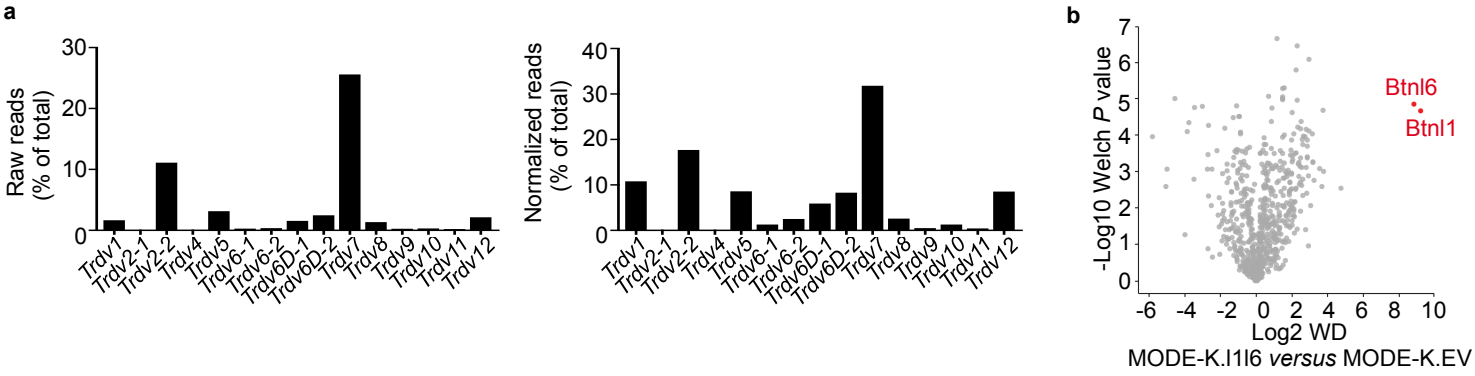
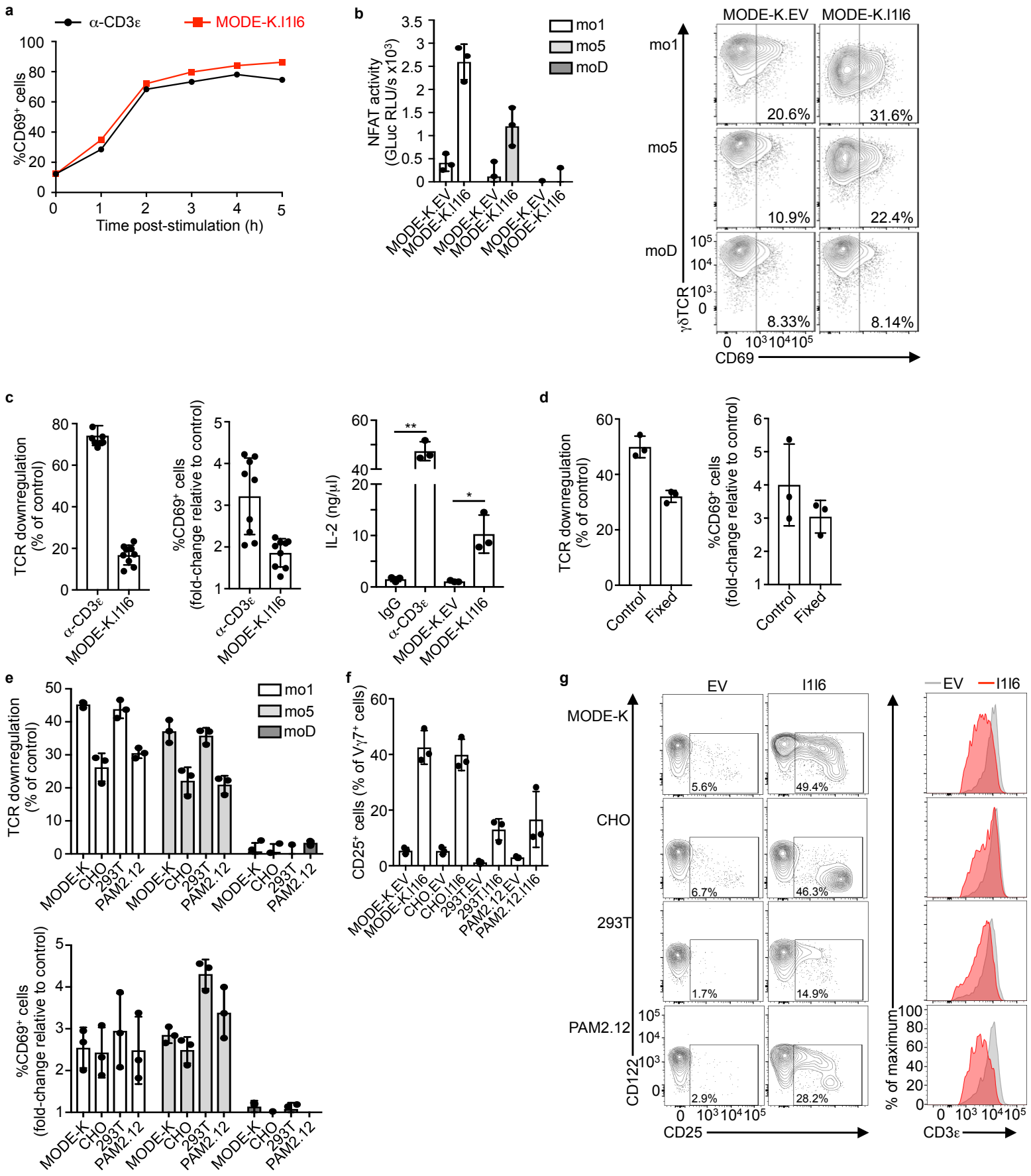


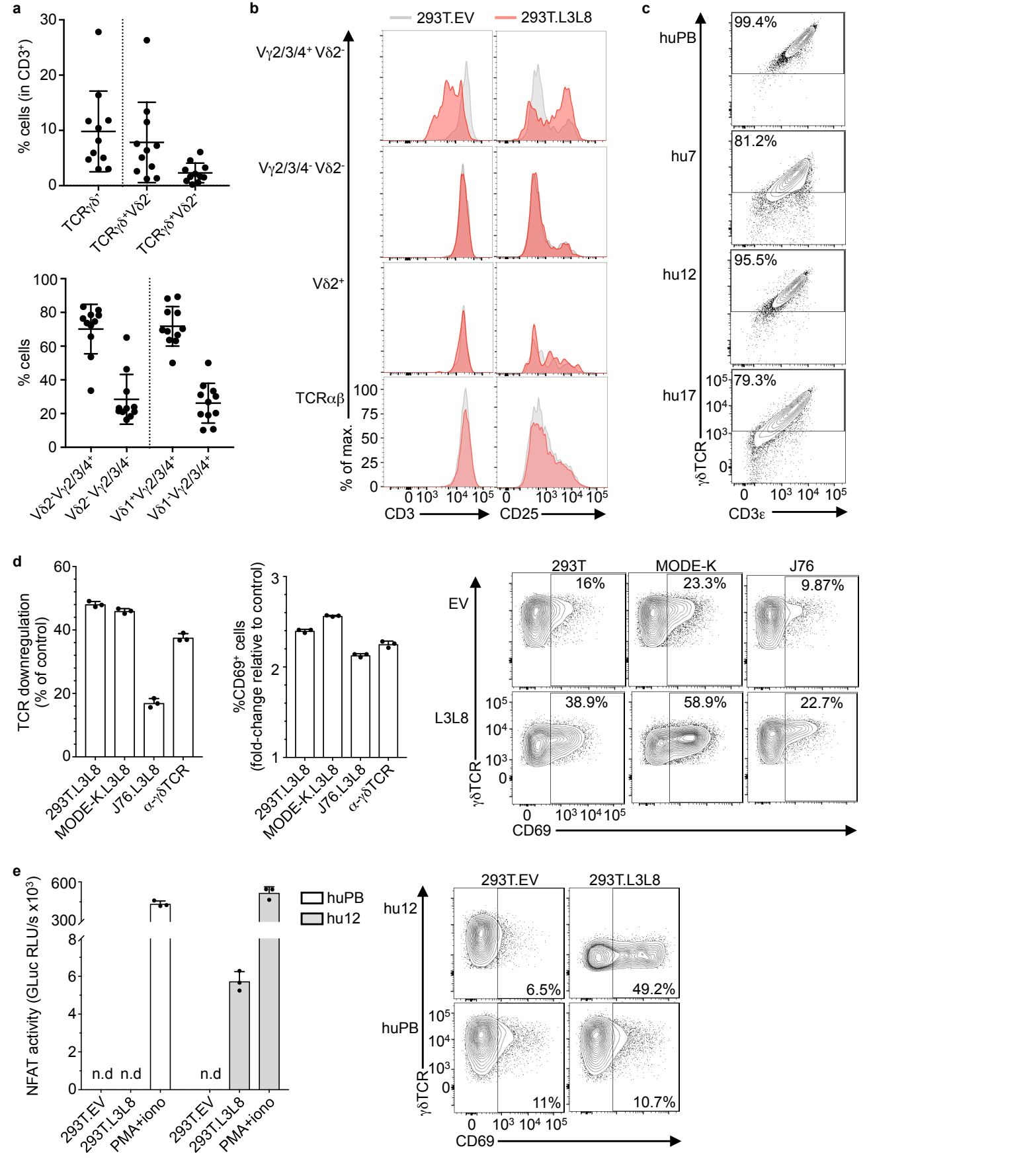
Supplementary Fig. 1. Delta chain usage of Vγ7⁻ IEL, and Btnl1+6 co-precipitation

a, TCR analysis of *Trdv* gene usage by Vγ7⁻ IEL sorted in parallel to the Vγ7⁺ IEL from Fig. 1a,b,c. Data derived from cells sorted from pooled mice IEL (n = 8). Representative of three independent sorts. **b**, Volcano plot representation of a mass-spectrometry analysis following anti-Flag pull-down on lysates from MODE-K.EV or MODE-K.l1l6 cells. Data expressed as the mean Welch difference in protein intensities in MODE-K.l1l6 *versus* MODE-K.EV samples (x-axis; Welch difference, WD) and associated *P* values (y-axis) for each protein (represented by dots), from multiple mass-spectrometry analysis replicates (n = 3)



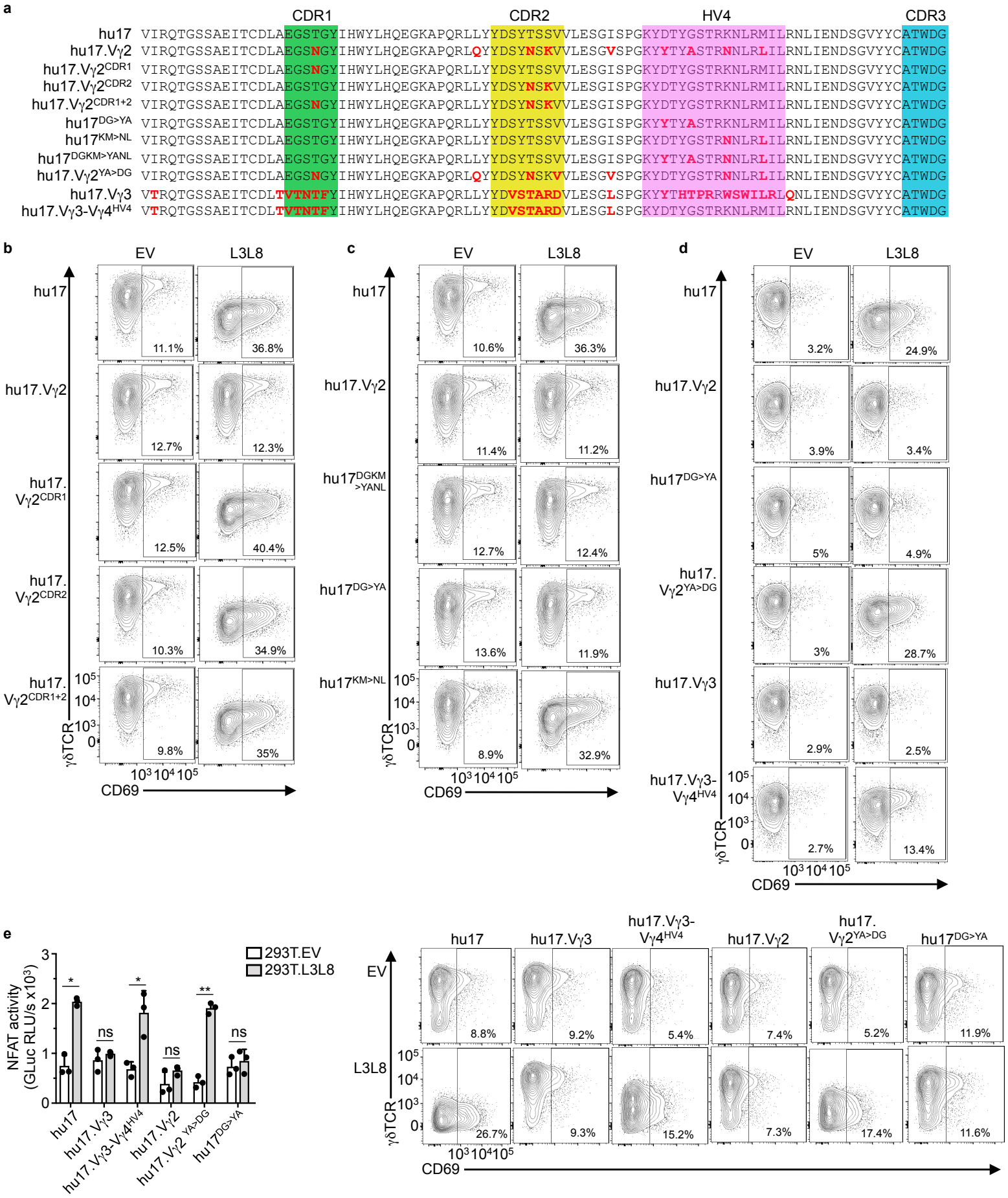
Supplementary Fig. 2. Requirements for a Btnl1+6-dependent response.

a, Flow cytometry analysis of the proportion of CD69⁺ J76-mo5 cells (y-axis) co-cultured with α-CD3ε or MODE-K.I1I6 over time (x-axis). Data expressed as mean of two experiments. **b,** Quantification of NFAT promoter activity (left) in JRT3.NFAT-GLuc cells transduced with the indicated TCRs and co-cultured with MODE-K.EV or MODE-K.I1I6 cells for 24 h. Gaussia Luciferase (GLuc) was measured in supernatants (RLU/s, relative light units per second). Data expressed as mean±s.d. (n = 3) and representative of two experiments. Cells were analysed in parallel by flow cytometry for γδTCR and CD69 expression (right) as a positive control of the response to MODE-K.I1I6 cells. **c**, Flow cytometry analysis of TCR downregulation (left), CD69 upregulation (centre) and ELISA quantification of IL-2 production (right) by E6.1-mo5 cells co-cultured with the indicated antibodies or cells lines. Flow cytometry data acquired after 5 h, expressed as mean±s.d., normalized to IgG or MODE-K.EV; pooled from nine independent experiments. ELISA data acquired after 24 h, expressed as mean±s.d. (n = 3); representative of three experiments. **d**, Flow cytometry analysis of TCR downregulation (left) and CD69 upregulation (right) by J76-mo5 cells co-cultured with untouched (control) or fixed MODE-K.I1I6 cells for 5 h. Data expressed as mean±s.d., normalized to MODE-K.EV; pooled from three independent experiments. **e**, Flow cytometry analysis of TCR downregulation (top) and CD69 upregulation (bottom) by J76-mo1, -mo5 or -moD cells co-cultured the indicated cell lines transduced with Btnl1+6. Data expressed as mean±s.d., normalized to corresponding cell line transduced with empty vector; pooled from three independent experiments. **f**, Flow cytometry analysis of the proportion of CD25⁺ cells in Vγ7⁺ IEL co-cultured with the indicated cell lines overnight. Data from individual mice expressed as mean±s.d. (n = 3). **g**, Flow cytometry analysis of CD25, CD122 and CD3ε expression by Vγ7⁺ IEL co-cultured with the indicated cell lines transduced with EV or Btnl1+6 (I1I6) overnight. Representative of co-cultures from individual mice (n = 3), related to (f).



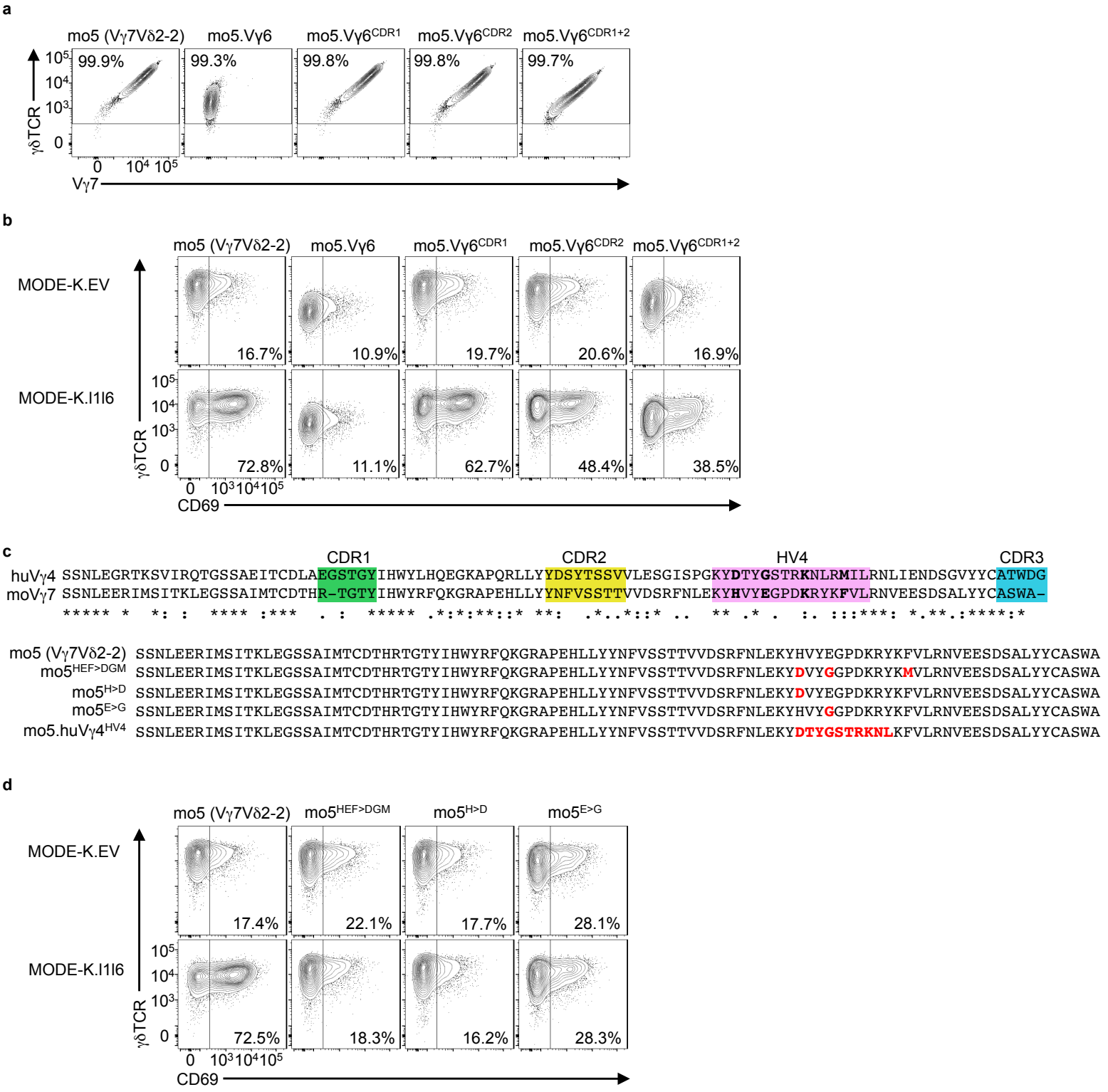
Supplementary Figure 3. The transfer of Vγ4⁺ TCRs from responding IELs confers reactivity to BTNL3+8.

a, Flow cytometry analysis of colonic γδ T cell composition, as proportion of total CD3⁺ cells (top); Vγ2/3/4 chain usage of TCRγδ⁺Vδ2⁻ cells and Vδ chain usage of Vδ2⁻Vγ2/3/4⁺ cells (bottom), after a 5 days culture. Data expressed as mean±s.d. of independent patient biopsies (n = 11). **b,** Flow cytometry analysis of CD3 and CD25 expression by the indicated human colonic lymphocyte subsets after co-culture with 293T.EV (grey) or .L3L8 (red) overnight. Representative of independent patient samples (n = 11). **c,** Flow cytometry analysis of CD3ε and γδTCR expression by J76 cells transduced with the indicated TCRs, 96h post-transduction. Representative of three independent experiments. **d,** Flow cytometry analysis of TCR downregulation (left) and CD69 upregulation (centre) by J76-hu12 cells co-cultured with the indicated cells lines or α-γδTCR antibody for 5 h. Data expressed as mean±s.d (n = 3), normalized to the matching cell lines transduced with empty vector, or control IgG. Corresponding raw flow cytometry plots are shown on the right. Representative of two (J76) or three (MODE-K) independent experiments (293T). **e,** Quantification of NFAT promoter activity (left) in JRT3.NFAT-GLuc cells transduced with the indicated TCRs and co-cultured with 293T.EV or 293T.L3L8 cells, or stimulated with PMA and ionomycin (PMA+iono) for 24 h. Gaussia luciferase (GLuc) was measured in supernatants (RLU/s, relative light units per second). Data expressed as mean±s.d (n = 3); n.d, non-detectable above background. Cells were analysed in parallel by flow cytometry for γδTCR and CD69 expression (right) as a positive control of the response to 293T.L3L8 cells. Representative of two independent experiments.



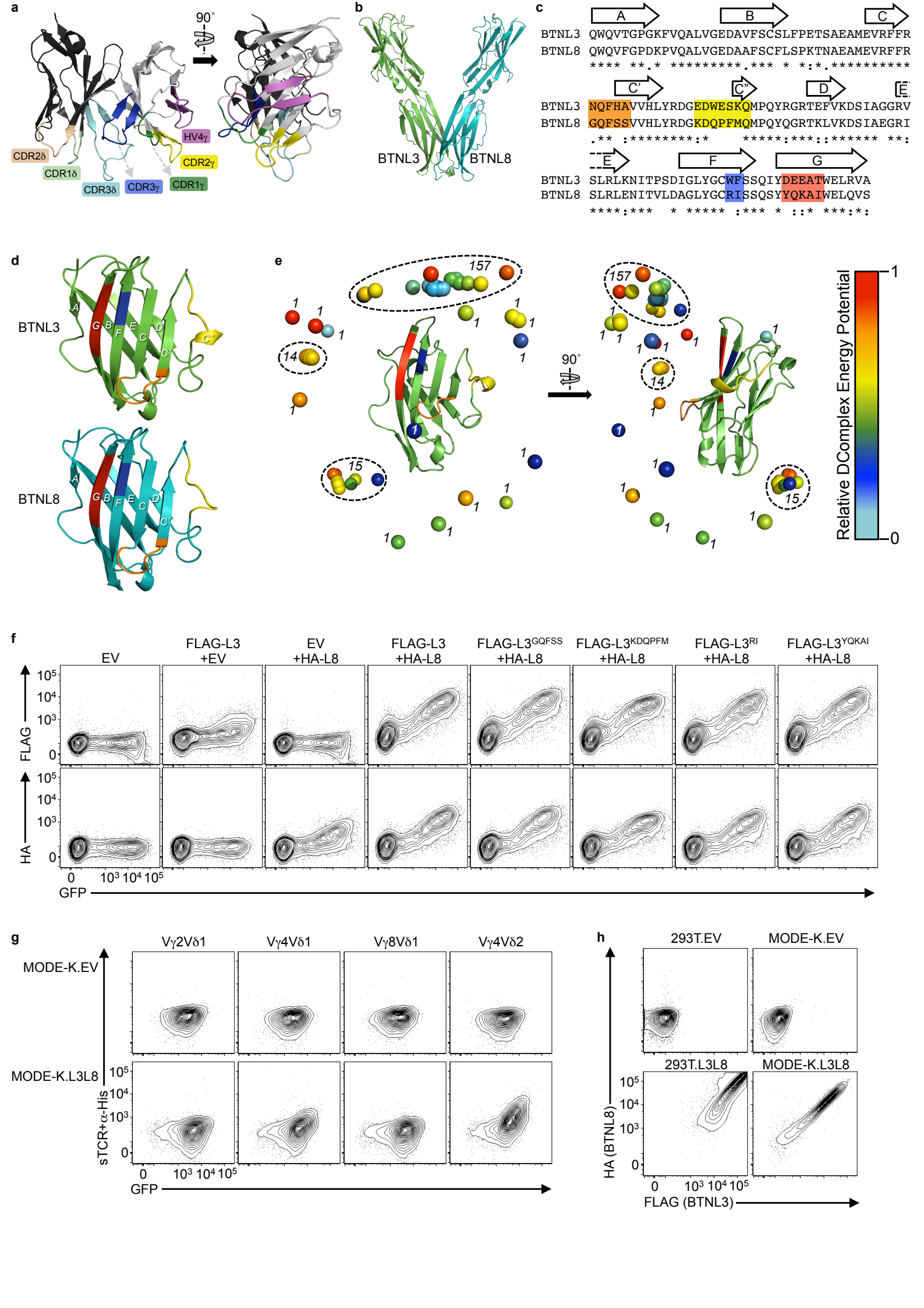
Supplementary Fig. 4. hu17 TCR variants and their responses to L3L8

a, Alignment of hu17 variants tested in Fig. 4b,c,d. Differences from the wild-type Vγ4 sequence are in bold red. CDR1/2/3 and HV4 are highlighted in green, yellow, cyan and pink, respectively. **b,c,d,** Flow cytometry analysis of γδTCR and CD69 expression by J76 cells transduced with the indicated hu17 TCR variants and co-cultured with 293T.EV or 293T.L3L8 cells for 5 h. Representative of individual co-cultures (n = 3), related to Fig. 4b,c,d. **e,** Quantification of NFAT promoter activity (left) in JRT3.NFAT-GLuc cells transduced with the indicated hu17 TCR variants and co-cultured with 293T.EV or 293T.L3L8 cells for 24 h. GLuc was measured in supernatants (RLU/s, relative light units per second). Data expressed as mean±s.d. of individual co-cultures (n = 3). *P < 0.05; **P < 0.01; ns, not significant. Cells were analysed in parallel by flow cytometry for γδTCR and CD69 expression (right) as a positive control of the response to 293T.L3L8 cells.



Supplementary Fig. 5. HV4 is required for responses to human and murine BTNL/Btnl

a, Flow cytometry analysis of Vγ7 and γδTCR expression by J76 cells transduced with the indicated TCRs and sorted to ensure equivalent expression. Representative of three independent experiments. **b,** Flow cytometry analysis of γδTCR and CD69 expression by J76 cells transduced with the indicated TCRs and co-cultured with MODE-K.EV or MODE-Kl1l6 cells for 5 h. Representative of individual co-cultures (n = 3), related to Fig. 5b. **c,** Alignments of mouse Vγ7 with human Vγ4 sequences (top; amino acids identified in Fig. 4c are bolded); and of mo5 variants tested in Fig. 5c,d (bottom; differences from wild-type Vγ7 sequence in bold red). **d,** Flow cytometry analysis of γδTCR and CD69 expression by J76 cells transduced with the indicated mo5 variants and co-cultured with MODE-K.EV or MODE-K.l1l6 cells for 5 h. Representative of individual co-cultures (n = 3), related to Fig. 5c.


a
90˚
CDR2δ
CDR1δ
CDR3δ
CDR3γ
CDR1γ
HV4γ
CDR2γ
b
BTNL3
BTNL8
c
A
B
C
C'
C"
D
E
F
G
BTNL3 QWQVTGPGKFVQALVGEDAVFSCSLFPETSAEAMEVRFFR
BTNL8 QWQVFGPDKPVQALVGEDAAFSCFLSPKTNAEAMEVRFFR
BTNL3 NQFHAVVHLYRDGEDWESKQMPQYRGRTEFVKDSIAGGRV
BTNL8 GQFSSVVHLYRDGKDQPFMQMPQYQGRTKLVKDSIAEGRI
BTNL3 SLRLKNITPSDIGLYGCWFSSQIYDEEATWELRVA
BTNL8 SLRLENITVLDAGLYGCRISSQSYYQKAIWELQVS
d
BTNL3
BTNL8
e
90˚
Relative DComplex Energy Potential
1
0
f
EV
FLAG-L3 +EV
EV +HA-L8
FLAG-L3 +HA-L8
FLAG-L3GQFSS +HA-L8
FLAG-L3KDQPFM +HA-L8
FLAG-L3RI +HA-L8
FLAG-L3YQKAI +HA-L8
FLAG
HA
GFP
g
Vγ2Vδ1
Vγ4Vδ1
Vγ8Vδ1
Vγ4Vδ2
MODE-K.EV
MODE-K.L3L8
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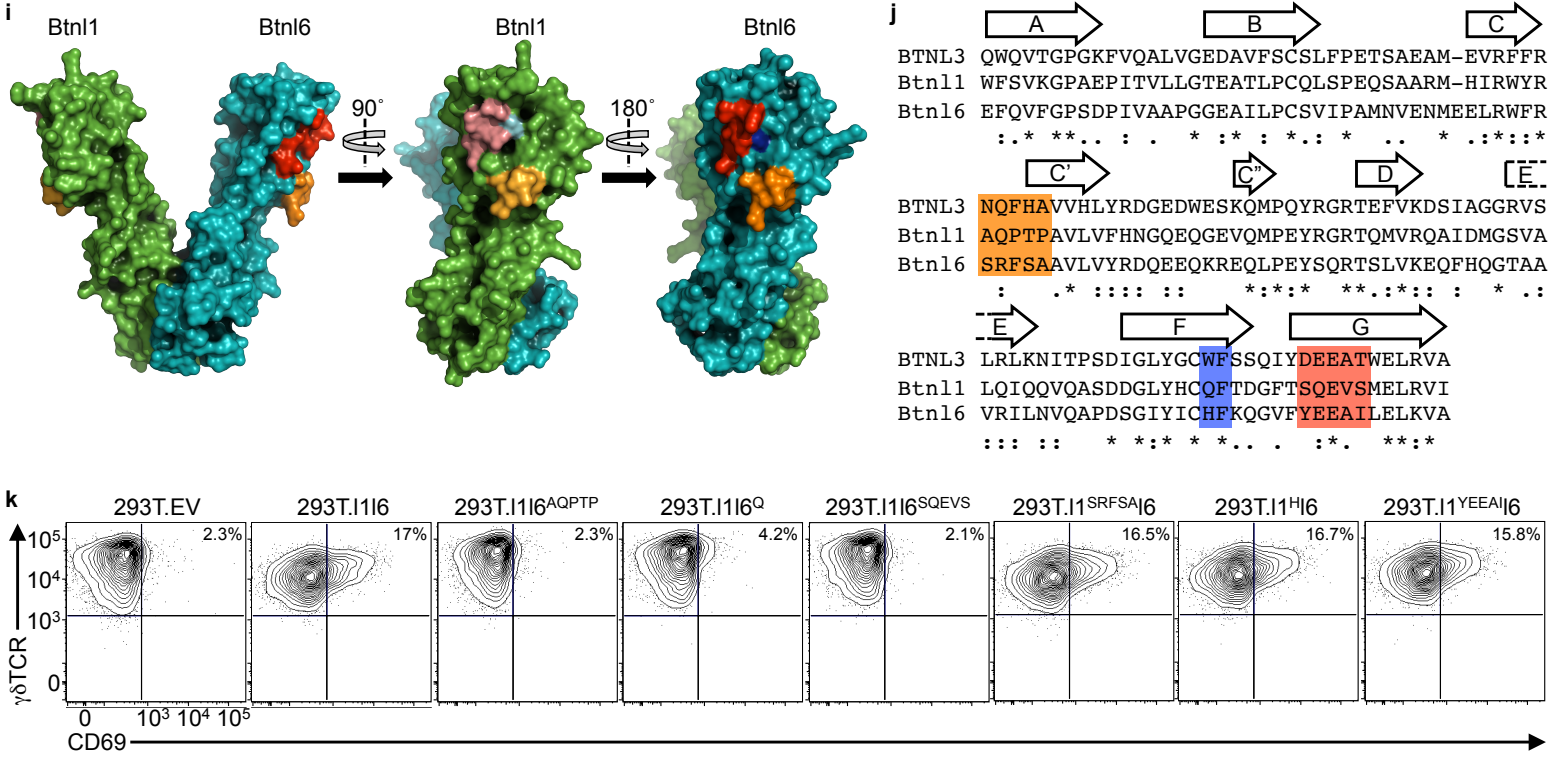
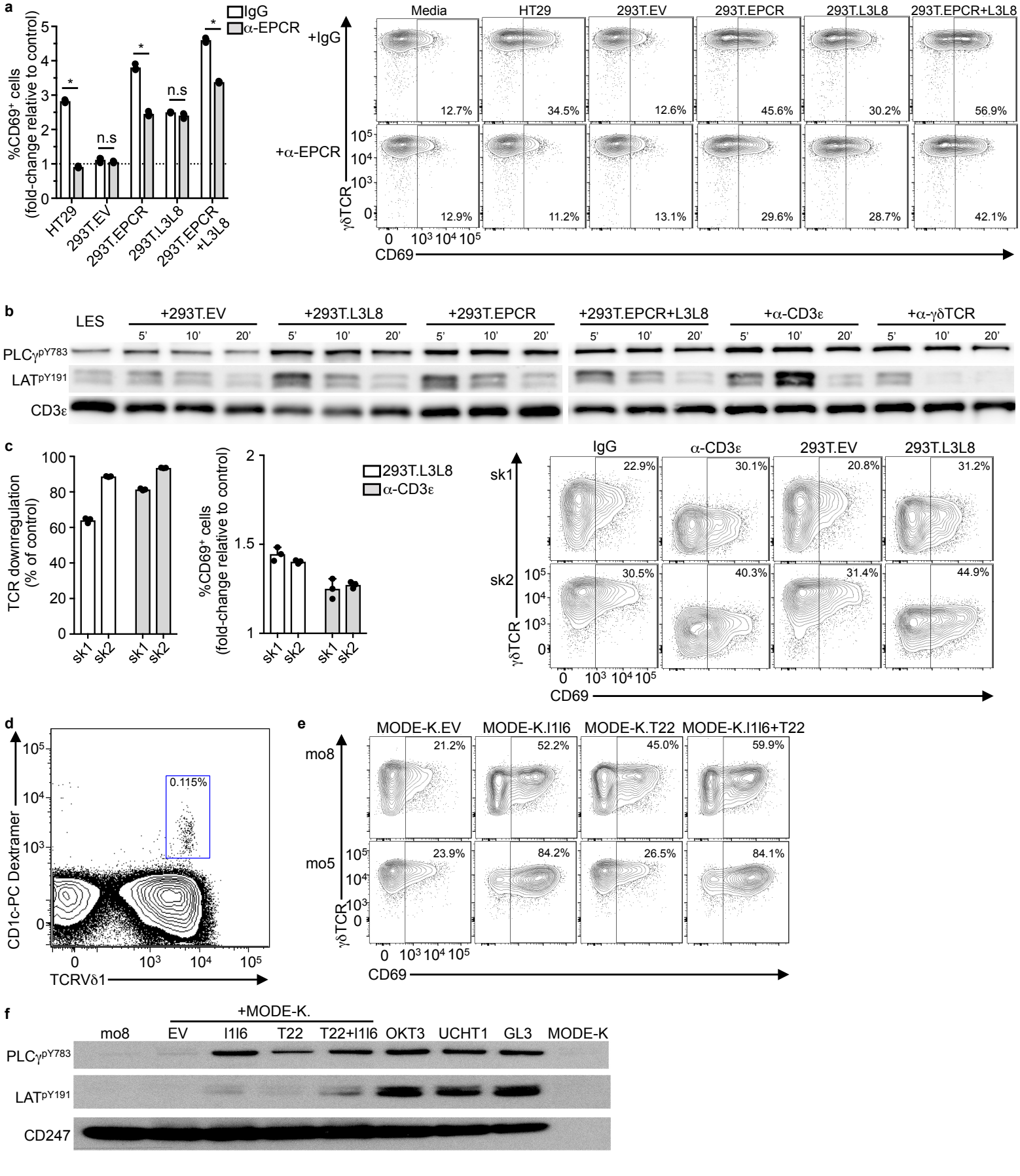
HA (BTNL8)
FLAG (BTNL3)



Supplementary Figure 6. Stepwise determination of the Vγ4 / BTNL3 interaction model

a, Cartoon representation of the crystal structure of a Vγ4Vδ1 TCR V-domains (from PDB 4MNG), with all CDRs and HV4γ highlighted. **b**, Cartoon representation of a BTNL3 (green) / BTNL8 (teal) heterodimer model, derived with 3D-JIGSAW from a BTN3A1 homodimer (PDB 4F80). **c**, Alignment of the IgV-domain sequences of BTNL3 and BTNL8. Canonical Ig-fold β-strands [A,B,C,C',C",D,E,F,G] are indicated with arrows. Solvent-exposed residues that are significantly different are highlighted in orange [NQFHA/GQFSS], yellow [EDWESK/KDQPFM], blue [WF/RI], and red [DEEAT/YQKAI]. **d**, Cartoon representation of the IgV-domains of BTNL3 (green) and BTNL8 (teal) from (b), with the same annotation as in (c). **e**, Cartoon representation of the results of 200 unrestricted docking simulation runs in SwarmDock, between the IgV-domain of BTNL3 (green) and the TCR Vγ4Vδ1 V-domains (PDB 4MNG). Each sphere represents the centroid of the TCR (numbers in italic indicate the number of solutions for groups) and are color-coded according to the relative energy between the docked poses (relative Docking Complex [DComplex] Energy Potential scale displayed on the right). Note that the IgV-domain of BTNL3 is rotated 45˚ counter-clockwise along the x-axis compared to (d). **f**, Flow cytometry analysis of FLAG-tagged BTNL3 and HA-tagged BTNL8 expression in 293T cells, 48 h post-transfection. Representative of three independent experiments. Related to Fig. 6c. **g**, Flow cytometry analysis of the binding of the indicated soluble TCRs (sTCR; pre-incubated with anti-6xHis tag antibody, α-His) after incubation with MODE-K.EV or MODE-K.L3L8 cells at 4˚C for 1 h. Representative of three independent experiments. Related to Fig. 6e. **h**, Flow cytometry analysis of FLAG-BTNL3 and HA-BTNL8 expression on the indicated cell lines that were used for the sTCR staining experiments. Related to (g) and Fig. 6e. **i**, Surface representation of a heterodimeric Btnl1 (green) / Btnl6 (teal) model, derived with 3D-JIGSAW from a BTN3A1 homodimer (PDB 4F80). Candidate CFG face motifs corresponding to the ones identified in BTNL3 (see Fig. 6b,c; and below) are highlighted in orange (AQPTP/SRFSA), blue (QF/HF), and red (SQEVS/YEEAI). **j**, Alignment of the IgV-domain sequences of BTNL3, Btnl1 and Btnl6. Canonical Ig-fold β-strands [A,B,C,C',C",D,E,F,G] are indicated with arrows. Candidates motifs are highlighted using the same colour-coding as in (i). **k**, Flow cytometry analysis of γδTCR and CD69 expression by J76-mo5 cells co-cultured with 293T cells expressed the indicated constructs for 5h. Representative of three independent experiments. Related to Fig. 6g.



Supplementary Figure 7. Dual reactivity of human and mouse γδTCRs

a, Flow cytometry analysis of CD69 upregulation (left) by JRT3-LES cells co-cultured with the indicated cell in the presence of control IgG or α-EPCR antibodies (10 μg/mL) for 3 h. Data expressed as mean±s.d. of the proportion of CD69⁺ cells in individual co-cultures (n = 3). Corresponding raw flow cytometry plots are shown (right). Representative of two independent experiments. *P < 0.001; n.s, not significant. **b,** Western blot analysis of PLCγ and LAT phosphorylation in JRT3-LES cells co-cultured with the indicated cell lines or antibodies at 37˚C for the indicated times. CD3ε, loading control. Representative of three independent experiments. **c,** Flow cytometry analysis of TCR downregulation (left) and CD69 upregulation (centre) by J76 cells transduced with the indicated TCRs and co-cultured with 293T.L3L8 cells or α-CD3ε. Data expressed as mean±s.d. of individual co-cultures (n = 3), normalized to 293T.EV or control IgG. Corresponding examples of raw flow cytometry plots are shown (right). Representative of three independent experiments. **d,** Flow cytometry analysis of CD1c-PC dextramer binding to human colonic γδTCR⁺ cells. The gate used for single-cell sorting is shown. **e,** Flow cytometry analysis of γδTCR and CD69 expression by J76-mo8 and J76-mo5 cells after co-culture with the indicated cell lines. Representative of three individual co-cultures, related to Fig. 7e. **e,** Western blot analysis of PLCγ and LAT phosphorylation in J76-mo8 cells co-cultured with the indicated cell lines or antibodies at 37˚C for 10 min. CD247, loading control. Representative of three (PLCγ) and two (LAT) independent experiments.

V usage	Clone #	CDR3γ	CDR3δ
Vγ7Vδ7	1	ASWRYSSGFHKV	AMLATDKLV
	2 (mo7)	**ASWGYSSGFHKV**	**AMGATDKLV**
	3	ASWGYSSGFHKV	AIYRFTDKLV
	4	ASWGRYSSGFHKV	AIYRSTDKLV
	5	ASWAQYSSGFHKV	AVGWGDKLV
	6	ASWAYSSGFHKV	AMGRDNAKLV
	7	ASWVYSSGFHKV	APSTTATDKLV
	8	**ASWGYSSGFHKV**	**AWGIRATDKLV**
	9	**ASWAGLSSGFHKV**	**AWGIRATDKLV**
	10	**ASWAEYSSGFHKV**	**AWGIRATDKLV**
	11	ASWAGYSSGFHKV	AMPRDATDKLV
	12	ASWAGYSSGFHKV	AMGRGTTDKLV
	13	ASWAPYSSGFHKV	ARISEGYDDKLV
	14	ASWEYSSGFHKV	AYRRDTSTDKLV
	15	ASWAEGGYSSGFHKV	ACLYRRDTDKLV
	16	ASWALYSSGFHKV	AMVGGIRVDKLV
	17 (mo6)	**ASWALSSGFHKV**	**AMGYRRDTDKLV**
	18	ASWAYSSGFHKV	AMLPRDTSSDKLV
	19	ASWGYSSGFHKV	ATYRRDTGTDKLV
	20	ASWAHSSGFHKV	AMVPYRRDTDKLV
	21	ASWAGRGSSGFHKV	AMVDIGGINTDKLV
	22	**ASWAYSSGFHKV**	**AMERISEGYELGKLV**
	23	ASWAEYSSGFHKV	AMEQVAGGIRTDKLV
	24 (mo8)	ASWAGYSSGFHKV	AMERWEGYELTDKLV
	25	ASWEYSSGFHKV	AMAIYRRDTRATDKLV
Vγ7Vδ2-2	26	ASWAGGGSSGFHKV	ALLEGPLSSDKLV
	27	ASWTYSSGFHKV	ALMASEGYADKLV
	28	ASWAGYSSGFHKV	ALMERGRGIADKLV
	29 (mo3)	**ASWARYSSGFHKV**	**ALMGIGGLATDKLV**
	30	ASWAGGGSGFHKV	ALMERGEGYEITDKLV
	31 (mo4)	**ASWAGYSSGFHKV**	**ALMERGTEGYATDKLV**
	32	ASWAVYSSGFHKV	ALMERGTEGYELSDKLV
	33 (mo5)	**ASWAGYSSGFHKV**	**ALMERGRRDTSLTDKLV**
	34	ASWAGPLYSSGFHKV	ALMERVGGIRAWSDKLV
	35	ASWAGGSSGFHKV	AIMEGGAYRRDTSSDKLV
	36	ASWAGDSSGFHKV	ALMERVGGIRVPCPDKLV
	37	ASWALYSSGFHKV	ALMERYIGGIRAWGTDKLV
	38	ASWAGYSSGFHKV	ALMERGLYRRDTSLGDKMV
Vγ7Vδ6D2	39	ASWADSSGFHKV	ALSEQGHIYTTDKFV
	40 (mo2)	ASWADSSGFHKV	ALSELSEGYEPATDKLV
	41	ASWAGYSSGFHKV	ALSELILTGGIRATDKLV
	42 (mo1)	**ASWAYSSGFHKV**	**ALSEPWHIGGIRATDKLV**
	43	ASWAGYSSGFHKV	ALSELIGAYRRDTSSDKLV

Supplementary Table 1. Murine γδTCR chain pairs identified through the single-cell analysis

Paired CDR3γ/δ amino acid sequences (red, non-germline-encoded) identified by single-cell TCR sequencing of Btnll+6-responding murine primary Vγ7^{+} IEL. Paired sequences for which both CDR3γ and δ were found in the deep sequencing analysis are depicted in bold.

Single cell PCR		
External primer sets		
mVγ7	For	ATGCTGTGGGCTCTGG
mCγ1	Rev	TTAGGATTTCTTCTCATTGCCACAG
mVδ2-2	For	ATGGTGCGGCCGTTC
mVδ6D	For	ATGGCTCCTCAGAGCCTG
mVδ7	For	ATGGAGAGGCTGCTGTGCTCTC
mCδ	Rev	TTACAAGAAAAATAACTTGGCAGTCAAGAG
hVγ2/3/4	For	ATG**G**AGTGGGCCCTAGCG
hCγ1/2	Rev	TTATGATTTCTCTCCATTGCAGCAG
hVδ1	For	ATGCTGTTCTCCAGCCTGCTG
hVδ3	For	ATGATTCTTACTGTGGGCTTTAGCTTTTTG
hCδ	Rev	TTACAAGAAAAATAACTTGGCAGTCAAGAG
Internal primer sets		
mVγ7	For	TGAAGGCCCGGACAAGAG
mCγ1	Rev	TGTGCTCTTTCCCCATTGC
mVδ2-2	For	TGCAACGTTAAACCGCTTCTC
mVδ6D	For	AAATCCATCAGCCTTGTCATTTC
mVδ7	For	AAGCACACTGGAAGACTGACATCC
mCδ	Rev	GTCGAATTCCACAATCTTCTTGG
hVγ2-8	For	CACTGGTACCTACACCAGGAGG
hCγ1/2	Rev	GGAGGAGGTACATGTAATATGCAGAG
hVδ1	For	GGTACAAGCAACTTCCCAGCAAAG
hVδ3	For	ACCGGATAAGGCAAGATTATTCC
hCδ	Rev	GGCAGCTCTTTGAAGGTTGC
Other cloning primer sets		
mVγ5	For	ATGTCAACCTCTTGGCTTTTTC
mVδ1	For	ATGCTTTGGAGATGTCCAGTC
hVγ9	For	ATGGTGTCACTGCTCCACACATC
hVδ2	For	ATGCAGAGGATCTCCTCCCTCAT
EPCR	For	GGAGCCTCAACTTCAGGATG
EPCR	Rev	TTAACATCGCCGTCCACC
T22	For	ATGTCCTGGGTCCTCAGGG
T22	Rev	TCAAGGTGACAGTAAAGACTCGCC
Sequencing primers		
mCγ1	Rev	CCAGGATAGTATTGCCATCC
mCδ	Rev	CAGACAAGCAACATTTGTTCC
hCγ	Rev	GCAGTAGTGTATCATTTGCATC
hCδ	Rev	GGTTTTACGTGATCTGTAGAATCTGTC

Supplementary Table 2. List of primers used in this study.
All sequences read 5' to 3'. For cloning of the human Vγ chains downstream of the IRES an NcoI restriction site (C^CATGG) was introduced by mutating the first nucleotide downstream of the start codon (bold red).