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Cross-reactivity of T cell-specific antibodies in the bank vole (*Myodes glareolus*)

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ABSTRACT

The bank vole is a common Cricetidae rodent that is a reservoir of several zoonotic pathogens and an emerging model in eco-immunology. Here, we add to a developing immunological toolkit for this species by testing the cross-reactivity of commercially available monoclonal antibodies (mAbs) to the bank vole lymphocyte differentiation molecules and a transcription factor. We show that a combination of mAbs against CD4, CD3, and Foxp3 allows flow cytometric distinction of the main subsets of T cells: putative helper CD4+, cytotoxic CD8+ (as CD3+CD4-) and regulatory CD4+Foxp3+. We also provide a comparative analysis of amino acid sequences of CD4, CD8αβ, CD3εγδ and Foxp3 molecules for a number of commonly studied Cricetidae rodents and discuss mAb cross-reactivity patterns reported so far in this rodent family. We found that in case of mAbs targeting the extracellular portions of commonly used T cell markers, sequence similarity is a poor prognostic of cross-reactivity. Use of more conserved, intracellular molecules or molecule fragments is a more reliable approach in non-model species, but the necessity of cell fixation limit its application in, e.g. functional studies.

1. Introduction

The rodent family Cricetidae, a sister taxon of Muridae, is the second largest mammalian family (Steppan and Schenk, 2017; Wilson and Reeder, 2005) and taken together they form the most speciose group of mammals. By the sheer numbers, wide geographic distributions and the prevalence of certain life history traits (Han et al., 2015, 2016), they include many reservoir species of zoonotic diseases, and thus both groups are the subject of intense ecological, parasitological and epidemiological studies (e.g., Jonsson et al., 2010; Grzybek et al., 2015; Occhibove et al., 2022). However, a major disparity remains in the field of immunology, where the vast majority of research focuses on two species of the family Muridae: the mouse (*Mus musculus*) and the rat (*Rattus norvegicus*). Although these are canonical model species in the life sciences (including immunology), there is a growing appreciation of the potential benefits that the inclusion of other species and genetically diverse populations would bring to the field (Flies, 2020). However, a major obstacle is the lack of specific immunological reagents, in particular monoclonal antibodies (mAbs), which remain essential for the qualitative and quantitative description of immunological parameters. As the production of specific mAbs is generally expensive and time-consuming, cross-reactivity screens between commercially available mAbs and cell markers or cytokines of non-model species are crucial to bridge this gap.

In the present study, we focused on the bank vole, *Myodes glareolus* (Schreber, 1780), one of the most widespread Cricetidae rodents in the Palearctic (Wilson and Reeder, 2005) and a common subject of ecological, evolutionary and behavioral studies (Kotlík et al., 2022; Lonn et al., 2017; Sadowska et al., 2008; Tschirren et al., 2013). More recently, the bank vole has attracted attention as a potential model in the study of prion diseases, as it is one of the few species readily susceptible to various prion infections (Larsen, 2016). Importantly, wild bank vole populations are known reservoirs of several zoonotic viruses, including lymphocytic choriomeningitis virus (LCMV), cowpox virus (CPXV), and

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Puumala virus (PUUV) (Grzybek et al., 2019), as well as the spirochete *Borrelia burgdorferi* s.l., the causative agent of Lyme disease in humans (Gomez-Chamorro et al., 2019). Consequently, numerous studies have investigated different components of both the innate and adaptive immune system of this species, such as cytokines (e.g., TNF-α), MHC molecules, and receptors (e.g., TLRs and TCRs) (Guivier et al., 2010a, 2010b; Migalska et al., 2018; Tschirren et al., 2013). At the same time, a budding immunological toolkit for bank voles is emerging, with the development of recombinant cytokines (IFN-gamma, Torelli et al., 2018) and permanent cell lines (Binder et al., 2019; Essbauer et al., 2011). However, to the best of our knowledge, no specific mAbs directed against the most important immunological markers have been developed for this species, nor have systematic cross-reactivity tests been performed.

Here, we set out to identify commercially available mAbs that would allow flow cytometric distinction of basic subsets of T cells: helper CD4+, cytotoxic CD8+ and regulatory CD4+Foxp3+ in the bank vole. To this end, we tested the cross-reactivity of several mAbs developed against CD4, CD8, CD3, and Foxp3 molecules of murid and cricetid rodents or humans. We then used an RT-qPCR assay to confirm that a combination of cross-reactive mAbs allowed the identification of the CD4 and CD8-expressing T cells in this species. Finally, we provided a comparative analysis of the amino acid sequences of CD4, CD8αβ, CD3εγδ and Fopx3 for a number of commonly studied Cricetidae rodents, putting into perspective mAb cross-reactivity patterns reported so far in this rodent family.

2. Materials and methods

2.1. Antibodies

Our primary goal was to identify commercially available mAbs that would specifically cross-react with bank vole T cell antigens. We focused our efforts mainly on anti-CD4 and anti-CD8 antibodies (Table 1; further details such as manufacturers and concentrations are provided in Suppl. Table 1), especially those that have been shown to cross-react with the antigens of the relatively closely related Syrian hamster, *Mesocricetus auratus* (Hammerbeck and Hooper, 2011) or have been developed specifically for members of the Cricetidae family (Green et al., 2013; Rees et al., 2017).We also tested a rat anti-human CD3 mAb (clone CD3–12 which targets an intracellular fragment of this molecule), an anti-MHC class II antibody that cross-reacts with Syrian hamster molecules (Hammerbeck and Hooper, 2011), and a highly cross-reactive mAb against the Foxp3 transcription factor (Table 1).

2.2. Animals and tissue processing

Bank voles were obtained from a laboratory colony maintained at the Institute of Environmental Sciences, Jagiellonian University, Krakow (Sadowska et al., 2008), in accordance with Resolution No. 258/2017 of the 2nd Local Institutional Animal Care and Use Committee in Kraków. Animals were cared for according to institutional guidelines and to avoid unnecessary killing, we used animals that were removed from the colony during routine maintenance procedures that control colony size. The culled voles were of both sexes and between 2 and 7 months of age. The animals were sacrificed by cervical dislocation and spleens were harvested immediately afterwards, into high-glucose DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific) and 1% penicillin/streptomycin. Tissues were minced and passed through a 100 μm cell strainer (Biologix) into 50 mL conical tubes and washed with the same medium used for the tissue collection. The single cell suspension was centrifuged at 300-400*g* for 5 min at 12°C before being resuspended in 3 mL of RBC Lysis buffer (eBioscence™) and incubated for 4 min at room temperature, with periodic gentle stroking to lyse red blood cells. The cells were then washed with PBS and counted.

2.3. Antibody cross-reactivity tests with flow cytometry

2.3.1. Initial screening of cross-reactive mAbs targeting extracellular antigens

Staining was performed on 96-well U-bottom plates. Cells (0.5×10^6 cells/well) were blocked for 15 min in 75 μL of PBS containing 2% FBS, 2% normal mouse serum (eBioscience™) and 2% normal rat serum (eBioscience™). Subsequently, antibodies targeting cell surface antigens were added in PBS containing 2% FBS, at the concentration recommended by the manufacturer (see Suppl. Table 1 for details). Cells were stained in the dark, on ice, for 30 min and washed twice with PBS. In the case of unconjugated antibodies (Table 1), cells were then stained with a FITC-conjugated secondary goat anti-mouse IgG (H+L) polyclonal antibody (Invitrogen, cat# 62–6511; 1:200) or PE-conjugated F(ab')2 goat anti-mouse IgG (H+L) secondary polyclonal antibodies (eBioscience™, cat# 12–4010-82), in a buffer containing 2% FBS or 1% BSA, and stained for 30 min, in the dark, on ice, and washed twice with PBS. In addition, a portion of cells without primary antibodies was stained with secondary antibodies only, as a specificity control. Finally, cells were resuspended in PBS with 0.5% FBS for analysis on an LSR Fortessa flow cytometer (BD Bioscience) equipped with CellQuest software (Becton Dickinson, San Diego, CA) or a CytoFLEX (Beckman Coulter) cytometer with CytExpert Acquisition and Analysis Software (Beckman Coulter, United States).

2.3.2. Extracellular and intracellular staining and cytometric analysis

Following the identification of cross-reactive mAbs against extracellular antigens, we tested the cross-reactivity of mAbs targeting intracellular antigens (Table 1). Staining was performed on 96-well U-bottom plates. Cells were first stained with either LIVE/DEAD™ Fixable Violet or Near IR (780) Viability Kit (Invitrogen, cat# L34963 or L34992, 1:1000). Staining was performed in a final volume of 1 mL, at RT, for 20 min, followed by a wash with 10 mL of PBS containing 2% FBS. Subsequently, $0.5–1 \times 10^6$ cells/well were kept in 100 μL PBS with 2% FBS for 15 min to block non-specific binding. For extracellular

Table 1
Antibodies screened for cross-reactivity in the Bank vole.

Type	Antigen	Clone	Target species	Host species	Isotype	Conjugate	Cross-reactivity
Extracellular	CD4	GK1.5	Mouse	Rat	IgG2b	FITC	+
	CD4	RM4–4	Mouse	Rat	IgG2b	FITC	–
	CD4	HAB1A	Syrian hamster	Mouse	IgG1	none	–
	CD4	HAL36A	Syrian hamster	Mouse	IgG2a	none	+
	CD4	695542	Cotton Rat	Mouse	IgG2b	none	–
	CD8α	JG12	Cotton Rat	Mouse	IgG2a	none	–
	CD8β	eBio341	Rat	Mouse	IgG1	FITC	–
	MHC class II (I-Ek)	14–4-4S	Rat	Mouse	IgG2a	PE	+
Intracellular	Foxp3	FJK-16s	Mouse	Rat	IgG2a	PE	+
	CD3ε	CD3–12	Human	Rat	IgG1	Alexa Fluor 647	+

staining, either FITC-conjugated anti-CD4 mAb (clone GK1.5, eBioscience™, 1:100) or PE-conjugated anti-MHC class II (I-Ek) (clone 14–4-4S, eBioscience™, 1:100) was added to the cells for 30 min on ice and then washed twice with PBS. Next, fixation/permeabilization was performed with Foxp3/Transcription Factor Staining Buffer Set (eBioscience™), following the manufacturer's instructions. Briefly, cells were fixed in 200 µL fixation/permeabilization working solution for 30 min at RT and then washed twice with permeabilization buffer working solution. The washed cells were resuspended in permeabilization buffer, supplemented with 2% normal mouse serum and 2% normal rat serum, and blocked for 15 min at RT. After blocking, PE-conjugated anti-Foxp3 mAb (clone FJK-16s, eBioscience™, 1:20) and/or Alexa Fluor 647-conjugated anti-CD3 mAb (clone CD3–12, Bio-Rad, 1:100) were added directly to the cells and incubated for 40 min, at RT. Corresponding fluorescence minus one (FMO) controls were prepared in the same manner. Additionally, unstained controls or cells stained only with viability dies were processed in parallel. Furthermore, UltraComp eBeads™ Plus Compensation Beads (Invitrogen™) were stained according to the manufacturer's protocol as single stained controls. After two final washes with permeabilization buffer, cells were resuspended in PBS with 0.5% FBS and analyzed on either CytoFLEX cytometer or LSRFortessa™.

Non stained controls were used to control for background signals that are derived from the autofluorescence of the cells and to identify negative populations of the bank vole cells. Lymphocytes were gated according to forward and side scatter patterns, live cells were gated based on LIVE/DEAD™ staining, and doublets were excluded based on FSC-A vs FSC-H patterns (Supp. Fig. 1). Further gating of lymphocyte populations was performed using FMOs.

2.4. Identification of CD4+ and CD8+ T cell subsets in bank vole

A combination of cross-reactive mAbs against CD3 and CD4 molecules should, in principle, allow differentiation of $CD4^+$ and $CD8^+$ T cell subsets, with the former identified as $CD3^+CD4^+$, and the latter as $CD3^+CD4^-$. To confirm this, we designed a qPCR assay to measure CD4 and CD8 expression in sorted populations of cell subsets. However, since our cross-reactive anti-CD3 antibody targets an intracellular portion of this antigen, harsh fixation and permeabilization would damage the RNA and prevent quantification. Therefore, a modification of the cell staining protocol designed for cell sorting was necessary to preserve the quality of RNA for subsequent processing (Channathodiyi and Houseley, 2021).

2.4.1. Cell sorting and RNA extraction

The staining protocol prior to cell sorting was simplified and changed based on modifications proposed by Channathodiyi and Houseley, 2021.

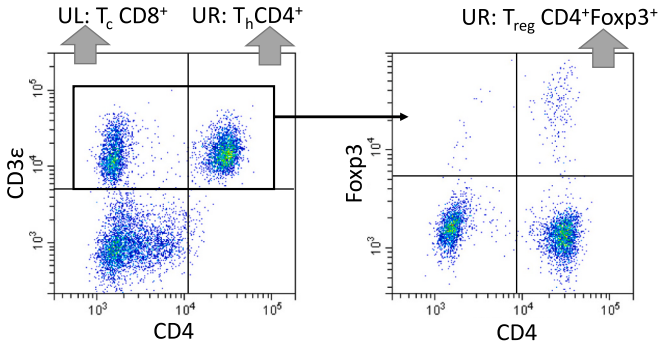

Fig. 1. Representative Bank vole lymphocyte staining with mAbs anti-CD3 (clone CD3–12, conjugated with Alexa Fluor 647), anti-CD4 (clone GK1.5, conjugated with FITC) anti-Foxp3 (clone FJK-16 s, conjugated with PE). The full gating strategy is shown in Supplementary Fig. 1, details on mAbs used are in the Table 1 and Supp. Table 1.

Most importantly, fewer staining steps were performed, glyoxal (Sigma Aldrich, cat. 50649) was used to fix cells (instead of the formaldehyde-based fixative available in the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set), and RNase inhibitors were added to the incubation and wash solutions. In addition, RNAse-free plasticware (including filtered tips) was used throughout, and all incubation steps were performed on ice. Otherwise, staining followed the protocol described above, with the modifications detailed below. For the initial extracellular staining, 5–7 $\times$ 10^6 cells were resuspended in a conical tube in 200 µL of PBS containing 2% FBS, supplemented with 1:200 RNase Inhibitor (RI, RNasin® Plus, Promega). Cells were blocked with mouse and rat sera for 10 min and subsequently stained with FITC-conjugated anti-CD4 mAb for 30 min, as described above. After two washes with PBS, cells were fixed in 3% glyoxal with 20% ethanol, supplemented with 1:25 RI, in a total volume of 250 µL, for 15 min. The glyoxal solution was prepared according to the Channathodiyi and Houseley, 2021.The cells were then washed twice with PBS supplemented with 1:150 RI and permeabilized in a working solution of a saponin-based Permeabilization Buffer (eBioscience™, cat# 00–8333), supplemented with 1:25 RI, in a total volume of 200 µL, for 30 min. Next, mouse and rat sera were added directly to the permeabilized cells, to a final concertation of 2% each, and cells were blocked for 15 min. After blocking, the cells were stained with Alexa Fluor 647-conjugated anti-CD3 mAb for 45 min. Finally, cells were washed twice with permeabilization buffer supplemented with RI (1:100) and resuspended in PBS supplemented with RI (1:100).

Target populations were isolated using FACS Aria IIIu (Becton Dickinson). Data were acquired and analyzed using BD FACSDiva™ software (Becton Dickinson). Lymphocytes were gated based on their FSC and SSC parameters. $CD4^+$ T cells were defined as $CD3^+CD4^+$; $CD8^+$ T cells were defined as $CD3^+CD4^-$; while the third population ("negative") was defined as $CD3^-CD4^-$, and likely contained B cells, NK cells, and certain minor subpopulations of innate-like lymphocytes (Supp. Fig. 2). 100,000 cells of each population were collected in tubes containing PBS supplemented with 1:100 RI. Cells were sorted to achieve purity greater than 96%. Immediately after sorting, cells were centrifuged at 1800*g* for 3 min at 4°C, and the supernatant was removed. Cells were then lysed with 350 µL of RLT buffer (Qiagen) and kept on ice until RNA extraction. RNA was extracted on the same day using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The protocol included an on-column DNase digestion step using the Rnase-Free DNase set (Qiagen). The RNA was eluted with RNAse-free H_2O in a final volume of 20 µL and stored at −80°C.

2.4.2. Gene expression analysis – RT-qPCR

The assay measured the expression of the *CD4* and *CD8α* genes in sorted T cell populations, as well as that of *LCK* (lymphocyte-specific protein tyrosine kinase), which was used as an additional control for T cell enrichment, since Lck is predominantly expressed in T cells and is required for T-cell receptor signaling (Artyomov et al., 2010). *TBP* (TATA box binding protein) was chosen as a reference gene because its expression has been shown to be among the most stable in mouse (Medrano et al., 2017) and bank vole (Němcová et al., 2020) spleens. The bank vole-specific target gene primers for *CD4, CD8α* and *LCK* were taken from Migalska et al., 2019 and for the reference *TBP* gene from Němcová et al., 2020.

Before the RT-qPCRs, 12 µL RNA was reverse transcribed using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and oligo(dT) primers, according to the manufacturer's protocol. The calibration sample was prepared by pooling RNA extracted from the three cell populations studied (i.e., $CD3^+CD4^+$; $CD3^+CD4^-$; $CD3^-CD4^-$) in equal proportions (before reverse transcription).

RT-qPCRs were performed on the Bio-Rad® CFX96 TM Real-Time PCR System (Bio-Rad) with SYBR-green-based detection of PCR products. Reactions were set up using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). The final reaction volumes were 20 µL, with

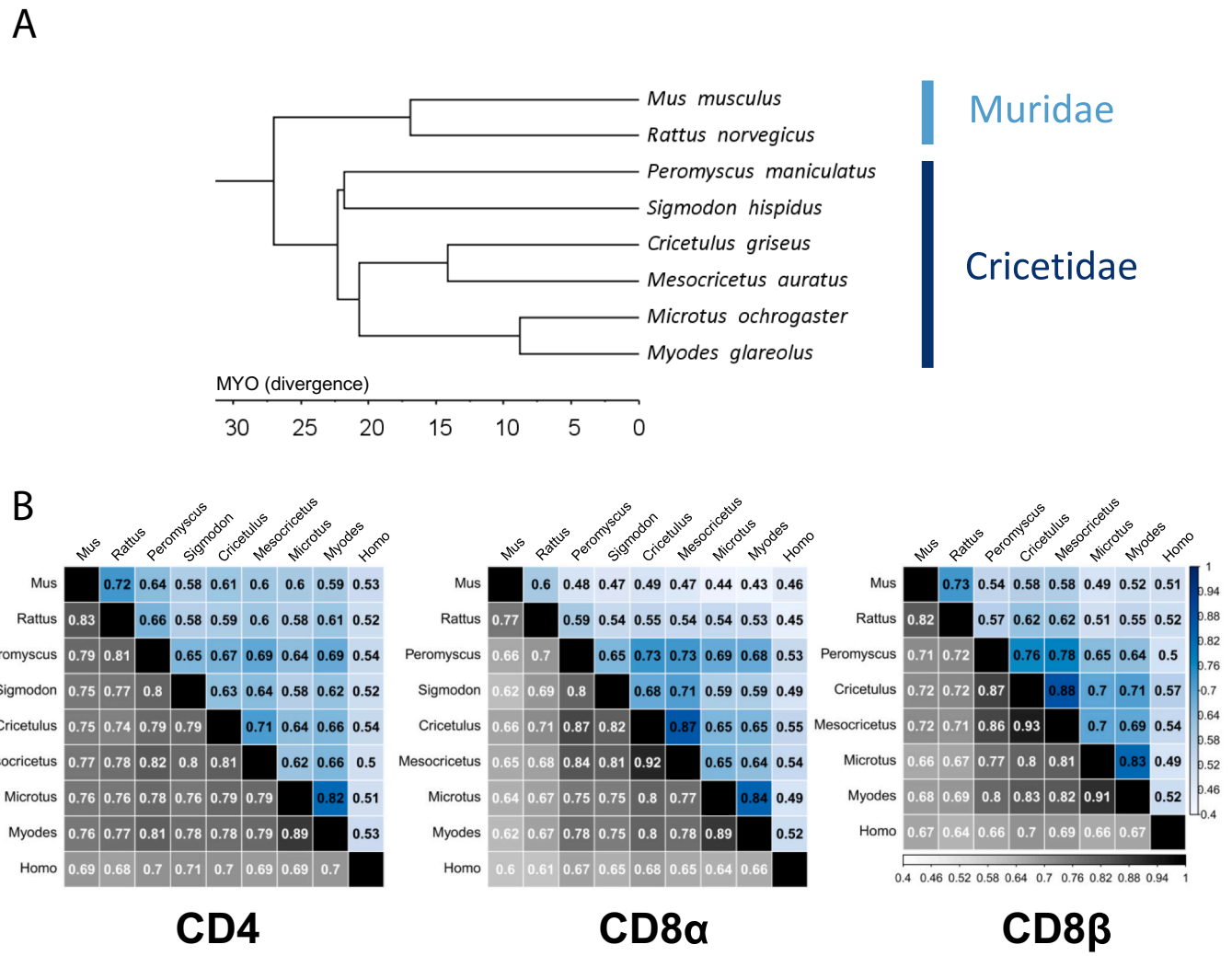

Fig. 2. A) Tree showing phylogenetic relationships among rodent species discussed in the article. Tree topology was based on a rodent phylogenetic tree published by Steppan and Schenk (2017), with root scaled to the median divergence time between Myodes and Mus reported on Timetree (see the main text). B) Pairwise amino acid sequence identity (upper-diagonal, in blue) and similarity (lower diagonal, in grey) matrices for CD4, CD8α and CD8β molecules. *Mus musculus* – mouse, *Rattus norvegicus* – rat, *Peromyscus maniculatus* - eastern deer mouse, *Sigmodon hispidus* - hispid cotton rat, *Cricetulus griseus* - Chinese hamster, *Mesocricetus auratus* - Syrian hamster, *Microtus ochrogaster* - prairie vole, *Myodes glareolus* – bank vole, and *Homo sapiens*, human. For brevity, only the generic name is provided on the figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

10 μL of Supermix, 0.5 μM of each primer, and 1 Ll of 1.5× diluted cDNA template. The cycling conditions were: 95°C for 30s, followed by 40 cycles at 95°C for 15s and 60°C for 30s. Each sample was run in triplicate and the mean Cq (quantification cycle value) was used for the calculations. Replicates never differed by more than one quantification cycle (mean SD for all assays: 0.047, range: 0.005–0.203). In addition, a non-template control for each primer pair, a duplicate of no-RT controls for each RNA isolate (using primers for the *CD8α* gene), and a triplicate of a calibrator sample were run. Melt curves of the products were inspected for each sample to confirm specific amplification. Normalized expression levels were calculated using the ΔΔCt method in the Bio-Rad CFX Maestro 1.1 program (v. 4.1.2433.1219).

2.5. Sequence analyzes

Finally, we examined whether the pattern of cross-reactivity we detected for the bank vole (or was previously reported in other cricetid rodents, Vaughn and Schountz, 2003; Hammerbeck and Hooper, 2011) correlated with sequence similarity between orthologous molecules. First, for each gene of interest (i.e., *CD3G, CD3D, CD3E, CD4, CD8A, CD8B, FOXP3*), high quality, annotated mRNA reference sequences were identified for mouse (*M. musculus*), rat (*R. norvegicus*), and human (*Homo sapiens*), using the HomoloGene and/or Genbank NCBI databases. The mouse and rat sequences were then used as queries in a Blastn search of a bank vole (*M. glareolus*) transcriptome (Kotlík et al., 2018). Next, the top hits were used as queries in a blastn search of the Genbank database, where deposited sequences from other Cricetidae rodents (mostly derived by automated computational analysis and annotated using the Gnomon gene prediction method) were identified. Sequences were available for the following common cricetid rodents: eastern deer mouse (*Peromyscus maniculatus*), Chinese hamster (*Cricetulus griseus*), Syrian hamster (*M. auratus*), prairie vole (*Microtus ochrogaster*), and the bank vole itself (Genbank sequence identifiers are in the Supp. Table 2). Coding sequences (CDSs) were extracted from these records (based on Genbank annotation), translated into amino acid sequences, aligned with the default settings using MAFFT v. 7 (Katoh and Standley, 2013), and visualized with BioEdit (Hall, 1999). In addition, because anti-CD4 and anti-CD8α cotton rat-specific mAbs are commercially available, *CD4* and *CD8A* hispid cotton rat (*Sigmodon hispidus*) sequences were obtained from commercially available constructs (Cotton Rat CD4 VersaClone cDNA, cat# RDC1063, R&D Systems; Cotton Rat CD8 alpha (AAL55392) VersaClone cDNA, cat# RDC0871, R&D Systems), which had been used

in the construction of said antibodies. Alignments of CD sequences, with annotations of signal peptides, transmembrane regions, and ITAMs (immunoreceptor tyrosine-based activation motives) are shown in the Supp. Fig. 3, *FOXP3* alignment is shown in the Supp. Fig. 4.

Next, we performed a pairwise comparison of the extracellular domains (ECD) of CD molecules (as the vast majority of mAbs against CDs commonly used in flow cytometry target extracellular parts of these molecules). For Foxp3, the entire CDS was used for comparison. The ECDs of CD molecules were extracted based on the features identified above (i.e., a fragment of the amino acid sequence between the signal peptide and the transmembrane domain), and a pairwise sequence identity and similarity were calculated with the MatGAT program (v2.01) (Campanella et al., 2003), using the BLOSUM62 scoring matrix for pairwise alignment.

3. Results

The primary focus of this article was the identification of cross-reactive antibodies to discriminate the major T cell subsets in the bank vole by flow cytometry. The test focused on four marker molecules: CD3, CD4, CD8 and Foxp3.

3.1. CD3

CD3 is required for the activation and signal transduction of both CD4+ and CD8+ T cells and is considered the major T cell marker. It is composed (in mammals) of four distinct chains, three of which (ε, γ, δ) dimerize as εγ and εδ transmembrane molecules, while the fourth chain (ζ) associates with a cytosolic portion of the T-cell receptor (Owen et al., 2013). Given the poor cross-reactivity of antibodies targeting the extracellular portion of CD3 across non-primate mammals (Conrad et al., 2007; Hammerbeck and Hooper, 2011) we only tested an antibody against a much more conserved, cytoplasmic portion of the CD3ε molecule (Supp. Fig. 3, area shaded in grey – 100% identity among analyzed species). This antibody stained ~48% of the splenocytes (42–56%, based on a sample of five, 2–3 month old, unrelated individuals from the laboratory colony) (Fig. 1). Furthermore, co-staining with mAb against rat MHC class II (clone 14–4-4S) showed mutually exclusive populations (Supp. Fig. 5) – consistent with the notion that MHC class II is not expressed on T cells (unless activated, and this phenomenon has only limited evidence from rodents; Holling et al., 2004). However, it is expressed on professional, antigen-presenting cells (Owen et al., 2013), and in the lymphocyte population analyzed here, these would most likely be B cells. Sequence identities between particular murine and cricetid CD3 chains ranged from 58 to 70% for CD3ε up to 69%–80% for CD3δ (Supp. Fig. 6).

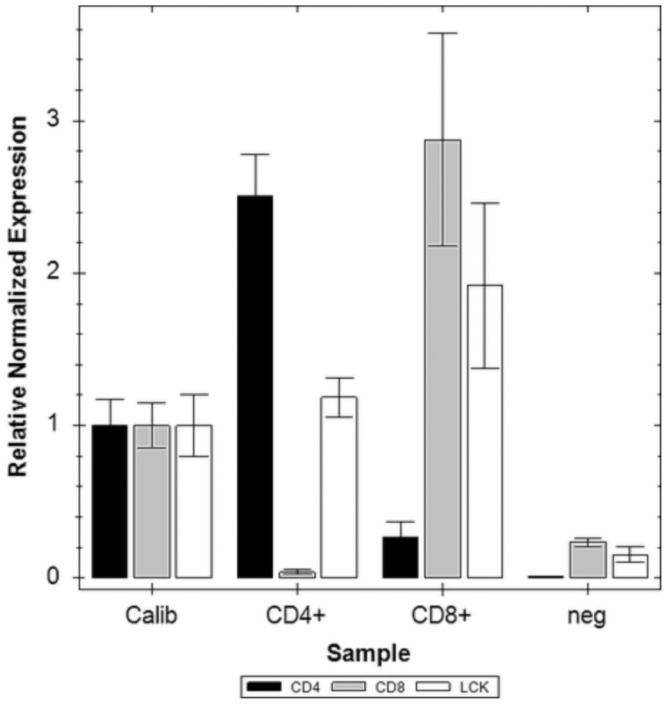

Fig. 3. Expression of the CD4, CD8α and LCK genes in three sorted populations of cells: "CD4+" - CD3 + CD4+; "CD8+" - CD3 + CD4-; "neg" - CD3-CD4-. Relative normalized expression levels were measured with ΔΔCt method, with calibrator sample ("Calib") prepared by mixing RNA from the three cell populations. TBP (TATA box binding protein) was used as a reference gene.

3.2. CD4

CD4 is a monomeric surface glycoprotein primarily expressed on T cells and thymocytes (but also on some macrophages and dendritic cells). On T cells, it serves as a co-receptor that coordinates MHC class II recognition and is considered a major marker of helper T cells (Owen et al., 2013). Only two of the five tested anti-CD4 mAbs stained bank vole splenocytes – one clone developed for the mouse, GK1.5 (Fig. 1) and one for the Syrian hamster- HAL36A. Both likely recognize the same (or similar) epitope, as suggested by the diagonal pattern of labeling reported by (Rees et al., 2017) in the Syrian hamster. In contrast, another mouse-specific antibody – clone RM4–4 (which likely targets a different epitope than GK1.5 as it does not block the binding of this mAb to CD4 – information provided by the manufacturer), did not stain bank vole cells, nor did the HAB1A clone against Syrian hamster CD4 or the 695542 clone developed for the cotton Rat CD4 (data not shown). The ECD sequence identity between the bank vole and mouse CD4 was 59%, compared to 62% for the cotton rat and 66% for the Syrian hamster (Fig. 2).

3.3. CD8

CD8 is a surface glycoprotein that can be formed by either a CD8αα homodimer or a CD8αβ heterodimer, the latter form being predominantly found on cytotoxic T cells, where it serves as a co-receptor for MHC class I recognition (Owen et al., 2013). Neither of the two tested mAbs (against rat CD8β or cotton Rat CD8α chain) stained bank vole cells. Sequence identity of bank vole CD8 chains compared to murid rodents was low – only 43% and 53% for CD8α (as compared to mouse and rat, respectively) and 52% - 55% for CD8β. Sequence identity with cricetid cotton rat CD8α was only slightly higher – 59% (Fig. 2).

Since none of the tested, commercially available mAbs against CD8 stained bank vole cells, and the low sequence similarity to murid CD8 chains gave little hope of identifying such an antibody in a broader test, we checked whether a co-staining with the above identified mAbs against CD3 and CD4 could be used to differentiate $CD4^+$ and $CD8^+$ T cell subsets (as $CD3^+CD4^+$ and $CD3^+CD4^-$, respectively). RT-qPCR of the sorted $CD3^+CD4^+$, $CD3^+CD4^-$ and $CD3^-CD4^-$ populations confirmed high expression of both *CD4* and *LCK*, but not *CD8A* in the $CD3^+CD4^+$ cells, and high levels of *CD8*A and *LCK*, but not *CD4* in the $CD3^+CD4^-$ cells, confirming that the combination of these mAbs can be used to identify (at least roughly) the $CD4^+$ and $CD8^+$ T cell subsets. Double negative cells ($CD3^-CD4^-$) showed minimal expression of *CD4*, and very low levels of *LCK* and *CD8A* (Fig. 3).

3.4. Foxp3

Foxp3 is a transcription factor that serves as a master regulator in the development and function of regulatory CD4+ T cells (Hori et al., 2017). Its sequence is highly conserved among mammals, and its identity exceeds 93% among the studied rodent species (Supp. Figs. 4 and 7). In our assays, we used a highly cross-reactive rat anti-mouse Foxp3 mAb clone FJK-16s, which has been shown to be useful for the identification of

regulatory T cells in a number of mammalian species (Gerner et al., 2010; Käser et al., 2008; Rocchi et al., 2011). According to the manufacturer, its epitope has been mapped to amino acids 75–125 of the mouse Foxp3. Along this fragment, the bank vole protein is 100% identical to the mouse reference (Sup. Fig. 4, shaded in grey). On average, it stained 8% of CD4+ cells from bank vole spleens (5–10% in the individuals tested), and the staining was restricted to this subset (i.e., was not found in $CD3^{+}CD4^{-}$ and $CD3^{-}CD4^{-}$ cells) (Fig. 1).

4. Discussion

Studies aimed at identifying cross-reactive antibodies in non-model rodents are rare, although they could easily add to the immunological toolbox available for many species recognized as reservoirs of zoonotic diseases. Moreover, when performed, they are rarely accompanied by a comparative sequence analysis, so it is unclear whether sequence similarity at the amino acid level can be informative when researchers are trying to find a cross-reactive mAb for their species of interest. Given the limited number of such studies, there is little intuition as to whether the successful identification of a cross-reactive mAb in one species is a good predictor of cross-reactivity in a close relative.

The bank vole belongs to the family Cricetidae, which split from its sister taxa, the Muridae (*M. musculus*, *R. norvegicus*), around 27 MYA (TimeTree.org, accessed: 25/04/2022, Kumar et al., 2022) (Fig. 2A). Within Cricetidae itself, the earliest split separated the Palearctic Cricetinae (*C. griseus*, *M. auratus*) and Holarctic Arvicolinae (*M. glareolus, M. ochrogaster*) from the New World clades of Neotominae (*P. maniculatus*), Sigmodontinae (*S. hispidus),* and Tylomyinae (Steppan and Schenk, 2017).

The amino acid sequence identity of the extracellular domains of the compared rodent CD molecules was typically well below 80% (Fig. 2, Supp. Fig. 4) – which is an often assumed threshold above which cross-reactivity to orthologous molecules can be expected. In addition, the overall similarity levels differed markedly between the CD molecules analyzed, or even between subunits of the same molecule. Considering their complex, often multimeric structure, glycosylation patterns, and conformational changes upon dimerization, the chances of cross-reactivity are rather low.

CD3 is a prime example, especially given that many widely used mAbs against CD3 recognize conformational epitopes on CD3ε, expressed when bound to either CD3γ or CD3δ (Salmerón et al., 1991). This may explain why, despite comparable or higher levels of sequence identity to that of CD4 molecules, finding mAbs that bind to the extracellular portion of this complex in Cricetidae can be challenging. Hammerbeck and Hooper (2011) tested nine different mAbs and were unable to identify any that were reactive with *M. auratus* T cells. At the same time, Vaughn and Schountz (2003) showed dimmed but apparently specific staining of CD3 in *P. maniculatus* with one of these mAbs (clone 145-2C11). Thus, the use of mAbs targeting a much more conserved cytosolic portion of CD3 molecules may be the most viable option in non-model rodents, as exemplified by our results in the bank vole. In general, reliance on transcription factors, such as Foxp3, and other intracellular molecules to identify immune cell populations, rather than far less conserved membrane molecules, seems to be the most promising approach for non-model species. It should be noted, however, that the cell fixation and permeabilization of the cell membrane required for intracellular staining will limit its application in e.g., functional studies.

The patchy patterns of cross-reactivity found for anti-CD4 mAbs highlight another aspect of the challenging task of identifying cross-reactive antibodies. While success in one non-target species suggests recognition of a more conserved epitope, overall it has little predictive power for successful staining in another, closely related species. For example, we successfully stained bank vole T cells with the rat anti-mouse CD4 clone GK1.5, which also stained Syrian hamster cells (Hammerbeck and Hooper, 2011), but not those of the cotton rat (Vaughn and Schountz, 2003). In contrast, another rat anti-mouse CD4 mAb, clone RM4–4, failed to stain cells from any of these three Cricetidae species. Unfortunately, although cross-reactivity is epitope-dependent, precise information on the antibody binding site is often not available, and only information on the immunogen used in rising the antibodies is provided. This also can be very broad, e.g., whole leucocytes (clones HAB1A, HAL36A), whole, recombinant proteins (clone 695542), and without more precise information, it remains impossible to narrow down the screens of available mAbs to those targeting sites with the highest local similarity. Thus, empirical testing remains key.

Finally, our inability to find a cross-reactive mAb against bank vole CD8 molecules shows that some markers may be too divergent to allow the use of commercially available mAbs from a related species. When developing specific antibodies against the species of interest is not an option, cell subsets can often be identified based on FSC/SSC patterns combined with lack of expression of certain markers. In our case, $CD3^{+}CD4^{-}$ gate was used to identify CD8+ T cells. However, researchers must remember that other cell types may be included in such defined gates, in this case, for example, certain rare populations of natural killer T cells (e.g., $CD3^{+}CD4^{-}NK1.1^{+}$, Godfrey et al., 2004) or double-negative $CD3^{+}CD4^{-}CD8^{-}$ T cells (Wu et al., 2022). The consequences of analyzing a more heterogeneous set of cells will depend on the specific goal of a given study and should be carefully considered during the experimental design phase.

5. Conclusions

In summary, we have identified three commercially available mAbs against CD3, CD4, and Foxp3 that allow the identification of putative helper CD4+, cytotoxic CD8+ and regulatory CD4+Foxp3+ T cells in a non-model rodent of the Cricetidae family: the bank vole. The information we provided will facilitate immunological studies in this reservoir of several zoonotic pathogens and an emerging model species in eco-immunology. We also discussed patterns of mAb cross-reactivity against these T-cell defining molecules in the context of the degree of amino acid sequence identity among murid and cricetid rodents, highlighting the continued need for and indispensability of empirical screens in the field of immunophenotyping.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2023.113524.

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