delta2^{+}$, while in solid organs and mucosal tissues non-$V\gamma9V\delta2^{+}$ TCR were more frequently expressed (Fichtner et al., 2020). In the epidermis of mice $V\gamma5^{+}$-expressing T cells were predominant, while $V\gamma7^{+}$ T cells were primarily located in the intestinal epithelium and $V\gamma1^{+}$ T cells were circulating in the periphery (Fichtner et al., 2020). While TCR repertoire sequencing is a powerful tool to study T cells, it does not enable the analysis of the spatial distribution of γδ T cells in tissues or the isolation of live cells based on TCR expression. The goal of this study was to develop new antibodies for specific γδ T cell subsets.

To our knowledge up until now there were no mAb available that could discriminate chicken gamma delta T cells based on the expression of specific Vγ or Vδ families. Here we describe a novel mAb, designated 8D2, that specifically recognizes a γδ T cell subset. We provide evidence that this mAb is specific for Vγ3-expressing γδ TCR. This novel mAb will be an important tool to further characterize chicken γδ T cell subsets.

2. Materials & methods

2.1. Animals and ethics statement

Chicken line M11 (B^2/B^2) was supplied by S. Weigend (Federal Research Institute for Animal Health, Mariensee, Germany). Fertilized eggs either from S. Weigend or from our own breeding were incubated, hatched and kept under conventional conditions at the Institute for Animal Physiology, University of Munich. The chickens had unrestricted access to food and water. Organs required for the experiments were taken from male and female chickens between 3 and 8 months of age. The animal experiments were authorized by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany (55.2-1-54-2532.0-60-2015; June 2019).

2.2. Cell culture and cell preparation

The chicken γδ T cell line D4 was cultured in RPMI 1640 cell culture medium containing 8% fetal bovine serum (FBS) and 2% chicken serum (ChS) in an incubator with 5% CO_2 at 40 °C. This cell line was generated in our laboratory by retroviral transformation of primary γδ T cells as described before (Marmor et al., 1993) and expressed a TCR comprised of the following genes: TRGV3-5, TRGJ3, TRDV1-4 and TRDJ3. To obtain a single cell suspension of splenocytes, the whole organ was passed through a stainless-steel mesh followed by density gradient centrifugation using Ficoll Histopaque-1077 (Sigma-Aldrich GmbH). Slow-speed centrifugation of blood was performed to obtain peripheral blood lymphocytes (PBL) (Viertlboeck and Göbel, 2007). Briefly, heparinized blood was mixed 1:1 with PBS and centrifuged at 60×*g* for 15 min at room temperature. After centrifugation the buffy coat was collected with a Pasteur pipette. Intraepithelial lymphocytes (IEL) were isolated from the ileum as described before (Göbel, 2000) with minor modifications. Briefly, the intestinal lumen was rinsed with PBS several times using a syringe, filled with PBS containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT), then the lumen was closed with yarn and the tissue was incubated in prewarmed PBS for 30 min at 37 °C. After incubation the intraluminal PBS containing IEL was removed and washed with additional PBS. IEL were further separated by density gradient centrifugation using Ficoll Histopaque-1077 (Sigma-Aldrich GmbH).

2.3. Antibodies for flow cytometry and fluorescence-activated cell sorting

Single-cell suspensions were incubated with antibodies diluted in PBS with 0.1% bovine serum albumin (BSA) and 0.01% sodium azide for 20 min on ice in the dark. After each incubation, the cells were washed with 200 μl PBS containing 0.1% BSA and 0.01% sodium azide prior to

analysis. Live and dead cells were discriminated with the Fixable Viability Dye eFluor™ 506 or 780 (Invitrogen). Indirect single-color flow cytometry staining was performed by using 8D2 mAb (unlabeled, mouse IgG1) or anti-TCRγδ mAb (unlabeled, clone TCR1, mouse IgG1, SBA), followed by incubation with goat-anti-mouse IgG1-APC (SBA). 8D2 was generated in our laboratory by immunizing mice with splenocytes stimulated with IL-2 and IL-12 and generation of hybridomas (Huhle et al., 2017). For multicolor flow cytometry staining, cells were first incubated with 8D2, then labeled with goat-anti-mouse IgG1-APC conjugate, followed by blocking with normal mouse serum (Jackson, JIM-015-000-120) for 20 min on ice in the dark, and a final staining with directly conjugated monoclonal antibodies obtained from Southern Biotechnology Associates (SBA): anti-TCRγδ (conjugated with FITC or PE, clone TCR1, mouse IgG1) and anti-CD3 (conjugated with Pacific blue, clone CT3, mouse IgG1). Anti-CD8 (unlabeled, clone CT8, mouse IgG1) was obtained from SBA and labeled with a PerCPCy5.5 Conjugation Kit (Biorad) in our laboratory following the manufacturer's instructions. IEL were additionally stained with anti-CD45 (unlabeled, clone 16-6, mouse IgG2a) (Viertlboeck and Göbel, 2007) and goat-anti-mouse IgG2a-FITC (SBA) as a secondary antibody. The measurements were conducted using a FACS Canto II (BD) and Fluorescence-activated cell sorting was performed on a FACSAria III instrument (BD) with an 80 μm nozzle. The purity of the sort-purified cells was higher than 96%. FlowJo v10.9.0 Software (Becton, Dickinson and Company, 2023) was utilized for data analysis. The gating strategy and an overview of all staining reagents is provided in the supplementary material (Supplementary Fig. 1, Supplementary Table 1).

2.4. RNA isolation, cDNA synthesis and semi-nested PCRs

The samples were prepared for Next-Generation Sequencing (NGS), performed by Eurofins Genomics (Ebersberg), as described before (Mamedov et al., 2013; Migalska et al., 2018; Linti et al., 2024). Briefly, total RNA was extracted from sort-purified cells (Relia Prep Cell RNA Cell Miniprep System, Promega) using 4.5×10^6 of each $TCR1^+8D2^-$ and $TCR1^+8D2^+$ cells and 5×10^5 of $TCR1^-8D2^+$ cells. Quantity and quality of the isolated RNA were checked using a Bioanalyzer 2100 Expert and a NanoDrop ND-1000. Only samples with RIN values above 7.7 and 260/280 and 260/230 ratios greater than 1.99 were used. cDNA was synthesized with a SMARTScribe Reverse Transcriptase (Takara) using 50 ng RNA, reverse primers specific for gamma and delta C-regions and a Template Switch Oligonucleotide containing a Unique Molecular Identifier (UMI) (Linti et al., 2024). γδ TCR were further amplified in two semi-nested PCR using the Advantage2 Polymerase from Takara and gene-specific reverse primers (Linti et al., 2024). Amplicons were sequenced by Illumina sequencing at 2×300 bp and a read depth of 30817–73557 reads per amplicon.

2.5. Bioinformatic analysis

Bioinformatic analysis was performed as previously described (Früh et al., 2024). FASTQ files were received from Eurofins. Quality control was performed with FastQC 0.12.0 (Andrew, 2010) and the alignment and gene annotation with MiXCR 4.2.0 (Bolotin et al., 2015). Finally, the graphical representations were generated in R v4.3.3 with Immunarch 0.9.1 (Nazarov et al., 2023).

2.6. Stable transfection

To establish a stable HEK293 cell line expressing chicken CD3 and a specific chicken TCR, the pSBbi-GP vector (Kowarz et al., 2015) was used. In a first step the EGFP gene in the vector was exchanged with the chicken CD3γ/δ (NCBI accession number: M59925), CD3ε (NCBI accession number: Y08918) and CD3ζ (NCBI accession number: AJ002317) genes separated by 2a ribosome skipping elements. Next a synthetic gene containing Vγ3-8, Jγ3 and Vδ1-2, Dδ2, Jδ1 (Geneart) was introduced into the cloning site of the vector under the control of an EF1a promotor. Following sequencing of the entire plasmid to check sequence integrity, it was transfected into HEK293 cells using Metafectene Pro (Biontex). After 24 h of incubation at 37 °C with 5% CO_2, the transfected cells were selected with Puromycin at a concentration of 1 mg/ml. To confirm successful transfection, flow cytometry analysis with the CT3 mAb was performed. A graphical representation of the plasmid was created in SnapGene 5.2.4 (GSL Biotech LLC, Dotmatics) (Supplementary Fig. 2).

2.7. Statistical analysis

Statistical analysis was conducted using R software (v4.4.1). A negative binomial generalized linear model (GLM) was fitted, with counts of T cell receptor sequences as the response variable and the cell population as predictor. This analysis was performed using the glm.nb() function from the MASS package (v7.3-61) (Venables and Ripley, 2002). For post-hoc pairwise comparisons among levels of the predictors, the emmeans package (v1.10.3) was employed, applying Tukey adjustment for multiple comparisons (Lenth et al., 2024). Values with $p \leq 0.05$ were considered statistically significant. To assess the linear relationship between 8D2 mAb and either TCR1 or CD3 mAb the Pearson correlation coefficients were calculated with Microsoft Excel.

3. Results and discussion

Our goal was to develop new markers against γδ T cell surface antigens that could be used to further characterize γδ T cells in chickens. We initially produced new mAb by creating hybridomas from mice that were immunized with *in vitro* expanded and highly activated γδ T cells. From this experiment we obtained several interesting candidate mAb that stained γδ T cells by flow cytometry surface staining.

3.1. The staining pattern of the 8D2 mAb reveals specificity for the γδ TCR

We tested these mAb initially on the γδ T cell line D4. The TCR surface expression on this cell line varied over time in cell culture as determined by staining with anti-TCRγδ clone TCR1. This phenomenon is known from retrovirally transformed cells and is due to genomic instability. Interestingly, when staining of the new candidate mAb clone 8D2 was compared with that of TCR1, we observed that the frequencies of positive cells at different time points were very similar between both mAb. At 14 days 58.8% of the cells were $TCR1^+$, while 58.6% of the cells were $8D2^+$. After 21 days in culture the amount of the positive cells for both mAb was about 47% (Fig. 1). Double staining of the cell line with TCR1 and 8D2 mAb showed that the same cells were positive for both mAb (Supplementary Fig. 3).

To determine whether 8D2 staining also mirrored TCR1 staining in primary cells, we stained leukocytes isolated from spleen, blood and intestine with 8D2 and TCR1 in combination with additional γδ T cell markers anti-CD3 clone CT3 and anti-CD8 clone CT8 mAb (Fig. 2, Supplementary Fig. 4). Surprisingly, in contrast to the staining of D4 cells, the staining pattern of 8D2 was distinct from that of TCR1 on primary cells. While 8D2 predominantly stained $TCR1^+$ γδ T cells, it only bound to a fraction of $CD3^+$ $TCR1^+$ γδ T cells. Overall, only few $8D2^+$ cells were not bound by TCR1 (Fig. 2, left panel). The frequency of $8D2^+$ cells was highest in the spleen when compared to PBL and IEL, as was the percentage of $8D2^+CD8^+$ double positive cells. Only a small percentage of PBL and IEL expressed 8D2 with variable CD8 co-expression (Fig. 2, right panel).

Since γδ T cell frequencies generally vary significantly between individual chickens, we next determined whether the observed pattern was consistent across animals. Splenocytes, PBL and IEL from four different individual chickens were stained with a combination of TCR1

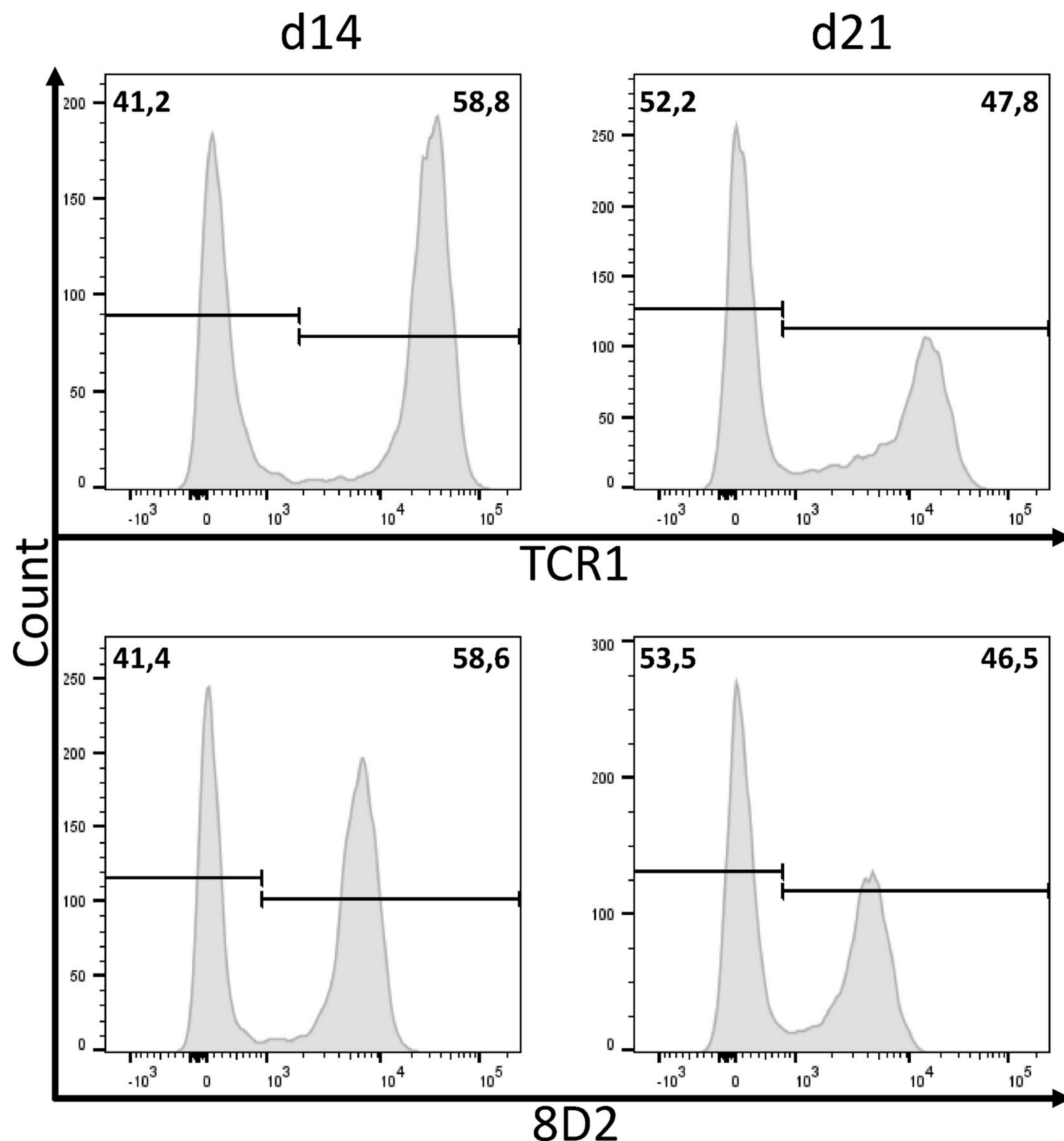

Fig. 1. Flow cytometry analysis of the γδ T cell line D4 reveals comparable reactivity with the 8D2 and TCR1 mAb.
Flow cytometry analysis of the γδ T cell line D4 stained with TCR1 (upper panel) and 8D2 mAb (lower panel) after 14 (left panel; d14) and 21 days (right panel; d21) in culture. Live single cells are shown with the percentage of cells indicated. Representative staining of n = 3 replicates.

and 8D2 mAb (Fig. 3). As expected, γδ T cell frequencies, as determined by TCR1 binding in these tissues, varied between 10% (animal 3, blood; Fig. 3) and 50% (animal 4, IEL; Fig. 3) of single live cells in splenocytes and PBL, and of CD45$^+$ single live cells in IEL. The frequency of 8D2$^+$ cells was consistently lower and ranged from ~2-3% (all animals, blood; Fig. 3) to 38% (animal 4, IEL; Fig. 3). The expression of the 8D2 epitope and the γδ TCR was positively correlated in all tissues where animals that had higher frequencies of TCR1$^+$ γδ T cells generally also exhibited increased frequencies of 8D2$^+$ cells, and vice versa (Fig. 3).

Together, these data indicated that both the TCR1 and 8D2 mAb may react with a cell surface molecule on the D4 cells that is either identical or co-regulated. Double staining of this Vγ3-type TCR cell line suggested that both mAb did not react with identical epitopes since no blocking effects could be observed (Supplementary Fig. 3). Overall, 8D2 only identified a subset of γδ T cells in splenocytes, PBL and IEL. Our analysis also showed that the frequency of 8D2$^+$ cells varied between individual chickens, as did the frequency of TCR1$^+$ cells, which is also influenced by age and sex of the animals. While the frequency of positive cells was variable between chickens in all organs, the two markers were positively correlated. Together, these data indicated that the 8D2 mAb is specific for a γδ T cell subset.

We also detected some 8D2$^+$TCR1$^-$ cells in all tissues. These cells were classified as TCR1-negative by gates set using the negative control staining (Supplementary Fig. 4). However, their fluorescent intensity was close to the threshold set by the gate, suggesting that they were poorly stained by the TCR1 mAb but should be considered γδ TCR$^+$ cells. These results indicate that staining of tissues with the TCR1-PE labeled mAb may fail to detect all γδ T cells, highlighting a note of caution for future staining experiments with this TCR1 mAb.

3.2. 8D2 mAb recognizes the Vγ3-and Vδ1-family

Based on the staining profile of 8D2 in combination with TCR1, we hypothesized that the 8D2 mAb recognizes an epitope on the TCR formed by specific TCRγ or TCRδ chains. To further test this hypothesis, splenocytes from two chickens were sorted into three populations by their TCR1 and 8D2 expression: TCR1$^+$8D2$^-$, TCR1$^+$8D2$^+$ and TCR1$^-$8D2$^+$. Each of these sorted subsets was analyzed for their V(D)J gene expression by TCR profiling using a recently established protocol (Früh et al., 2024).

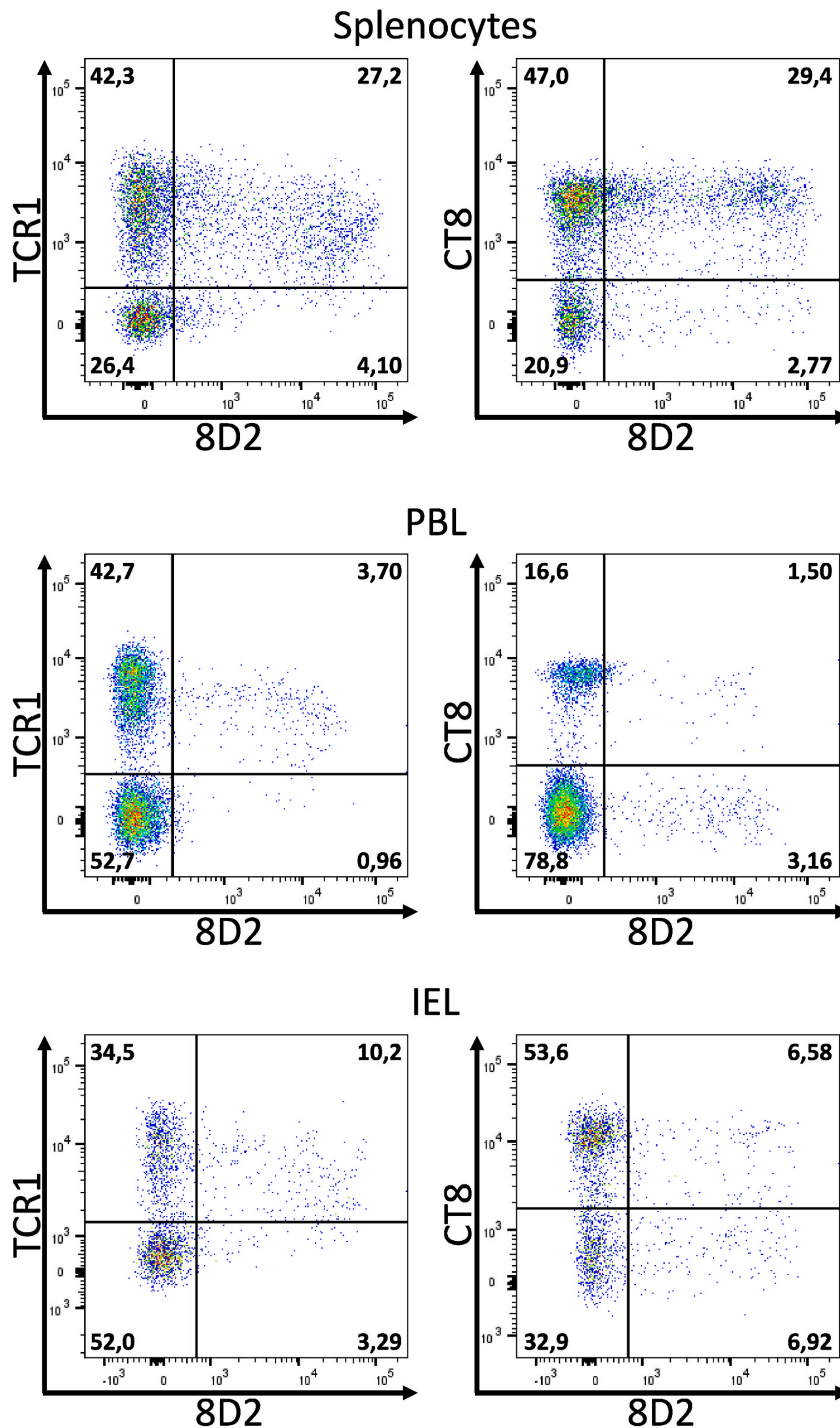

Fig. 2. The 8D2 mAb specifically reacts with γδ T cell subsets in various tissues.
Flow cytometry staining showing 8D2 expression on primary chicken cells compared to TCRγδ (clone TCR1, left panel) or CD8 (clone CT8, right panel) expression, with percentages of live single $CD3^+$ cells (splenocytes and PBL, top and middle panel), or of live single $CD45^+CD3^+$ cells (IEL, bottom panel) indicated. For each organ, one representative staining of four animals is shown.

The DJ gene usage was similar across all three populations. The most prevalent J gene in all six samples was Jγ3 comprising roughly 60% of the whole Jγ repertoire (Supplementary Fig. 5A). The Jγ2 gene was expressed in the remaining ~40%, while Jγ1 was rarely expressed (Supplementary Fig. 5A). In delta chains the most prevalent J gene was Jδ1 comprising ~82% of the whole Jδ repertoire in all samples (Supplementary Fig. 5B). The most frequent Dδ gene was Dδ2 which was expressed in about 90% of Dδ repertoire across all six samples (Supplementary Fig. 5C).

Strikingly, however, V gene usage was strongly biased in $8D2^+$ cells.

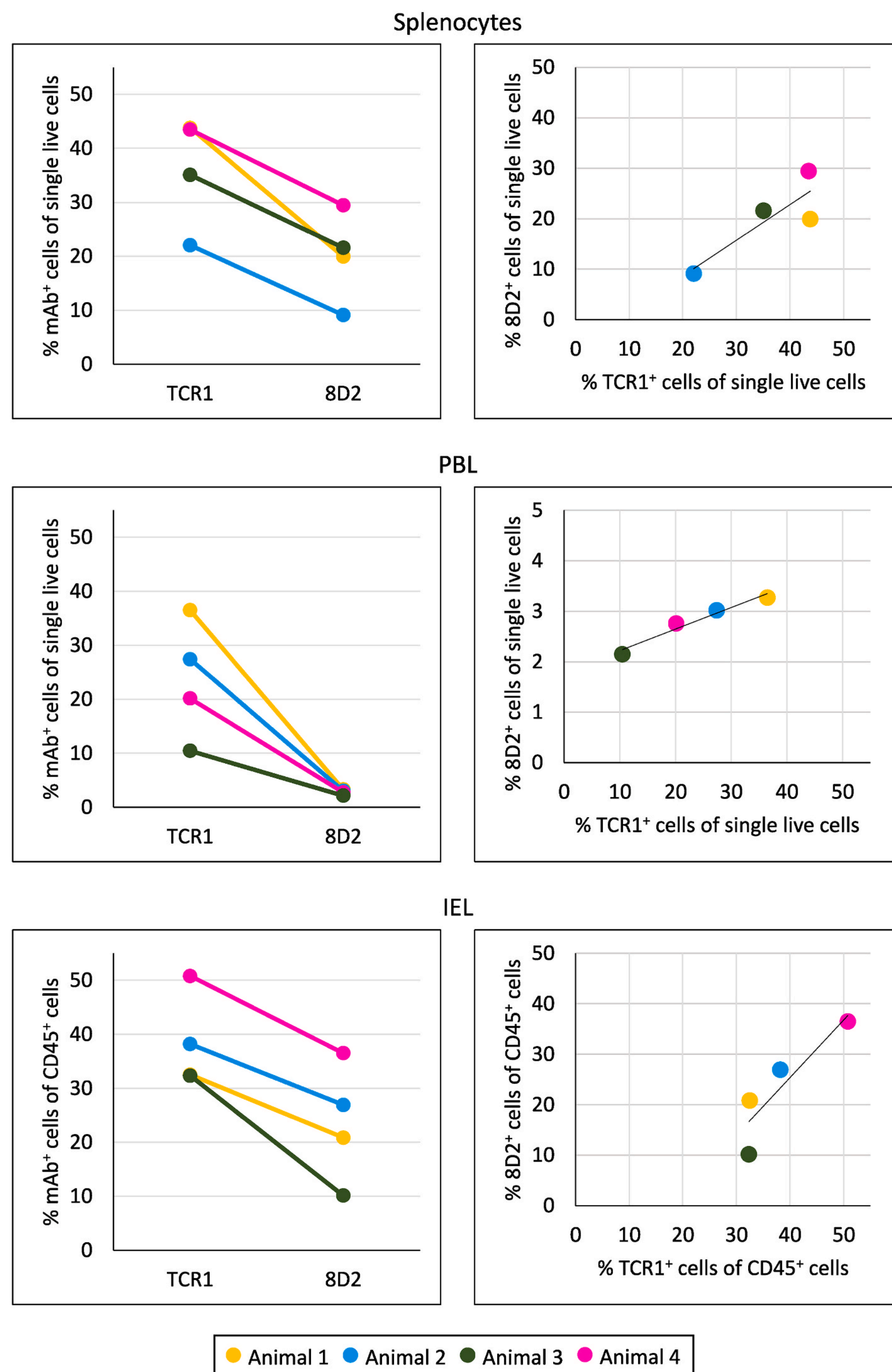

Fig. 3. The frequencies of $8D2^{+}$ and $TCR1^{+}$ cells are positively correlated on splenocytes, PBL and IEL.
Flow cytometry staining showing the frequencies of positive cells in splenocytes, PBL and IEL stained with clones 8D2 and TCR1. The data are presented as percentage of positive cells grouped by the marker (left panel) and as a scatterplot of frequencies (right panel); n = 4 biological replicates.

While Vγ- and Vδ-expression was comparable in $TCR1^{+}8D2^{+}$ and $TCR1^{-}8D2^{+}$ cells, the pattern differed significantly from the V gene distribution in $TCR1^{+}8D2^{-}$ cells (Fig. 4). In $TCR1^{+}8D2^{-}$ cells the four Vγ-families (Vγ1, Vγ2, Vγ3 and Vγ4) were represented at varying frequencies (Fig. 4) that are consistent with previous TCRγ expression data from the spleen (Früh et al., 2024). In this population, TRGV2-26 and the TRGV3-6 were most frequently used (Fig. 5A, Supplementary Fig. 6A). In $TCR1^{+}8D2^{+}$ cells, however, expression of Vγ1, Vγ2 and Vγ4 family members was strongly reduced, and most V genes belonged to the Vγ3-family (Fig. 4), with TRGV3-5 being predominant (Fig. 5A, Supplementary Fig. 6B). Likewise, in $TCR1^{-}8D2^{+}$ cells the TCRγ repertoire was almost exclusively comprised of Vγ3-family members (Figs. 4 and 5A, Supplementary Fig. 6C), with TRGV3-5 and TRGV3-8 being among the most frequent genes (Fig. 5A, Supplementary Fig. 6C). A similar bias was observed in the expression of Vδ genes in both $TCR1^{-}8D2^{+}$ and $TCR1^{+}8D2^{+}$ cells, where TRDV1-2 was most frequently used (Fig. 5B, Supplementary Figs. 7B and 7C). This gene was also present in $TCR1^{+}8D2^{-}$ cells but less frequently. TRDV1-17 was frequently expressed in $TCR1^{+}8D2^{-}$ cells, but not in $8D2^{+}$ cells (Fig. 5B, Supplementary Fig. 7A).

Similar to the individual bias in the prevalence of Vγ/Vδ and Jγ/Jδ, specific combinations of V and J genes were also preferentially used in

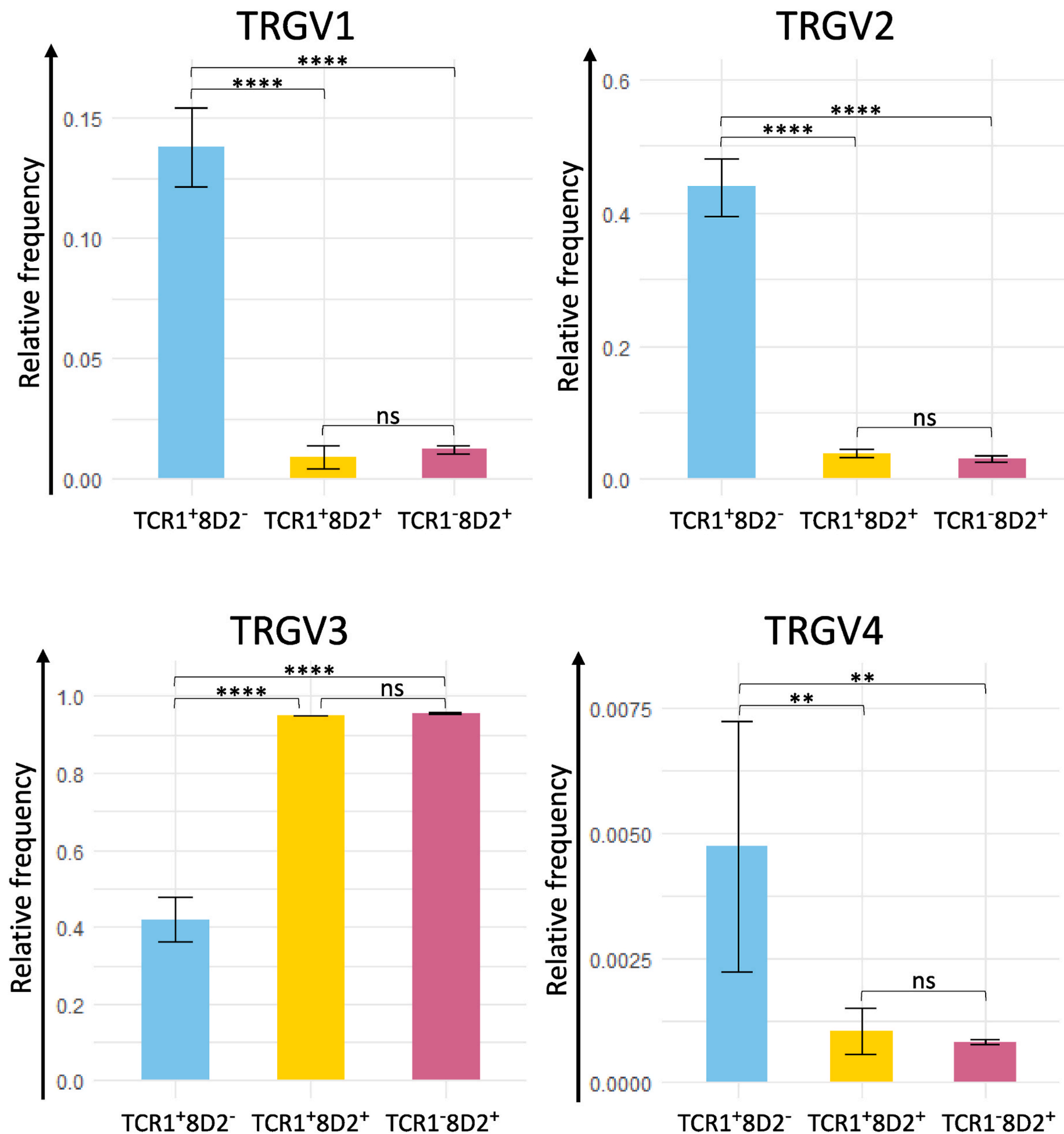

Fig. 4. 8D2$^+$ cells primarily express Vγ3 TCR.
Splenocytes from two animals were sorted according to their TCR1 and 8D2 reactivity into three distinct populations, as indicated. The relative frequency of V gene family usage within these three subsets is shown. Note: each scale is different. Animals = 2. Mean ± SD; p-values as indicated, **** = $p \leq 0.0001$, ** = $p \leq 0.01$, ns = not significant = $p > 0.05$.

8D2$^+$ vs. 8D2$^-$ cells (Fig. 5). TCR1$^-$8D2$^+$ and TCR1$^+$8D2$^+$ cells were remarkably similar, with the most prevalent combinations being TRGV3-5, TRGV3-8 and TRGV3-10, each paired with TRGJ3 (Fig. 5A). In TCR1$^+$8D2$^+$ cells, the combination of TRGV3-6 with TRGJ3 also occurred frequently. This VJ pairing was also found in TCR1$^+$8D2$^-$ cells, however, the most prevalent combination in these cells was TRGV2-26 with TRGJ3, a combination which was rare in 8D2$^+$ cells (Fig. 5A).

Similar to the γ chain, the pairing of Vδ and Jδ genes in TCR1$^-$8D2$^+$ and TCR1$^+$8D2$^+$cells was comparable. In both populations, the following combinations were most frequently used: TRDV1-2, TRDV1-5, TRDV1-9 and TRDV1-25, each preferentially paired with TRDJ1 (Fig. 5B). TRDV1-2 – TRDJ1 was most prevalent, but this pairing was also frequently found in the TCR1$^+$8D2$^-$ cells along with the following combinations: TRDV1-17 with TRDJ1 and TRDV1-25 with the TRDJ1 (Fig. 5B).

There have been only a few previous reports about the γδ TCR repertoire expressed in chickens (Dixon et al., 2021; Zhang et al., 2021; Früh et al., 2024; Linti et al., 2024). Zhang et al. examined the repertoire in the thymus and discovered that multiple genes from different families are preferentially expressed, with members of the Vγ3 subgroup dominating (Zhang et al., 2021). Dixon et al. described the TRGV3 family as the dominant family in all tissues tested, identifying TRGV3.3 as the most frequent gene, comprising 30–40% of the entire TCR gamma repertoire (Dixon et al., 2021). In our study we identified several frequently occurring genes in TCR1$^+$8D2$^-$ cells, rather than a single prominent gene. Dixon et al. and Zhang et al. used different chicken lines and protocols for TCR analysis, and another nomenclature, which may explain the differences highlighted here.

The results from previous work from our laboratory by Früh et al. and Linti et al., using the same chicken genome sequences, however,

Fig. 5. Analysis of the most common TCRγ and TCRδ V-J gene combinations in sorted γδ T cell subsets.
Splenocytes from two animals (A1, A2) were sorted according to their TCR1 and 8D2 reactivity into three distinct populations, as indicated. The bubble grid plots show the frequency of different combinations of Vγ and Jγ genes (A) and Vδ and Jδ genes (B), with J genes depicted on the y-axis and V genes on the x-axis. The color and size of the symbols correspond to the frequency of each specific combination. The Unique Molecule Fraction (UMF) was calculated as the fraction of the unique molecule count of a single clonotype relative to the total sum of unique molecule counts within the sample. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

were overall consistent with the data presented in this study (Früh et al., 2024; Linti et al., 2024). In all studies a bias towards TRGJ3 and TRDJ1 was observed. In addition, TRGV2-26, TRGV3-5 and TRGV3-6 were the most prevalent genes reported in these two studies. Likewise, in this study TRGV2-26 and TRGV3-6 were by far the most frequently used genes in TCR1^{+}8D2^{-} cells. In TCR1^{-}8D2^{+} cells, TRGV3-5 was the most common Vγ gene together with TRGV3-8, that also occurred at varying frequencies in the other two studies. The two previous papers reported different Vδ genes as most prevalent. Linti et al. identified TRDV1-2 as the most prevalent, while Früh et al. reported TRDV1-25 as the most common Vδ gene. Both, TRDV1-2 and TRDV1-25 were among the most frequent V genes in the TCR1^{+}8D2^{-} cells in our study.

In summary, 8D2^{+} cells primarily, though not exclusively, expressed genes of the Vγ3-family and were enriched for particular Vδ genes, while TCR with Vγ1-, Vγ2- and Vγ4-family genes were poorly bound by 8D2.

3.3. 8D2 mAb recognizes a Vγ3 cell line, but no Vγ1 and Vγ2 cell lines

To confirm that 8D2 specifically binds the chicken γδ TCR bearing a Vγ3 gene, we established a stable cell line expressing a TCR comprised of TRGV3-8, TRGJ3, TRDV1-2 and TRDJ1 (Fig. 6A), since these combinations were noticeably more prevalent in the TCR1⁻8D2⁺ cells compared to the other two populations (Fig. 5). For this purpose, a plasmid was constructed that expresses all elements needed for surface expression of the TCR/CD3 complex, namely the CD3γδ, CD3ε and CD3ζ genes, each separated by ribosomal skipping 2a elements. The TCRγ and TCRδ genes were expressed under a second promotor (Supplementary Fig. 2). Stable clones after selection were analyzed for their CD3 expression and one cell line was further propagated.

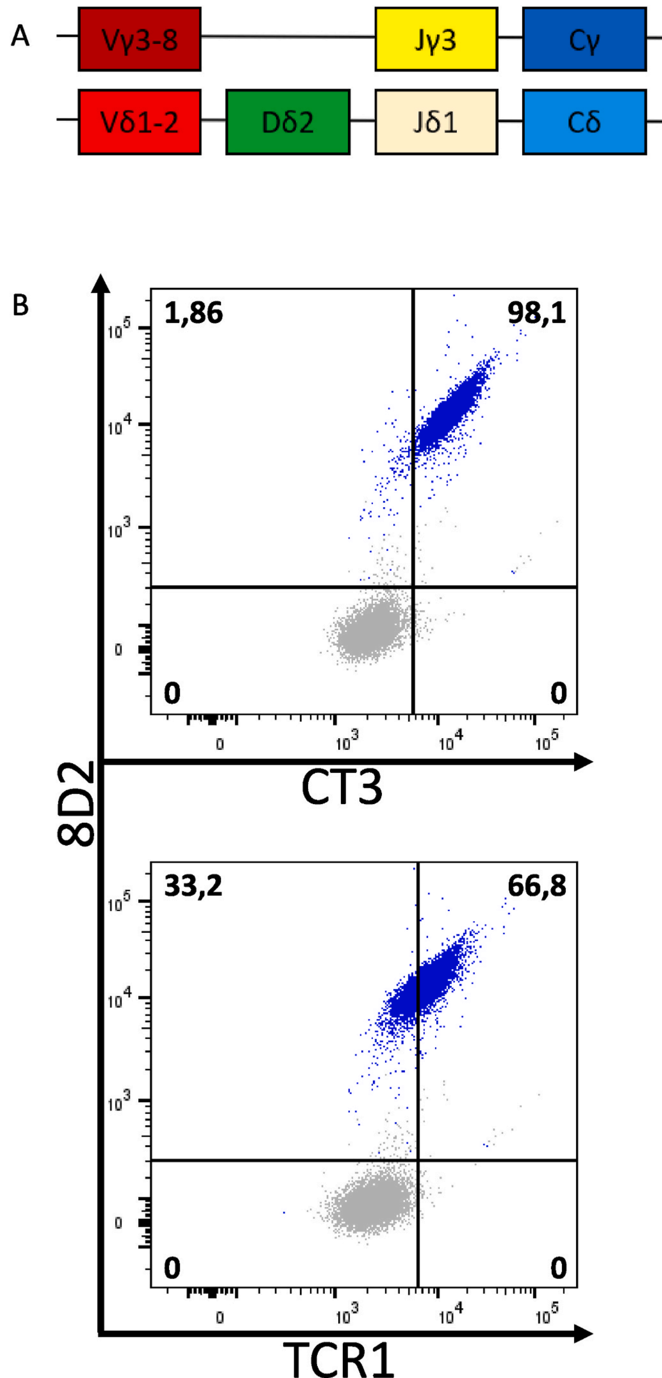

Fig. 6. 8D2 reacts with a stable cell line expressing a Vγ3 TCR.
(A) Schematic representation of the selected γ and δ chains used for transfection. (B) Flow cytometry staining of Vγ3-expressing HEK293 cells using 8D2 in combination with either CT3 (upper panel) or TCR1 (lower panel). Live single cells are shown with the percentage of positive cells indicated. One representative experiment of n = 3 replicates is shown. Blue = stained sample, grey = unstained control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Double staining with 8D2 and either CT3 or TCR1 was performed on this cell line (Fig. 6B). All CT3⁺ and TCR1⁺ cells were also positive for 8D2. The mean correlation coefficient was 0,78 for 8D2 with TCR1 and 0,86 for 8D2 with CT3 (n = 2). Thus, the expression of 8D2 and either TCR1 or CT3 were strongly correlated on this cell line, confirming the trend observed on splenocytes, PBL and IEL (Fig. 3).

We also stained cell lines expressing a Vγ1 or Vγ2 family member γδ TCR previously generated in our laboratory (Fenzl et al., 2017) with 8D2 and TCR1. Approximately 26–28% of the cells reacted with the TCR1 mAb, whereas they barely reacted with the 8D2 mAb (Fig. 7). However, nearly 100% of the Vγ3 cell line were TCR1⁺ and 8D2⁺ (Fig. 7).

These results indicated that the chicken 8D2 mAb specifically reacted with γδ T cells utilizing Vγ3 family members. Specific γδ T cell markers also exist for humans (e.g. clone 5A6.E9) and mice (e.g. clone UC7-13D5), which are utilized in many studies (Peterman et al., 1993; Dutta et al., 2017). Furthermore, several Vγ and Vδ TCR-specific mAb are available for humans (Jitsukawa et al., 1987; Miossec et al., 1990; Moretta et al., 1991), including Vγ9-specific (e.g. clone TiγA), Vδ1-specific (e.g. clone A13) and Vδ2-specific (e.g. clone anti-TiVδ2) mAb. Please note that human Vγ9 is also referred to as human Vγ2 in another nomenclature. Unfortunately, mAb for specific Vγ and Vδ TCR have not been available for chickens until now. However, this study identifies the first Vγ3 TCR-specific mAb.

This novel mAb was also evaluated in techniques beyond flow cytometry surface staining. Preliminary data indicated that the antibody can be used in immunohistochemistry and in intracellular stainings. 8D2 can therefore be used in combination with mAb against intracellular antigens, as fixation and permeabilization of splenocytes did not alter the preceding surface staining. Additionally, the mAb also stained intracellularly after fixation and permeabilization. Whether 8D2 can be used for Western blots or fluorescence microscopy has not yet been investigated.

In conclusion, here we describe a novel mAb 8D2 that specifically stains a γδ T cell subset expressing Vγ3 family TCR. Some of these cells may be poorly detected by the TCR1 mAb. This mAb could be instrumental in further delineating γδ T cell subsets across various organs and in characterizing the dynamics of these subsets during infections.

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Ethics statement

The animal study was approved by Government of Upper Bavaria, identification code: 55.2-1-54-2532.0-60-2015; June 2019. The study was conducted in accordance with the local legislation and institutional requirements.

CRediT authorship contribution statement

Veronika E.M. Drexel: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Thomas W. Göbel:** Writing – review & editing, Funding acquisition, Formal analysis, Conceptualization. **Simon P. Früh:** Writing – review & editing, Formal analysis, Conceptualization.

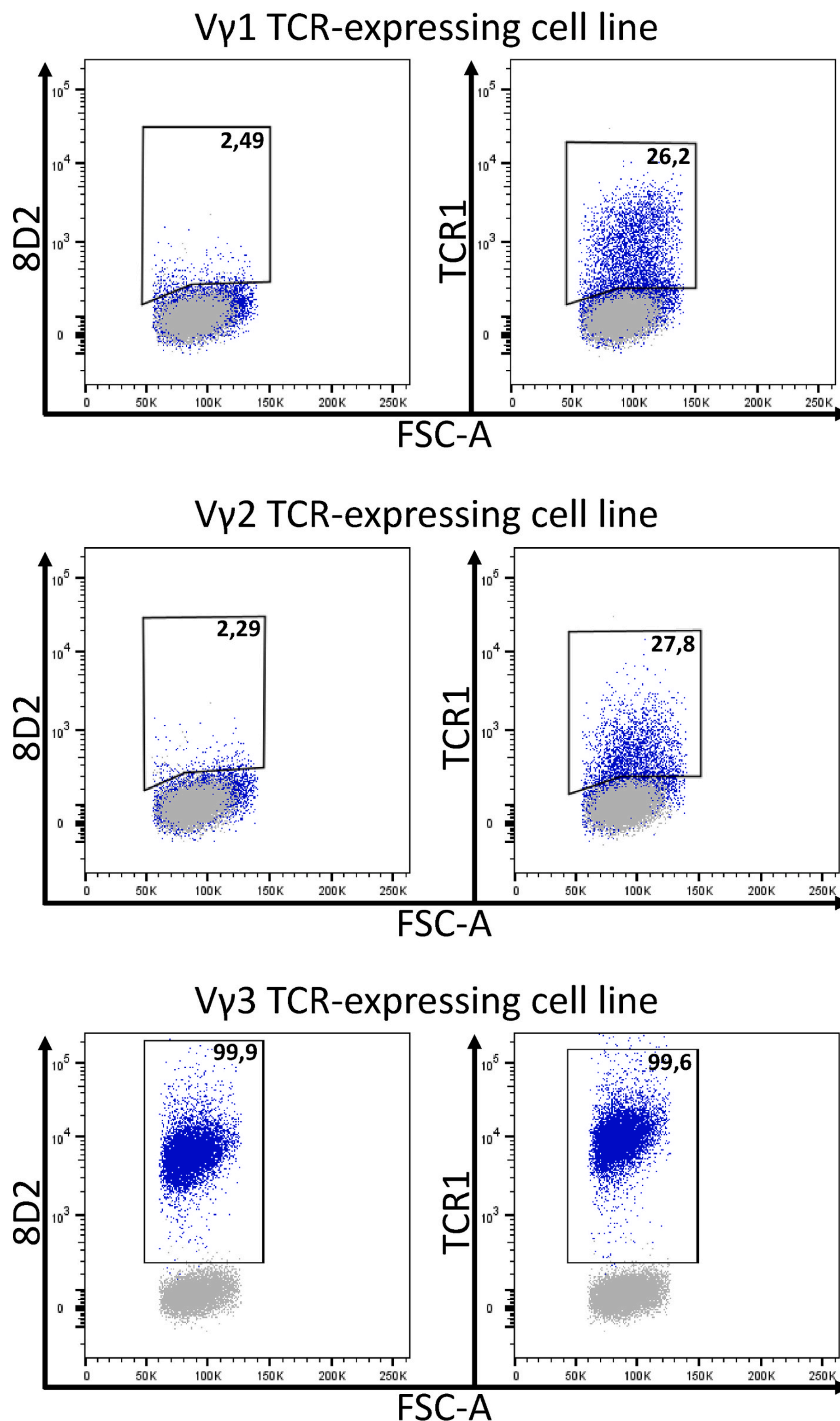

Fig. 7. Reactivity of Vγ1-, Vγ2- and Vγ3-expressing cell lines with 8D2 and TCR1.
Vγ1-, Vγ2- and Vγ3-expressing cell lines were stained with 8D2 (left panel) and TCR1 (right panel) mAb. The percentage of positive cells is indicated. Blue = stained sample, grey = unstained control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT (https://chat.openai.com/) in order to improve readability and language, with no contribution to the scientific content. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2024.105250.

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