# Supplementary Information for

## Alpaca (Vicugna pacos), the first non-primate species with a phosphoantigen-reactive Vγ9Vδ2 T cell subset

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**This PDF file includes:**

Fig. S1 to S13

Tables S1 to S8

References for SI reference citations: 1-10

**Other supplementary materials for this manuscript include the following:** none

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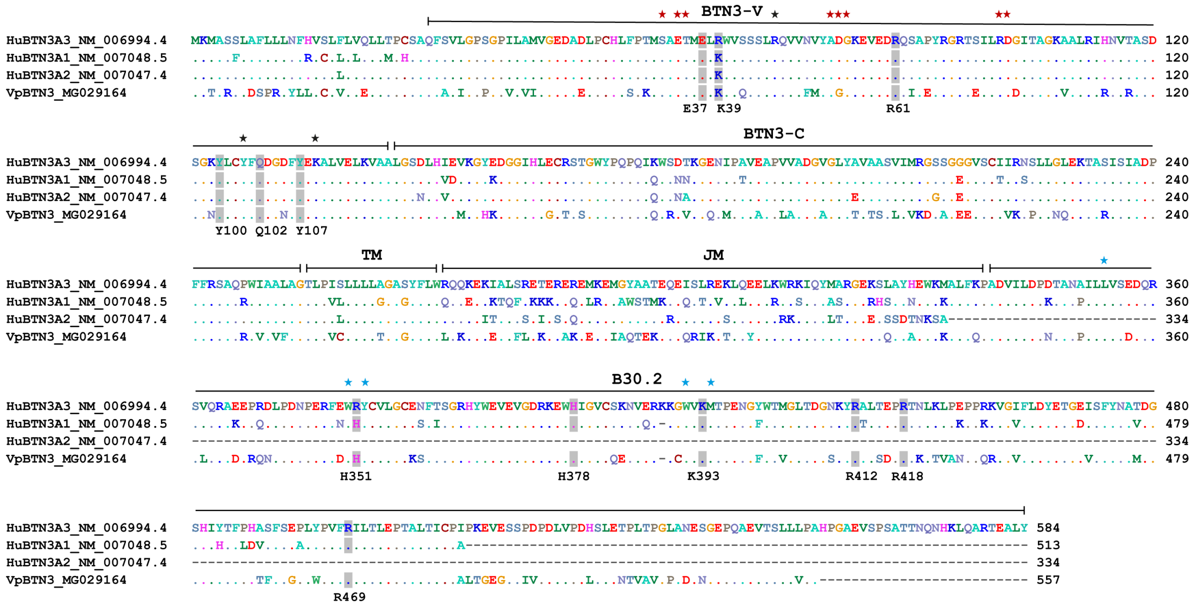


**Fig. S1: Amino acid alignment of BTN3 sequences of human and alpaca.** Calculated with Clustal Omega and modified with BioEdit (color-code: default). Identical amino acids (dots) and gaps (dashes) are indicated. The domains of BTN3 were determined according to human BTN3A1 as described before by Palakodeti et al. for BTN3-V and Nguyen et al. **(1, 2)** and proposed phosphoantigen binding residues are highlighted in gray with numbering according to Vavassori et al. (BTN3-V) and Sandstrom et al. (B30.2) **(3, 4)**. Additional residues proposed to be involved in PAg binding (blue asterisks) **(5)** and BTN3 function (black asterisks) **(6)** are highlighted. Binding sites of the 103.2scFv are indicated with red asterisks **(2)**. Abbreviations: TM (transmembrane), JM (juxtamembrane).

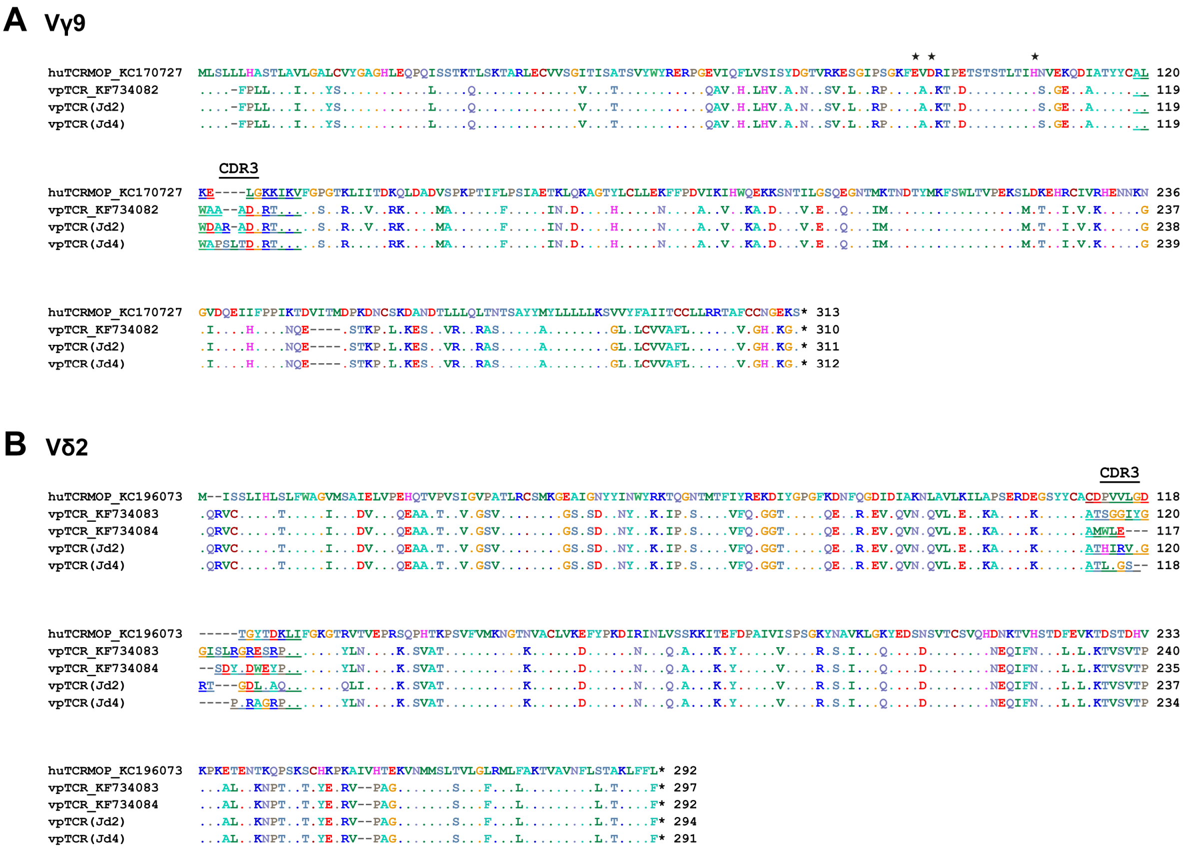


**Fig. S2: Amino acid alignment of TCR sequences of human and alpaca.** Clustal Omega and modified with BioEdit (color-code: default). Identical amino acids (dots) and gaps (dashes) are indicated. The CDR3 regions of Vγ9 (**A**) and Vδ2 chains (**B**) are indicated. Black asterisks indicate residues outside the CDRs shown to affect PAg-mediated activation by site-directed mutagenesis by Willcox et al **(6)**.

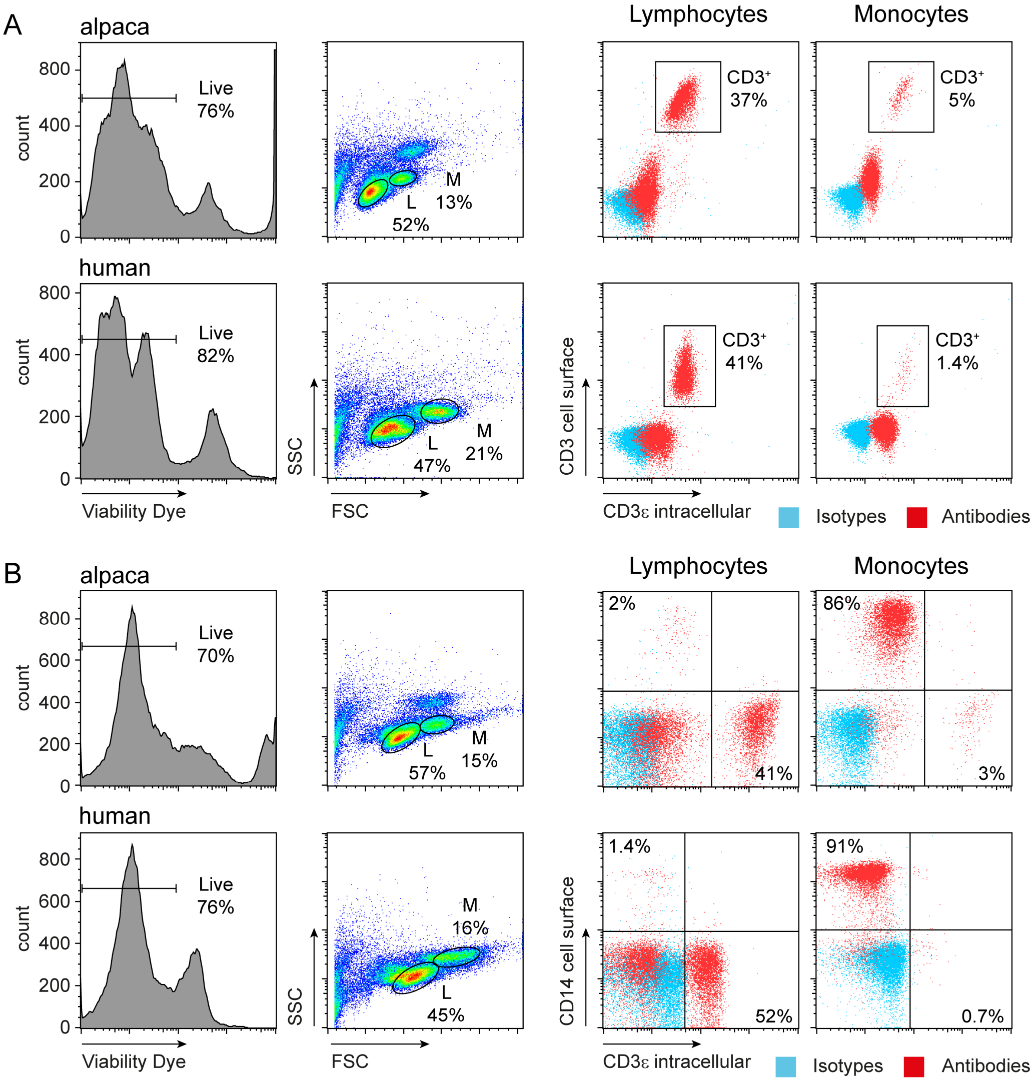


**Fig. S3: Gating strategies for identification of T cells and monocytes.** (**A and B**) Living cells were identified by staining with viability dye (first panel) and frequencies of live cells are indicated. Subsequently, lymphocyte and monocyte gates were set based on FSC/SCC (second panel) and gated cells were analyzed for antibody staining (third and fourth panel). (**A**) Identification of mAb LT97A as presumably alpaca CD3-specific. Cells were stained with LT97A (alpaca) or SK7 (human) mAb and a PE-labeled secondary antibody, fixed, permeabilized and stained with the mAb CD3-12 binds to a common epitope of CD3ε **(7)**. Antibody-stained cells are depicted in red and overlaid with isotype controls in blue. Isotype control staining varied significantly between species and cell type but in all cases positive vs. negative cells could be clearly distinguished. Cells stained for cell surface and intracellular CD3 overlapped completely. Conversely, none of the cells stained for intracellular CD3 expressed CD14. Thus, the alpaca CD3-12 antibody can be regarded as reliable marker for T cells and the majority of cells identified by FSC/SSC gating can be considered as lymphocytes or monocytes, respectively.

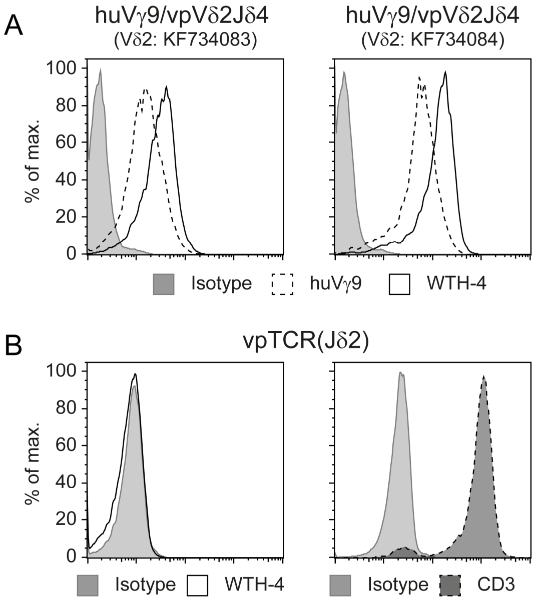


**Fig. S4: WTH-4 binds to Vδ2Jδ4-containing alpaca TCR**s. (**A**) Staining of TCR-negative BW58 mouse hybridoma cells transduced with the human Vγ9 chain (TCR MOP) and alpaca Vδ2Jδ4 chains (this paper). GenBank accession numbers of Vδ2 chains are indicated. Cells were stained with mAb WTH-4 (line) or mouse-anti human Vγ9 clone (dotted line) and isotype control mAb (filled histogram) with the secondary antibody DαM R-PE. (**B**) Staining of BW58 mouse hybridoma cells transduced with an alpaca Vγ9 ("ALWDARADGRTIKVFGSGTRLIVT") and alpaca Vδ2Jδ2 ("ATHIRVGGRTGDLTAQLIFGKGTQLIVEP") were stained with WTH-4 the secondary antibody DαM R-PE (left) or with biotinylated anti-mouse CD3ε and Streptavidin PE (right). CD3 expression shows TCR at the cell surface, while WTH-4 is not detecting the TCR with Vδ2Jδ2.

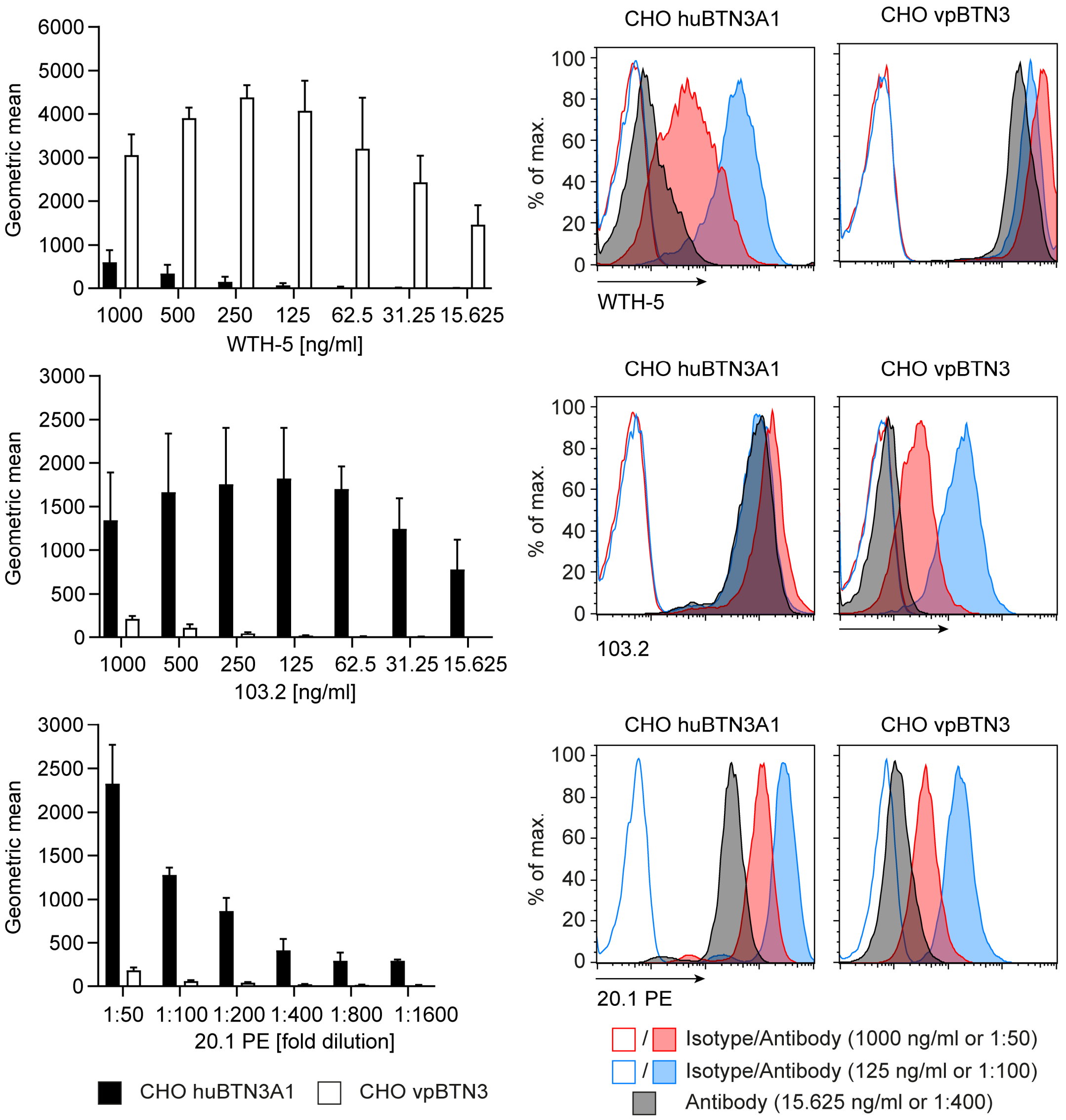
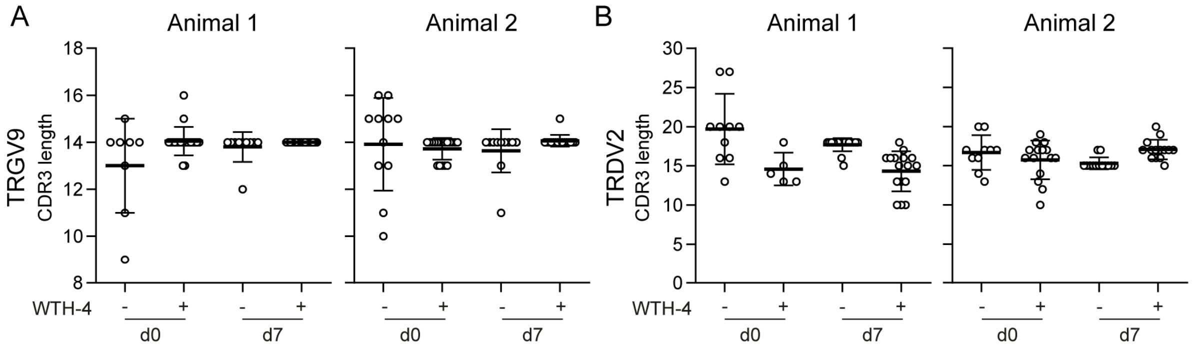


**Fig. S5: Comparison of binding of BTN3-specific mAbs to huBTN3A1- or vpBTN3 transduced hamster cells (CHO).** CHO cells were transduced with fusion proteins containing huBTN3A1 or vpBTN3 with mCherry, respectively. Cells were sorted for similar mCherry fluorescence analogous to Fig. S9 (293T cells) and stained with a serial dilution (2-fold) of the indicated mAbs. Geometric means (GMs) were plotted against mAb concentration. Starting concentrations were 1000 ng/ml for 103.2 and WTH-5 and 1:50 for mAb 20.1 PE. Histograms depict selected concentration of specific mAbs (filled histogram) and isotype controls (lines).



C

| TCR clone | Vγ9 | Vδ2 | CDR3 length |
|---|---|---|---|
| scTCR1 | ALWDARADGRTIKVFGSGTRLIVT | ALPGWSGISKSLIFGKGTYLNVEP | 14/13 |
| scTCR2 | ALWVSLRGRTIKVFGSGTRLIVT | ATPVRYTLESRDPLIFGKGTYLNVEP | 13/15 |
| scTCR3 | ALWAPWTDGRTIKVFGSGTRLIVT | AARVGVGTIYGRGRPLIFGKGTYLNVEP | 14/17 |
| scTCR4/vpTCR(Jδ4) | ALWAPSLTDGRTIKVFGSGTRLIIT | ATLVGSPTRAGRPLIFGKGTYLNVEP | 15/15 |
| scTCR5 | ALWDARGDGRTIKVFGSGTRLIIT | ATLIRSGWRRGKGPLIFGKGTYLNVEP | 14/16 |
| scTCR6 | ALWDAQADGRTIKVFGSGTRLIIT | AAAGSRMSPLIFGKGTYLNVEP | 14/11 |
| scTCR7 | ALWEPLTDGRTIKVFGSGTRLIIT | ATAVSSLRAGPLIFGKGTYLNVEP | 14/13 |
| scTCR8 | ALWDPLADGRTIKVFGSGTRLIIT | ATTMGLGDRALIFGKGTYLNVEP | 14/12 |
| scTCR9 | ALWARRADGRTIKVFGSGTRLIIT | ATDIRLGDRPLIFGKGTYLNVEP | 14/12 |

**Fig. S6:** Homogenization of CDR3 lengths in alpaca Vγ9JP chains and evidence for pairing of Vγ9 and Vδ2 in vivo. CDR3 lengths of TRGV9/TRGC (**A**) and TRDV2/TRDC (**B**) amplicons from unstimulated/stimulated (50 U/ml hIL-2 and 1 μM HMBPP for 7 days) sorted WTH-$4^+$ or WTH-$4^-$ cells were compared. CDR3s were assigned and underscored as reported by **(8)** and lengths are indicated. Each symbol marks a single clone, mean+SD indicated. (**C**) Single-cell PCR was performed on WTH-$4^+$ cells and pairs of Vγ9 and Vδ2 CDR3+TRJ are shown. scTCR1-3 were obtained from unstimulated cells of animal 1 and scTCR4-9 from stimulated (50 U/ml hIL-2 and 1 μM HMBPP for 7 days) of animal 3. Frequencies of nucleotide-identical TCR amplicons were as follows: scTCR1-3: 1/21 single cells each, scTCR4: 4/17, scTCR5: 3/17, scTCR6: 2/17, scTCR7: 3/17, scTCR8: 1/17, scTCR9: 1/17.

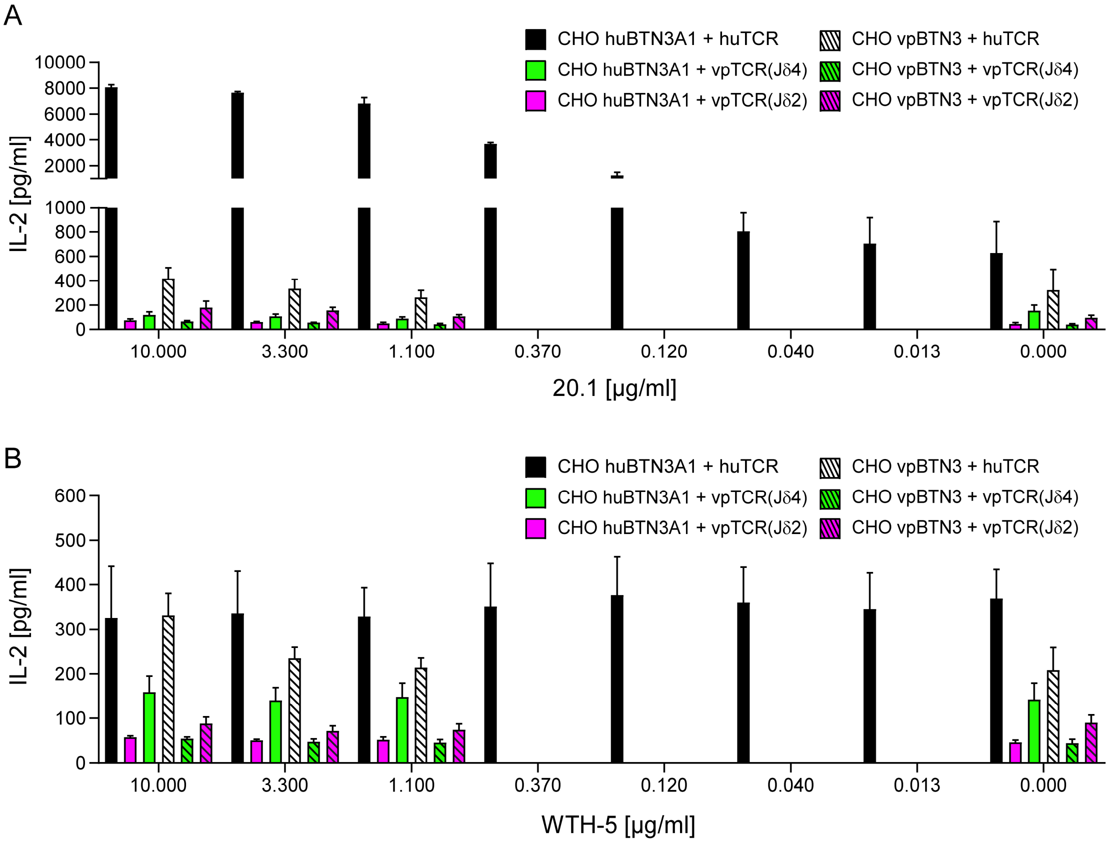


**Fig. S7: Lack of mAb 20.1 or WTH-5 induced IL-2 Production by vpTCR transductants.** Indicated TCR transductants and BTN3-transduced cells were cultured with the indicated concentrations of mAb 20.1 (**A**) or WTH-5 (**B**). Data was collected from 3 independent experiments and plotted with SEM.

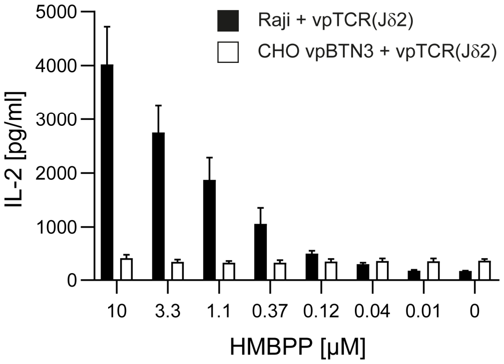


**Fig. S8: vpBTN3-transduced CHO cells do not support HMBPP-mediated stimulation of vpTCR(Jδ2) transductants**. vpTCR(Jδ2) TCR transductants were cultured with vpBTN3-transduced CHO cells or RAJI cells (50.000 cells/well) and indicated HMBPP concentrations and subsequently tested for mIL-2 production.

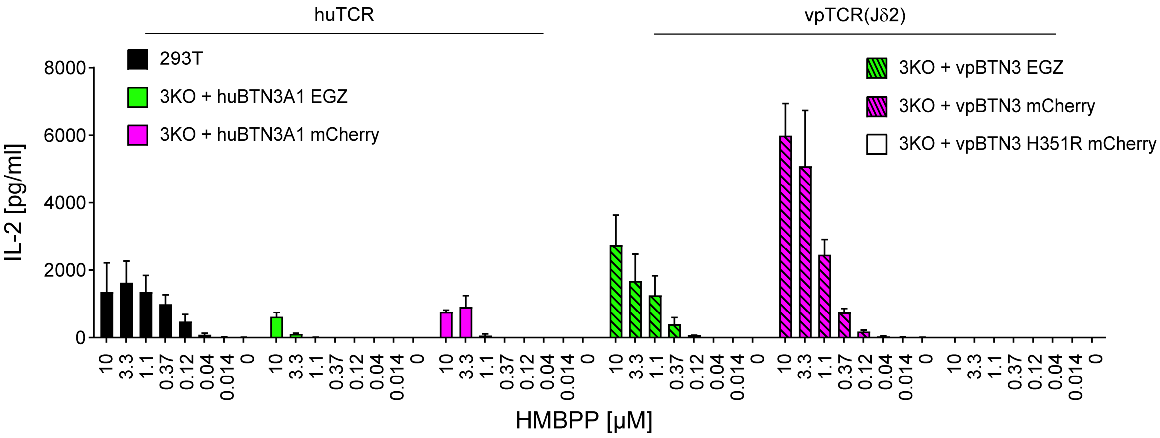


**Fig. S9: Comparison of BTN3 constructs and loss-of-function of H351R mutant.** Comparison of PAg-mediated activation in the presence of BTN3KO cells transduced with huBTN3A1 or vpBTN3 wildtype (EGZ reporter gene after internal ribosome binding site or mCherry fusion constructs) and of the H351R vpBTN3 mutant (mCherry with internal stop codon). BTN3KO cells transduced with huBTN3A1 were stained with mAb 103.2 and vpBTN3 transductants with WTH-5 and sorted for equal levels of BTN3 expression. Internal controls were untransduced 293T cells. IL-2 production by TCR MOP (huTCR) or vpTCR(Jδ2)-transduced 53/4 cells is plotted against the concentration of HMBPP in the culture. Results are shown as mean±SEM of three independent experiments. There was no HMBPP-induced stimulation of the H51R mutant (max. IL-2 production: 6 pg/ml).

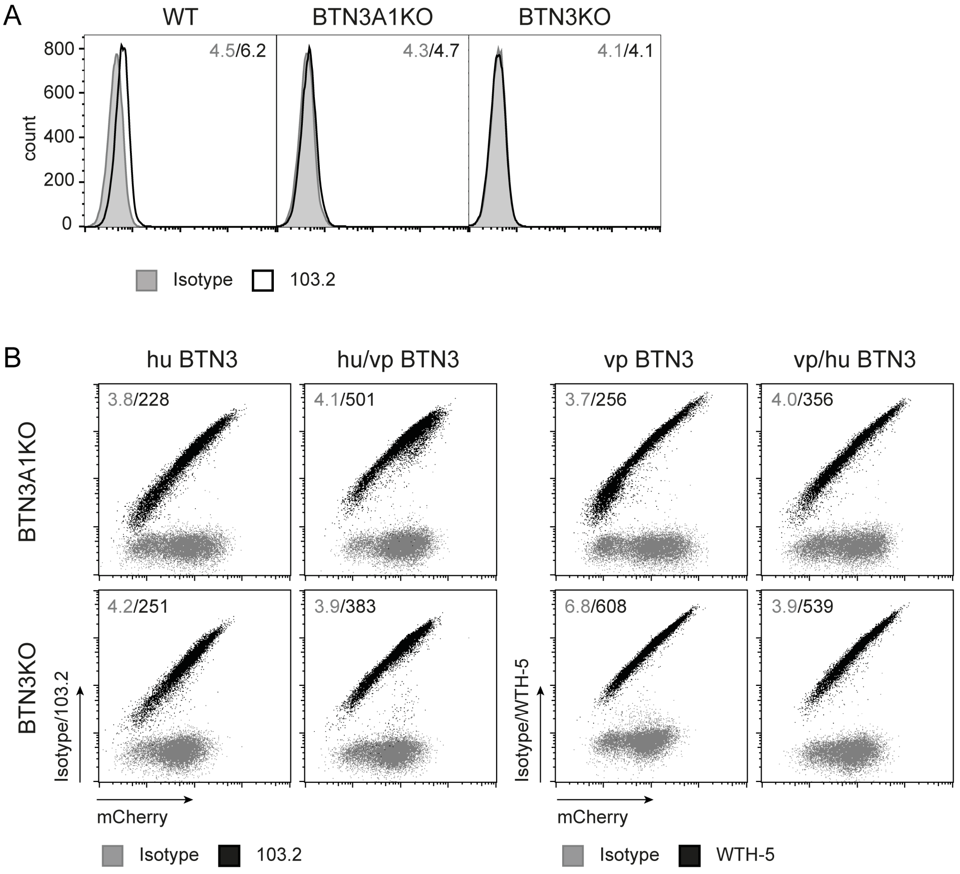


**Fig. S10: BTN3 expression by 293T cells, 293T BTN3 knock out cells and BTN3-transduced knock out cells**. Wild type and knock out 293 T cells (**A**) as well as BTN3A1KO (upper panel of **B**) or BTN3KO (lower panel of **B**) were transduced with the indicated BTN3 constructs (see also **Fig. 5**) and stained with antibodies specific for the respective extracellular domain: mAb 103.2 for huBTN3A (BTN3A1/2/3) and mAb WTH-5 for vpBTN3 (secondary antibody: DαM R-PE). Transductants were previously sorted for similar mCherry levels and instrument setting for analysis of **A** and **B** were identical, to allow a direct comparison of BTN3 cell surface expression of wildtype/KO cells and transductants.

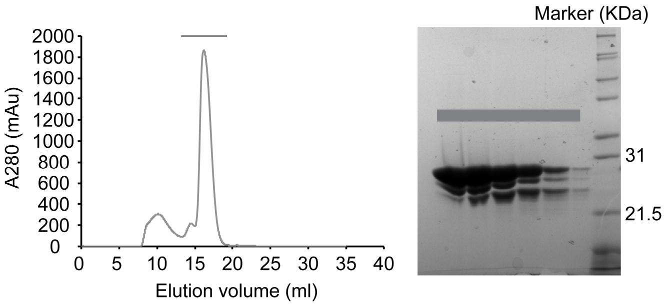


**Fig. S11: Alpaca B30.2 protein purification.** The gel filtration curve for alpaca B30.2 domain in ITC buffer is shown on the left and the SDS-PAGE of the protein after gel filtration purification is shown on the right.

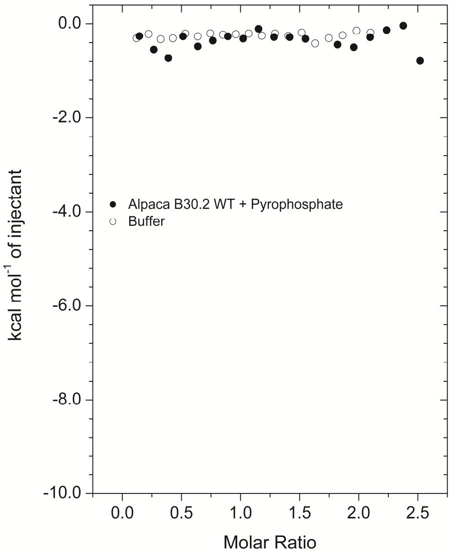


**Fig. S12: The prenyl ester of pAg is critical for binding to the alpaca B30.2 domain**. ITC binding isotherm traces of alpaca B30.2 domains with pyrophosphate show no interaction between the protein and the diphosphate molecule. This measurement was performed with 30 µM protein in the cell and 600 µM ligand in the syringe to maintain the same ratio between sample and ligand as in Figure 6. Buffer control is shown as an open circle and the protein interaction is depicted as a filled in circle. The reported molar ratio is the ratio of pAg:B30.2 domain.

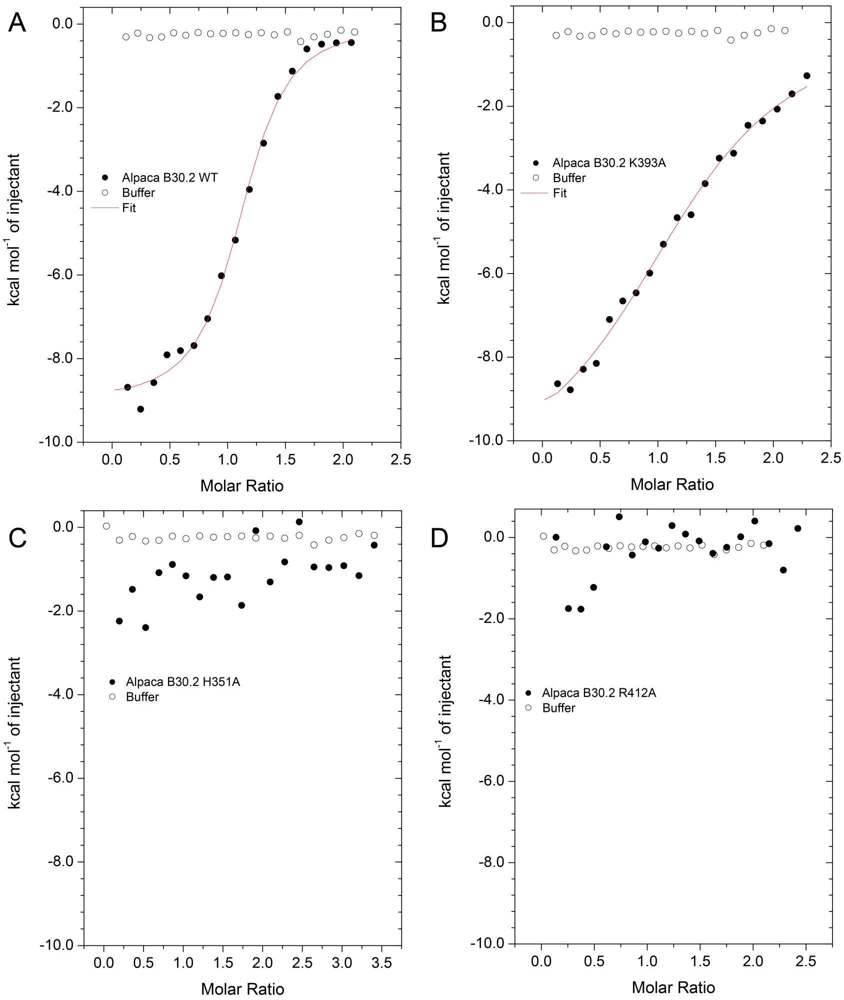


**Fig. S13: Mutations of the putative alpaca B30.2 pAg-binding pocket show conserved roles of individual residues.** ITC binding isotherm traces of mutated alpaca B30.2 domains between HMBPP and (**A**) wild-type, (**B**) K393A mutant, (**C**) H351A mutant, and (**D**) R412A mutant alpaca B30.2 domains show differential binding affinities are dependent upon which residue is being mutated. All measurements were performed using 30 µM protein and 300 µM. The buffer controls are represented by an open circle, and the protein-ligand data are represented by a filled circle. The binding curves in red were fit assuming one-site binding. H351A and R412A mutant fits are not shown. The reported molar ratio is the ratio of pAg:B30.2 domain.

**Table S1:** Primers (Sigma-Aldrich) used for the amplification and cloning of TCR and BTN3 sequences.

| Primer name | 5’ to 3’ sequence |
|---|---|
| TRGV9-fwd | GTGCAGGTCATCTAGAGCAACCGC |
| TRGC-rev | TCCATTGACTTTTCAGGCACGGTCA |
| TRDV2-fwd | GTCAGCAGATGTGTTGGTGCCTCA |
| TRDC-rev | CCAGCACCGAGAGGGACATCATGT |
| scTRGV9-fwd | TCGAGAAAGGCCCGGTCAAG |
| scTRGC-rev | GGGGCCATGTCTGCATCAAG |
| scTRDV2-fwd | ATCAAGTAAACAACCAGGTCCT |
| scTRDC-rev | TGACAAAGACAGATGGTGTGG |
| vpBTN3-MfeI-fwd | CTATCAATTGGCCGCCACCATGAAAACGGCCAGGTCCCTAGA |
| vpBTN3-MfeI-rev | ATAGCAATTGCTAGGCAGGGACAAGCAAGGAT |
| huBTN3-EcoRI-fwd | CTATGAATTCGCCGCCACCATGAAAATGGCAAGTTTCCTGGC |
| huBTN3-BglII-rev | CTATAGATCTCGCTGGACAAATAGTCAGGGC |
| vpBTN3-BglII-rev | ATAGAGATCTGGCAGGGACAAGCAAGGATGTTA |
| hu/vpBTN3-rev | GGCGCTCCTGAAGAAGGGGTCTGC |
| hu/vpBTN3-fwd | GCAGACCCCTTCTTCAGGAGCGCC |
| VpBTN3_H351RmutRev | CAAGCACACAGTA**ACG**CCAATCAAATCTC |
| VpBTN3_H351RmutFw | GAGATTTGATTGG**CGT**TACTGTGTGCTTG |
| Vγ9ins1-EcoRI-fwd | GGACCATCCTCTAGAGAATTCGCCGCCACCATGCTGTCACTGTTCCCGC |
| Vγ9ins1-rev | TGGTGAGGGTAGACGTCGAAGTGTCAGGCG |
| Vγ9ins2-fwd | TTCGACGTCTACCCTCACCATTCACAGTGT |
| Vγ9ins2-rev | TGATGGTGTTTCCCTGCTGGGATTCCAGA |
| Vγ9ins3-fwd | CCAGCAGGGAAACACCATCATGACGAATGATAC |
| Vγ9ins3-BamHI-rev | GGGGGAGGGAGAGGGGGATCCTCACGAGCCCTTCCCGTG |
| Vδ2ins1-EcoRI-fwd | GGACCATCCTCTAGAGAATTCGCCGCCACCATGCAGAGGGTCTGCTCCC |
| Vδ2ins1-rev | TCTCTGATGCCTTGAGGATCTCCAGCAGGACC |
| Vδ2ins2-fwd | GATCCTCAAGGCATCAGAGAGAGACAAAGGATC |
| Vδ2ins2-rev | GACCGAGCTTGATGGCACTGTACCTCCCAC |
| Vδ2ins3-fwd | CAGTGCCATCAAGCTCGGTCAGTATGAAGATTCCG |
| Vδ2ins3-BamHI-rev | GGGGGAGGGAGAGGGGGATCCTTAGAAGAAAAATAACTTGGTAGTC |
| Vδ2ins1-3-EcoRI-fwd | GATCGAATTCGCCGCCACCATGCAGAGGGTCTGCTCCCT |
| Vδ2ins1-3-BamHI-rev | GATCGGATCCTTAGAAGAAAAATAACTTGG |
| scVγ9ins1-rev | TGCACGGCTTGACCGGGCCTTTCTCGATACC |
| scVγ9ins2-fwd | AGGCCCGGTCAAGCCGTGCAGCACCTGC |
| scVγ9ins2-rev | GCTTTCTGTCTGTAATAATGAGCCGTGTGCC |
| scVγ9ins3-fwd | CATTATTACAGACAGAAAGCTTGATGCAGACATGG |
| scVδ2ins1-rev | CCAGCAGGACCTGGTTGTTTACTTGATCAACTTC |
| scVδ2ins2-fwd | AAACAACCAGGTCCTGCTGGAGATCCTCAAGG |
| scVδ2ins2-rev | ACAAAGACAGATGGTGTGGCAACAGACTGC |
| scVδ2ins3-fwd | GCCACACCATCTGTCTTTGTCATGAAAAATGGG |
| scVδ2ins3-BglII-rev | GGGGGAGGGAGAGGGAGATCTTTAGAAGAAAAATAACTTGGTAGTC |

**Table S2: Homology of alpaca BTN3 to human BTN3 isoforms.** The percentage of identical amino acids was calculated using NCBI blastp.

| Homology to vpBTN3 (aa identity %/query cover %) | BTN3 | BTN3-V | BTN3-C | TM | JM | B30.2 |
|---|---|---|---|---|---|---|
| **huBTN3A1** | 71/92 | **81**/100 | 66/100 | **88**/100 | 49/98 | **79**/79 |
| **huBTN3A2** | 68/58 | **81**/100 | 66/100 | 76/100 | 48/79 | - |
| **huBTN3A3** | **73**/100 | 80/100 | **68**/100 | 76/100 | **64**/98 | 76/100 |

**Table S3: List of TRGV9 CDR3 and TRGJ regions obtained by TRGV9/TRGC PCR.** The frequencies of unique CDR3+TRGJ sequences (nucleotide-identical) among translatable clones is indicated as well as CDR3 lengths and TRGJ types according to **(9)** were assigned. [1-4] CDR3+TRGJ appearing in more than one condition, [4a, 4b] CDR3 region similar to [4]. * rearrangement used by scTCR 1, ** rearrangement used by scTCR 2. Identical colors indicate identical amino acid and nucleotide sequences.

| **Animal 1** | | | | **Animal 2** | | | |
|---|---|---|---|---|---|---|---|
| ***TRGV9*: CDR3+*TRGJ*** | **CDR3 length** | ***TRGJ*** | **# Clones** | ***TRGV9*: CDR3+*TRGJ*** | **CDR3 length** | ***TRGJ*** | **# Clones** |
| **WTH-4$^{-}$ unstimulated** | | | | **WTH-4$^{-}$ unstimulated** | | | |
| ALWDTLTDGKTIKVFGSGTRLIIT | 14 | *JP-B* | 3/8 | ALWDALRKDGRTIKVFGSGTRLIIT | 15 | *JP-C* | 2/11 |
| ALWERLGTDGKTIKVFGSGTRLIIT | 15 | *JP-B* | 1/8 | ALWDARLLTDGRTIKVFGSGTRLIIT | 16 | *JP-C* | 1/11 |
| ALWDALTDGKTIKVFGSGTRLIIT | 14 | *JP-B* | 1/8 | ALWDPRTDGRTIKVFGSGTRLIIT [5] | 14 | *JP-C* | 1/11 |
| ALWDVIDGKTIKVFGSGTRLIIT | 13 | *JP-B* | 1/8 | APTDGRTIKVFGSGTRLIIT | 10 | *JP-C* | 1/11 |
| ALWEDGRTIKVFGSGTRLIVT | 11 | *JP-A* | 1/8 | ALWAARDGRTIKVFGSGTRLIIT | 13 | *JP-C* | 1/11 |
| APPPRTIKVFGSGTRLIVT | 9 | *JP-A* | 1/8 | ALWDAPVTDGRTIKVFGSGTRLIIT | 15 | *JP-C* | 1/11 |
| | | | | ALWDAQNGRTIKVFGSGTRLIVT | 13 | *JP-A* | 1/11 |
| | | | | ALWDAPRLTDGRTIKVFGSGTRLIVT | 16 | *JP-A* | 1/11 |
| | | | | ALWDARINGGRTIKVFGSGTRLIVT | 15 | *JP-A* | 1/11 |
| | | | | ALWNTSGWIKIFGEGTKLIVIPP | 11 | *JP1* | 1/11 |
| **WTH-4$^{-}$ HMBPP-stimulated** | | | | **WTH-4$^{-}$ HMBPP-stimulated** | | | |
| ALWDALPDGRTIKVFGSGTRLIVT | 14 | *JP-A* | 5/10 | ALWDRWADGRTIKVFGSGTRLIIT [6] | 14 | *JP-C* | 6/11 |
| ALWDLMTDGRTIKVFGSGTRLIVT | 14 | *JP-A* | 4/10 | ALWDARTDGRTIKVFGSGTRLIIT | 14 | *JP-C* | 2/11 |
| ALWDADGKTIKVFGSGTRLIIT | 12 | *JP-B* | 1/10 | ALWDHGRTIKVFGSGTRLIIT | 11 | *JP-C* | 1/11 |
| | | | | ALWAGQPTGRTIKVFGSGTRLIIT | 14 | *JP-C* | 1/11 |
| | | | | ALCRGPTSGWIKIFGEGTKLIVIPP | 13 | *JP1* | 1/11 |
| **WTH-4$^{+}$ unstimulated** | | | | **WTH-4$^{+}$ unstimulated** | | | |
| ALWDARSDGRTIKVFGSGTRLIVT [1] | 14 | *JP-A* | 4/20 | ALWDARDGRTIKVFGSGTRLIIT | 13 | *JP-C* | 5/18 |
| ALWEPLTDGRTIKVFGSGTRLIVT [2] | 14 | *JP-A* | 3/20 | ALWAPQTDGRTIKVFGSGTRLIIT | 14 | *JP-C* | 4/18 |
| ALWDARADGRTIKVFGSGTRLIVT [3] * | 14 | *JP-A* | 3/20 | ALWDPLPDGRTIKVFGSGTRLIIT | 14 | *JP-C* | 2/18 |
| ALWVSLRGRTIKVFGSGTRLIVT ** | 13 | *JP-A* | 2/20 | ALWDRWADGRTIKVFGSGTRLIIT [6] | 14 | *JP-C* | 2/18 |
| ALWDALADGRTIKVFGSGTRLIVT | 14 | *JP-A* | 2/20 | ALWDSLTDGRTIKVFGSGTRLIIT | 14 | *JP-C* | 2/18 |
| ALWDALTDGRTIKVFGSGTRLIVT | 14 | *JP-A* | 1/20 | ALWDVRTDGRTIKVFGSGTRLIIT | 14 | *JP-C* | 1/18 |
| ALWDARLTDGRTIKVFGSGTRLIVT | 15 | *JP-A* | 1/20 | ALWDPRTDGRTIKVFGSGTRLIIT [5] | 14 | *JP-C* | 1/18 |
| ALWDPLTDGRTIKVFGSGTRLIVT | 14 | *JP-A* | 1/20 | ALWDGITDGRTIKVFGSGTRLIVT [7] | 14 | *JP-A* | 1/18 |
| ALWDSTGLTDGRTIKVFGSGTRLIVT | 16 | *JP-A* | 1/20 | | | | |
| ALWDARTDGRTIKVFGSGTRLIVT | 14 | *JP-A* | 1/20 | | | | |
| ALWDAAADGKTIKVFGSGTRLIIT [4] | 14 | *JP-B* | 1/20 | | | | |
| **WTH-4$^{+}$ HMBPP-stimulated** | | | | **WTH-4$^{+}$ HMBPP-stimulated** | | | |
| ALWEPLTDGRTIKVFGSGTRLIVT [2] | 14 | *JP-A* | 5/17 | ALWAPQTDGRTIKVFGSGTRLIIT | 14 | *JP-C* | 7/16 |
| ALWDAAADGKTIKVFGSGTRLIIT [4] | 14 | *JP-B* | 4/17 | ALWDGITDGRTIKVFGSGTRLIVT [7] | 14 | *JP-A* | 3/16 |
| ALWDARSDGRTIKVFGSGTRLIVT [1] | 14 | *JP-A* | 2/17 | ALWDALADGRTIKVFGSGTRLIIT | 14 | *JP-C* | 2/16 |
| ALWDAAADGKTIKVFGSGTRLIVT [4a] | 14 | *JP-A/B* | 1/17 | ALWDPRTDGRTIKVFGSGTRLIIT [5] | 14 | *JP-C* | 2/16 |
| ALWDSRSDGRTIKVFGSGTRLIVT | 14 | *JP-A* | 1/17 | ALWDARRADGRTIKVFGSGTRLIVT | 15 | *JP-A* | 1/16 |
| ALWDPRSDGRTIKVFGSGTRLIVT | 14 | *JP-A* | 1/17 | ALWEPLTDGRTIKVFGSGTRLIVT [2] | 14 | *JP-A* | 1/16 |
| ALWDSRADGRTIKVFGSGTRLIVT | 14 | *JP-A* | 1/17 | | | | |
| ALWDARADGRTIKVFGSGTRLIVT [3] * | 14 | *JP-A* | 1/17 | | | | |
| ALWDAAADGRTIKVFGSGTRLIIT [4b] | 14 | *JP-B/C* | 1/17 | | | | |

**Table S4: List of TRDV2, CDR3 and TRDJ regions.** CDR3 regions (underscored) together with TRDJ amino acid sequences are listed for each condition. The frequencies of nucleotide-identical CDR3+TRDJ sequences among translatable clones is indicated as well as CDR3 lengths and TRDJ were named according to homologies to human TRDJ. The amino acid in position δ97 is marked in bold **(10)**. [1-11] CDR3+TRDJ appearing in more than one condition. Identical colors indicate identical amino acid and nucleotide sequences.

| **Animal 1** | | | | **Animal 2** | | | |
|---|---|---|---|---|---|---|---|
| ***TRDV2*: CDR3+*TRDJ*** | **CDR3 length** | ***TRDJ*** | **# Clones** | ***TRDV2*: CDR3+*TRDJ*** | **CDR3 length** | ***TRDJ*** | **# Clones** |
| **WTH-4$^{-}$ unstimulated** | | | | **WTH-4$^{-}$ unstimulated** | | | |
| ATR**M**RTGGIITGGNSVNPLIFGKGTYLNVEP [1] | 20 | J4 | 2/10 | ARS**T**IYGREYPLIFGKGTYLNVEP [4] | 13 | J4 | 1/10 |
| AAP**L**ESGIPRWRHPLIFGKGTYLNVEP [2] | 16 | J4 | 2/10 | AAP**L**ESGIPRWRHPLIFGKGTYLNVEP [2] | 16 | J4 | 2/10 |
| ATR**Q**GYGWSRDWIRVTAQLIFGKGTQLIVEP | 20 | J2 | 2/10 | ATR**I**RYTDVETRLRPLIFGKGTYLNVEP [7] | 17 | J4 | 3/10 |
| ASR**G**GYDIRLESGWETEWGSWDTRQMFFGAGTKLYVEH | 27 | J3 | 2/10 | ATR**M**RTGGIITGGNSVNPLIFGKGTYLNVEP [1] | 20 | J4 | 2/10 |
| ATH**I**RVGGRTGDLTAQLIFGKGTQLIVEP [3] | 18 | J2 | 1/10 | ATR**A**VRYRLGGAGRNLIFGKGTYLNVEP [8] | 17 | J4 | 1/10 |
| ARS**T**IYGREYPLIFGKGTYLNVEP [4] | 13 | J4 | 1/10 | AAY**V**GVGRGTAQLIFGKGTQLIVEP | 14 | J2 | 1/10 |
| **WTH-4$^{-}$ HMBPP-stimulated** | | | | **WTH-4$^{-}$ HMBPP-stimulated** | | | |
| ATH**I**RVGGRTGDLTAQLIFGKGTQLIVEP [3] | 18 | J2 | 15/17 | ATR**I**AYTGAGRRELIFGKGTQLIVEP | 15 | J2 | 11/13 |
| ATK**A**RWSRLGDRPLIFGKGTYLNVEP [5] | 15 | J4 | 1/17 | ATR**I**RYTDVETRLRPLIFGKGTYLNVEP [7] | 17 | J4 | 2/13 |
| ATT**C**VGVGYDIRTPLIFGKGTYLNVEP | 16 | J4 | 1/17 | | | | |
| **WTH-4$^{+}$ unstimulated** | | | | **WTH-4$^{-}$ unstimulated** | | | |
| ASL**M**VGGGGRGELIFGKGTYLNVEP | 14 | J4 | 1/5 | ATR**I**RYTDVETRLRPLIFGKGTYLNVEP [7] | 17 | J4 | 3/15 |
| ATA**V**RSGWSRDGRPRPLIFGKGTYLNVEP [6] | 18 | J4 | 1/5 | AAP**L**ESGIPRWRHPLIFGKGTYLNVEP [2] | 16 | J4 | 3/15 |
| ARS**T**IYGREYPLIFGKGTYLNVEP [4] | 13 | J4 | 1/5 | ATK**V**GFRERGASLIFGKGTYLNVEP | 14 | J4 | 1/15 |
| ATS**G**WSRGFEPLIFGKGTYLNVEP | 13 | J4 | 1/5 | AST**I**YGRGETPLIFGKGTYLNVEP | 13 | J4 | 1/15 |
| ASK**V**GWSRLGDRPLIFGKGTYLNVEP | 15 | J4 | 1/5 | ATS**V**RVGGFRETKGPLIFGKGTYLNVEP [9] | 17 | J4 | 1/15 |
| | | | | ATR**M**TTGVVGVGLVRGPLIFGKGTYLNVEP [10] | 19 | J4 | 1/15 |
| | | | | ATT**G**LKRGGPLIFGKGTYLNVEP | 12 | J4 | 1/15 |
| | | | | ATK**I**RRGPLIFGKGTYLNVEP | 10 | J4 | 1/15 |
| | | | | ATP**I**RSGWRDGRRPLIFGKGTYLNVEP | 16 | J4 | 1/15 |
| | | | | AIK**M**VGFRYTDWEPRPLIFGKGTYLNVEP | 18 | J4 | 1/15 |
| | | | | ANP**I**VRYTPGLGVRGPLIFGKGTYLNVEP [11] | 18 | J4 | 1/15 |
| **WTH-4$^{+}$ HMBPP-stimulated** | | | | **WTH-4$^{+}$ HMBPP-stimulated** | | | |
| ATK**A**RWSRLGDRPLIFGKGTYLNVEP [5] | 15 | J4 | 3/16 | ATR**I**RYTDVETRLRPLIFGKGTYLNVEP [7] | 17 | J4 | 4/14 |
| ATK**L**VVGIRTWETPLIFGKGTYLNVEP | 16 | J4 | 3/16 | AAP**L**ESGIPRWRHPLIFGKGTYLNVEP [2] | 16 | J4 | 3/14 |
| ATA**L**RRGRLIFGKGTYLNVEP | 10 | J4 | 2/16 | ATR**A**VRYRLGGAGRNLIFGKGTYLNVEP [8] | 17 | J4 | 2/14 |
| ATT**I**YGRGRYPLIFGKGTYLNVEP | 13 | J4 | 2/16 | ATR**M**TTGVVGVGLVRGPLIFGKGTYLNVEP [10] | 19 | J4 | 1/14 |
| ARA**I**TAYGQTPSERLIFGKGTYLNVEP | 16 | J4 | 2/16 | ATS**V**RVGGFRETKGPLIFGKGTYLNVEP [9] | 17 | J4 | 1/14 |
| ATG**V**RYTASGWRSGPLIFGKGTYLNVEP | 17 | J4 | 1/16 | ATR**V**GVGVRGQYPLIFGKGTYLNVEP | 15 | J4 | 1/14 |
| AAR**A**RETPLIFGKGTYLNVEP | 10 | J4 | 1/16 | ATR**M**RTGGIITGGNSVNPLIFGKGTYLNVEP [1] | 20 | J4 | 1/14 |
| ATT**I**RSRGRDILIFGKGTYLNVEP | 13 | J4 | 1/16 | ANP**I**VRYTPGLGVRGPLIFGKGTYLNVEP [11] | 18 | J4 | 1/14 |
| ATA**V**RSGWSRDGRPRPLIFGKGTYLNVEP [6] | 18 | J4 | 1/16 | | | | |

**Table S5: Overview of phosphoantigen reactivity of alpaca Vγ9Vδ2 TCR pairings.** CDR3 (underscored) and TRJ regions of alpaca Vγ9 and Vδ2 chains are indicated as well as phosphoantigen reactivity. Reactivity of murine responder cells transduced with TCR pairings to HMBPP stimulation in co-culture with 293T cells was rated according to magnitude of IL-2 produced by responder cells (-: no IL-2 production, +/++/+++: slight to high IL-2 production, n.a.: TCR pairing not tested).

| **Phosphoantigen reactivity of alpaca Vγ9Vδ2 TCR pairings**<br>Vγ9<br>Vδ2 | GenBank: KF734082 (9)<br>ALWAAADGRTIKVFGSGTRLIVT | vpTCR(Jδ2) (CDR3γ: 14aa)<br>ALWDARADGRTIKVFGSGTRLIVT | scTCR4/vpTCR(Jδ4)<br>ALWAPSLTDGRTIKVFGSGTRLIIT |
|---|---|---|---|
| GenBank: KF734083 (9)<br>ATSGGIYGGISLRGRESRPLIFGKGTYLNVEP | - | + | n.a. |
| GenBank: KF734084 (9)<br>AMWLESDYTDWEYPLIFGKGTYLNVEP | - | n.a. | n.a. |
| vpTCR(Jδ2)<br>ATHIRVGGRTGDLTAQLIFGKGTQLIVEP | + | +++ | n.a. |
| scTCR4/vpTCR(Jδ4)<br>ATLVGSPTRAGRPLIFGKGTYLNVEP | n.a. | n.a. | ++ |

**Table S6: Summary statistical analysis of Figure 4A.** A two-way ANOVA (repeated measures) was carried out with a Bonferroni post-hoc test (ns: p > 0.05, *: p < 0.05, **: p < 0.01, ***: p < 0.001 and ****: p < 0.0001). Results are shown for 0/0.014/0.04/0.12/0.37/1.1/3.3/10 µM HMBPP in this order.

| **Statistics summary vpTCR(Jδ2)** | **293T** | **293T A1-3 KO** | **293T A1-3 KO vpBTN3** |
|---|---|---|---|
| **293T** | x | ns/ns/ns/ns/ns/****/****/**** | ns/ns/ns/ns/ns/*/****/**** |
| **293T A1-3 KO** | ns/ns/ns/ns/ns/****/****/**** | x | ns/ns/ns/ns/ns/****/****/**** |
| **293T A1-3 KO vpBTN3** | ns/ns/ns/ns/ns/*/****/**** | ns/ns/ns/ns/ns/****/****/**** | x |

| **Statistics summary vpTCR(Jδ4)** | **293T** | **293T A1-3 KO** | **293T A1-3 KO vpBTN3** |
|---|---|---|---|
| **293T** | x | ns/ns/ns/ns/ns/**/****/**** | ns/ns/ns/ns/ns/****/****/**** |
| **293T A1-3 KO** | ns/ns/ns/ns/ns/**/****/**** | x | ns/ns/ns/ns/ns/****/****/**** |
| **293T A1-3 KO vpBTN3** | ns/ns/ns/ns/ns/****/****/**** | ns/ns/ns/ns/ns/****/****/**** | x |

| **Statistics summary huTCR** | **293T** | **293T A1-3 KO** | **293T A1-3 KO vpBTN3** |
|---|---|---|---|
| **293T** | x | ns/ns/ns/ns/ns/****/****/**** | ns/ns/ns/ns/ns/****/****/**** |
| **293T A1-3 KO** | ns/ns/ns/ns/ns/****/****/**** | x | ns/ns/ns/ns/ns/ns/ns/ns |
| **293T A1-3 KO vpBTN3** | ns/ns/ns/ns/ns/****/****/**** | ns/ns/ns/ns/ns/ns/ns/ns | x |

**Table S7: Summary statistical analysis of Figure 5.** A two-way ANOVA (repeated measures) was carried out with a Bonferroni post-hoc test (ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ and ****: $p < 0.0001$). Results are shown for 0/0.014/0.04/0.12/0.37/1.1/3.3/10 µM HMBPP in this order.

| **Statistics summary huTCR** | **293T** | **293T A1-3 KO** | **293T A1-3 KO huBTN3A1** | **293T A1-3 KO vpBTN3** | **293T A1-3 KO hu/vpBTN3** | **293T A1-3 KO vp/huBTN3** |
|---|---|---|---|---|---|---|
| **293T** | x | ns/ns/ns/ns/*/****/****/**** | ns/ns/ns/ns/*/****/*/ns | ns/ns/ns/ns/*/****/****/**** | ns/ns/ns/ns/ns/**/***/**** | ns/ns/ns/ns/*/****/****/**** |
| **293T A1-3 KO** | ns/ns/ns/ns/*/****/****/**** | x | ns/ns/ns/ns/ns/ns/ns/**** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/**/****/****/**** | ns/ns/ns/ns/ns/ns/ns/ns |
| **293T A1-3 KO huBTN3A1** | ns/ns/ns/ns/*/****/*/ns | ns/ns/ns/ns/ns/ns/ns/**** | x | ns/ns/ns/ns/ns/ns/ns/**** | ns/ns/ns/ns/**/****/****/**** | ns/ns/ns/ns/ns/ns/ns/**** |
| **293T A1-3 KO vpBTN3** | ns/ns/ns/ns/*/****/****/**** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/ns/ns/ns/**** | x | ns/ns/ns/ns/**/****/****/**** | ns/ns/ns/ns/ns/ns/ns/ns |
| **293T A1-3 KO hu/vpBTN3** | ns/ns/ns/ns/ns/**/***/**** | ns/ns/ns/ns/**/****/****/**** | ns/ns/ns/ns/**/****/****/**** | ns/ns/ns/ns/**/****/****/**** | x | ns/ns/ns/ns/**/****/****/**** |
| **293T A1-3 KO vp/huBTN3** | ns/ns/ns/ns/*/****/****/**** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/ns/ns/ns/**** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/**/****/****/**** | x |

| **Statistics summary huTCR** | **293T** | **293T A1 KO** | **293T A1 KO huBTN3A1** | **293T A1 KO vpBTN3** | **293T A1 KO hu/vpBTN3** | **293T A1 KO vp/huBTN3** |
|---|---|---|---|---|---|---|
| **293T** | x | ns/ns/ns/ns/ns/***/****/**** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/ns/***/****/**** | ns/ns/ns/ns/ns/***/**/ns | ns/ns/ns/ns/ns/ns/ns/ns |
| **293T A1 KO** | ns/ns/ns/ns/ns/***/****/**** | x | ns/ns/ns/ns/ns/****/***/*** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/***/****/****/**** | ns/ns/ns/ns/ns/ns/**/**** |
| **293T A1 KO huBTN3A1** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/ns/****/***/*** | x | ns/ns/ns/ns/ns/****/****/**** | ns/ns/ns/ns/ns/***/*/ns | ns/ns/ns/ns/ns/ns/ns/ns |
| **293T A1 KO vpBTN3** | ns/ns/ns/ns/ns/***/****/**** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/ns/****/****/**** | x | ns/ns/ns/ns/***/****/****/**** | ns/ns/ns/ns/ns/ns/**/**** |
| **293T A1 KO hu/vpBTN3** | ns/ns/ns/ns/ns/***/**/ns | ns/ns/ns/ns/***/****/****/**** | ns/ns/ns/ns/ns/***/*/ns | ns/ns/ns/ns/***/****/****/**** | x | ns/ns/ns/ns/ns/****/****/ns |
| **293T A1 KO vp/huBTN3** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/ns/ns/**/**** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/ns/ns/**/**** | ns/ns/ns/ns/ns/****/****/ns | x |

| **Statistics summary vpTCR** | **293T** | **293T A1-3 KO** | **293T A1-3 KO huBTN3A1** | **293T A1-3 KO vpBTN3** | **293T A1-3 KO hu/vpBTN3** | **293T A1-3 KO vp/huBTN3** |
|---|---|---|---|---|---|---|
| **293T** | x | ns/ns/ns/ns/ns/**/****/**** | ns/ns/ns/ns/ns/**/***/ns | ns/ns/ns/ns/ns/ns/ns/**** | ns/ns/ns/*/***/****/****/**** | ns/ns/ns/ns/ns/**/****/**** |
| **293T A1-3 KO** | ns/ns/ns/ns/ns/**/****/**** | x | ns/ns/ns/ns/ns/ns/ns/**** | ns/ns/ns/ns/ns/**/****/**** | ns/ns/ns/**/***/****/****/**** | ns/ns/ns/ns/ns/ns/ns/ns |
| **293T A1-3 KO huBTN3A1** | ns/ns/ns/ns/ns/**/***/ns | ns/ns/ns/ns/ns/ns/ns/**** | x | ns/ns/ns/ns/ns/***/****/**** | ns/ns/ns/**/****/****/****/**** | ns/ns/ns/ns/ns/ns/ns/**** |
| **293T A1-3 KO vpBTN3** | ns/ns/ns/ns/ns/ns/ns/**** | ns/ns/ns/ns/ns/**/****/**** | ns/ns/ns/ns/ns/***/****/**** | x | ns/ns/ns/*/****/****/****/**** | ns/ns/ns/ns/ns/**/****/**** |
| **293T A1-3 KO hu/vpBTN3** | ns/ns/ns/*/***/****/****/**** | ns/ns/ns/**/***/****/****/**** | ns/ns/ns/**/****/****/****/**** | ns/ns/ns/*/****/****/****/**** | x | ns/ns/ns/**/****/****/****/**** |
| **293T A1-3 KO vp/huBTN3** | ns/ns/ns/ns/ns/**/****/**** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/ns/ns/ns/**** | ns/ns/ns/ns/ns/**/****/**** | ns/ns/ns/**/****/****/****/**** | x |

| **Statistics summary vpTCR** | **293T** | **293T A1 KO** | **293T A1 KO huBTN3A1** | **293T A1 KO vpBTN3** | **293T A1 KO hu/vpBTN3** | **293T A1 KO vp/huBTN3** |
|---|---|---|---|---|---|---|
| **293T** | x | ns/ns/ns/ns/ns/ns/ns/**** | ns/ns/ns/ns/ns/ns/ns/**** | ns/ns/ns/ns/ns/ns/ns/**** | ns/ns/ns/ns/*/****/****/**** | ns/ns/ns/ns/ns/ns/ns/* |
| **293T A1 KO** | ns/ns/ns/ns/ns/ns/ns/**** | x | ns/ns/ns/ns/ns/***/****/**** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/***/****/****/**** | ns/ns/ns/ns/ns/ns/****/**** |
| **293T A1 KO huBTN3A1** | ns/ns/ns/ns/ns/ns/ns/**** | ns/ns/ns/ns/ns/***/****/**** | x | ns/ns/ns/ns/ns/***/****/**** | ns/ns/ns/ns/ns/****/****/* | ns/ns/ns/ns/ns/ns/ns/ns |
| **293T A1 KO vpBTN3** | ns/ns/ns/ns/ns/ns/ns/**** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/ns/***/****/**** | X | ns/ns/ns/ns/***/****/****/**** | ns/ns/ns/ns/ns/ns/****/**** |
| **293T A1 KO hu/vpBTN3** | ns/ns/ns/ns/*/****/****/**** | ns/ns/ns/ns/***/****/****/**** | ns/ns/ns/ns/ns/****/****/* | ns/ns/ns/ns/***/****/****/**** | x | ns/ns/ns/ns/ns/****/****/**** |
| **293T A1 KO vp/huBTN3** | ns/ns/ns/ns/ns/ns/ns/* | ns/ns/ns/ns/ns/ns/****/**** | ns/ns/ns/ns/ns/ns/ns/ns | ns/ns/ns/ns/ns/ns/****/**** | ns/ns/ns/ns/ns/****/****/**** | x |

**Table S8: Thermodynamic binding parameters determined by ITC of pAgs for Alpaca B30.2 domain.**

| Ligand | Stoichiometry (N) | $K_D$ (μM) | ΔH (kCal/mol) | ΔS (cal/mol/deg) |
|---|---|---|---|---|
| HMBPP | 1.00 ± 0 | 6.17 ± 0.15 | -7.53 ± 0.42 | -1.42 |
| IPP | 1 | 113.4 ± 15 | -1.63 ± 0.14 | 12.6 |

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