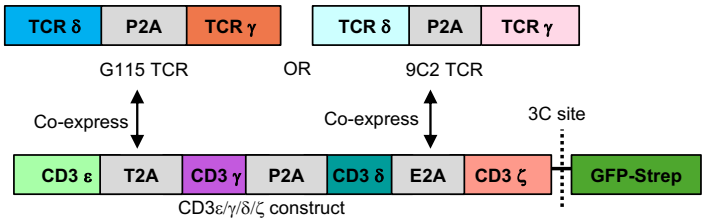
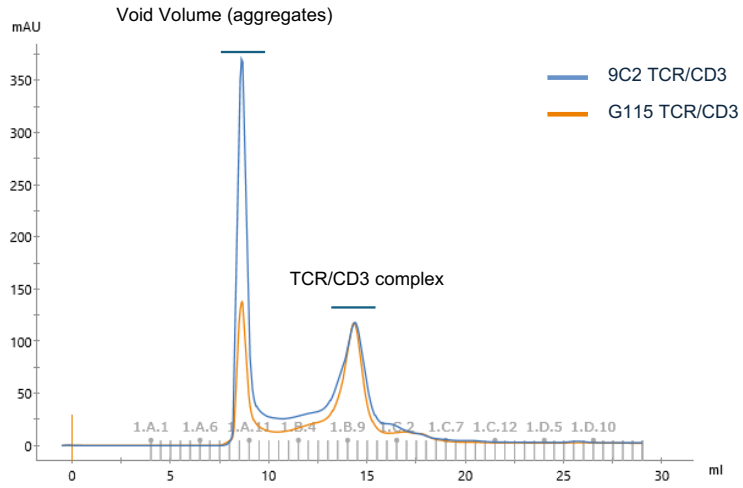
a


TCR δ
P2A
TCR γ
TCR δ
P2A
TCR γ
G115 TCR
OR
9C2 TCR
Co-express
Co-express
3C site
CD3 ε
T2A
CD3 γ
P2A
CD3 δ
E2A
CD3 ζ
GFP-Strep
CD3ε/γ/δ/ζ construct

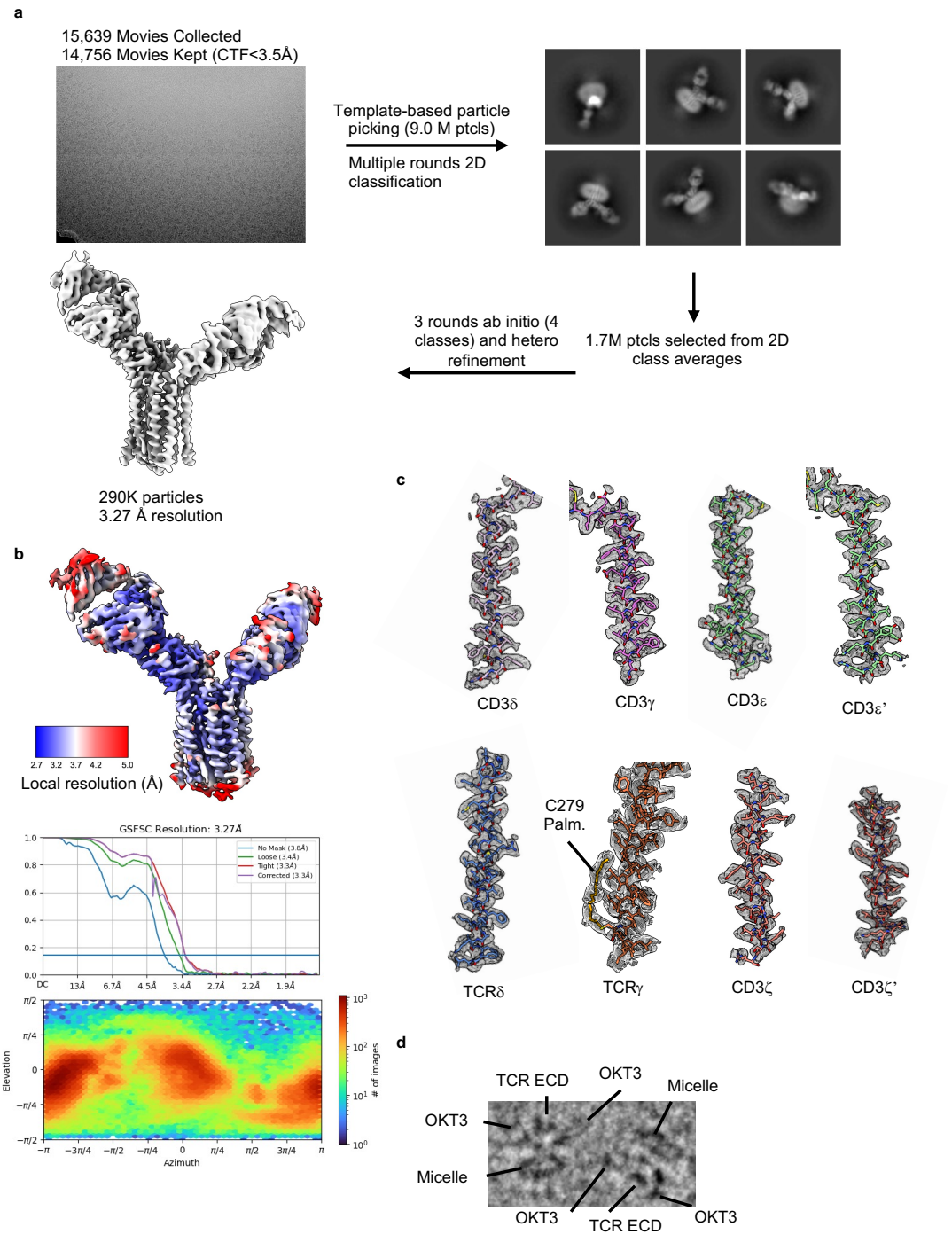
b


Void Volume (aggregates)
mAU
350
300
250
200
150
100
50
0
9C2 TCR/CD3
G115 TCR/CD3
TCR/CD3 complex
1.A.1
1.A.6
1.A.11
1.B.4
1.B.9
1.C.2
1.C.7
1.C.12
1.D.5
1.D.10
0
5
10
15
20
25
30
ml

Supplementary Figure 1. Purification of two clonotypic γδ TCR/CD3 complexes.

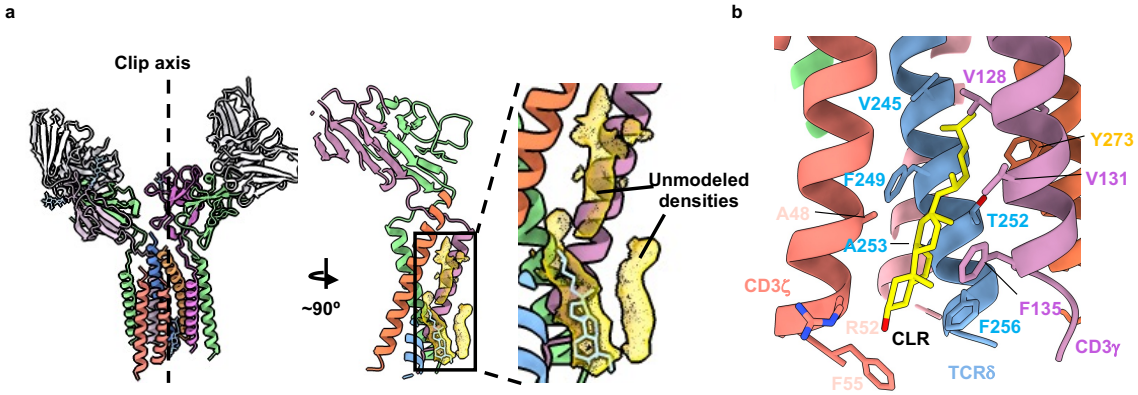
a) Schematic representation of constructs used to express the γδ TCR/CD3 complexes.

b) Size exclusion chromatography profile using Superose 6 Increase 10/300 GL column of detergent-solubilized γδ TCR/CD3 complexes following GFP-strep tag cleavage.


a
15,639 Movies Collected
14,756 Movies Kept (CTF<3.5Å)
Template-based particle picking (9.0 M ptcls)
Multiple rounds 2D classification
1.7M ptcls selected from 2D class averages
3 rounds ab initio (4 classes) and hetero refinement
290K particles
3.27 Å resolution
b
2.7 3.2 3.7 4.2 5.0
Local resolution (Å)
GSFSC Resolution: 3.27Å
No Mask (3.8Å)
Loose (3.4Å)
Tight (3.3Å)
Corrected (3.3Å)
Elevation
Azimuth
# of images
c
CD3δ
CD3γ
CD3ε
CD3ε'
TCRδ
C279
Palm.
TCRγ
CD3ζ
CD3ζ'
d
TCR ECD
OKT3
Micelle
OKT3
Micelle
OKT3
TCR ECD
OKT3

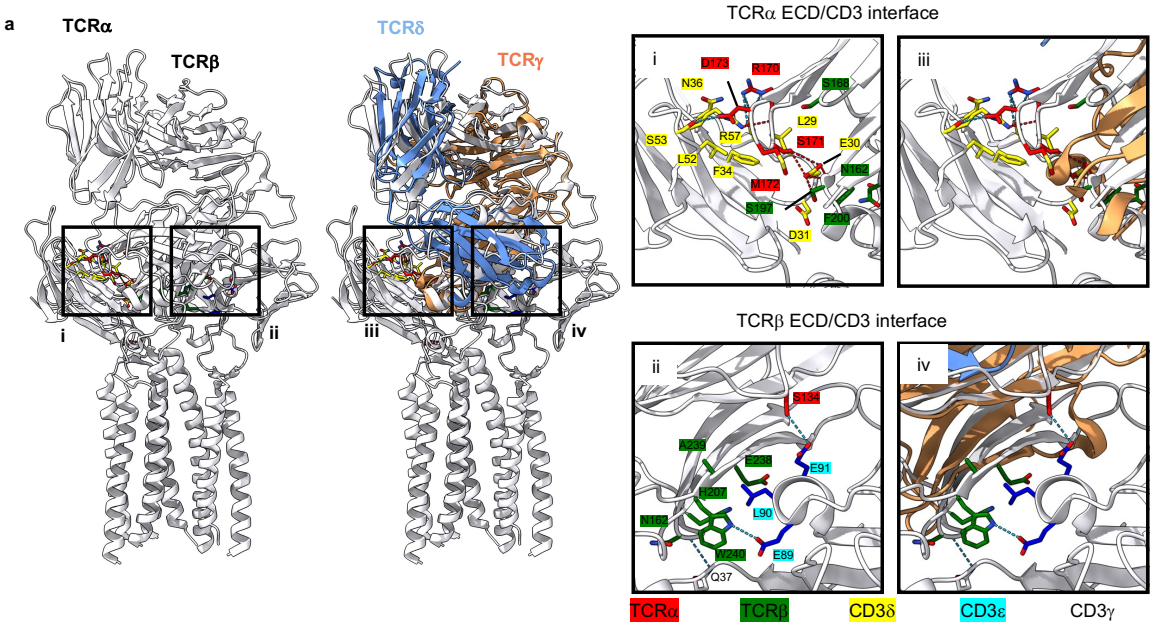
Supplementary Figure 2. CryoEM structure of OKT3 Fab-bound G115 TCR/CD3.

a) Data processing pipeline. Only representative 2D class averages are shown. **b**) CryoEM map filtered and colored according to local resolution, gold standard FSC curve, and particle orientation distribution as produced by CryoSPARC. **c**) Density fit of indicated TCR/CD3 chain TM domains into cryoEM densities. The cryoEM density protruding out of TCRγ C279 resembles a palmitoylated cysteine. This is shown for illustration but is not included in the deposited model. **d**) Two individual particles displaying the ECDs of the $\gamma\delta$ TCR chains.

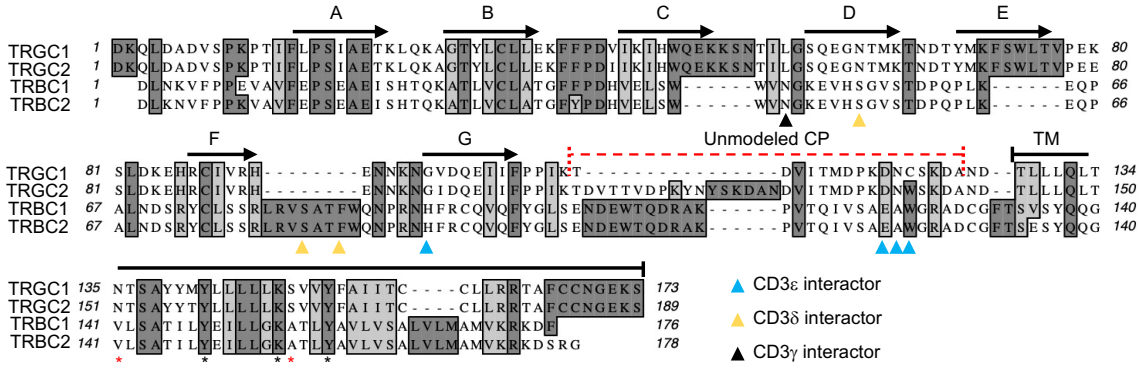

a
Clip axis
~90°
Unmodeled densities
b
V128
V245
Y273
F249
V131
A48
T252
A253
CD3ζ
F135
R52
CLR
F256
CD3γ
TCRδ
F55

Supplementary Figure 3. Sterol-shaped densities in γδTCR TM domain.

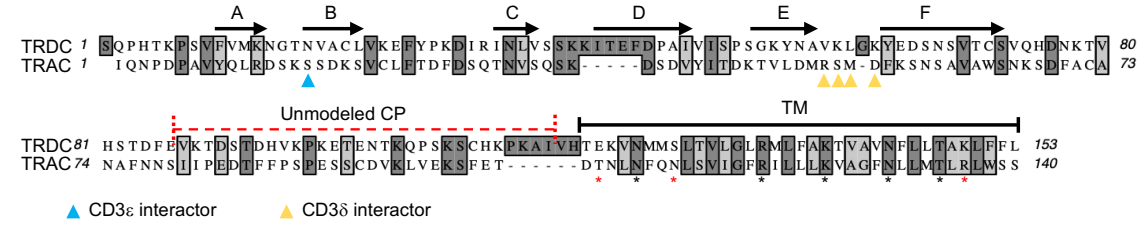
a) Analysis of sterol like densities in the TM region of the TCR. Boxed region in the middle is enlarged in the right panel. **b**) van der Waals interactions between a putative cholesterol (CLR) and residues in the TCR/CD3 chains.


a
TCRα
TCRβ
TCRδ
TCRγ
i
ii
iii
iv
TCRα ECD/CD3 interface
TCRβ ECD/CD3 interface
D175
R170
N36
S168
L29
S53
R57
S171
E30
L52
F34
M172
S169
F203
D31
S132
K233
P235
E91
T237
L90
N115
P241
E89
Q37
TCRα
TCRβ
CD3δ
CD3ε
CD3γ

b


A
B
C
D
E
F
G
TM
Unmodeled CP
TRGC1
TRGC2
TRBC1
TRBC2
CD3ε interactor
CD3δ interactor
CD3γ interactor

c

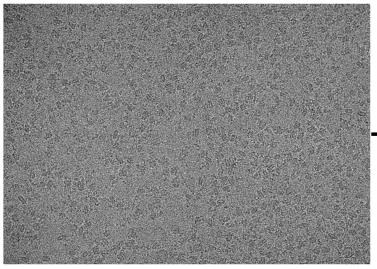

A
B
C
D
E
F
TM
Unmodeled CP
TRDC
TRAC
CD3ε interactor
CD3δ interactor

Supplementary Figure 4. αβ TCR ECD/CD3 ECD interface residues are not conserved in γδ TCR

a) Left, structure of αβ TCR/CD3 complex (PDB:8ES7) with interface residues between TCR ECDs and CD3 ECDs shown as sticks. Right, superposition of G115 γδ TCR ECD (TCRγ, orange; TCRδ, blue) onto the αβ TCR/CD3 structure. Interface residues that mediate TCR chain-CD3 chain interactions are colored as follows: Red, TCRα; Green, TCRβ; Yellow, CD3δ; Blue, CD3ε; Ghost White, CD3γ. Boxed regions on the left are enlarged on the right as indicated. **b**) Multisequence alignment of TRGC1/2 and TRBC1/2 protein sequences. **c**) Multisequence alignment of TRDC and TRAC protein sequences. Red dashed lines represent the flexible portions of the connecting peptides that are not resolved in our ECD or TM domain-containing structures. Asterisks are used to label γδTCR residues involved in apparent hydrogen bonds with CD3. Black asterisks represent residues that are conserved between αβ and γδ TCR chains. Red asterisks represent residues that are specific to γδ TCR/CD3 complexes. Arrowheads represent residues involved in TCR-CD3 contacts, colored based on which CD3 chain they interact with, as shown in A. Note: sequences in **a**) are numbered as they appear in PDB 8ES7 while sequences in **b**) and **c**) are numbered as they appear in Uniprot, using the following accession codes: TRGC1: P0CF51; TRGC2: P03986; TRBC1: P01850; TRBC2: A0A5B9; TRDC: B7Z8K6; TRAC: P01848.

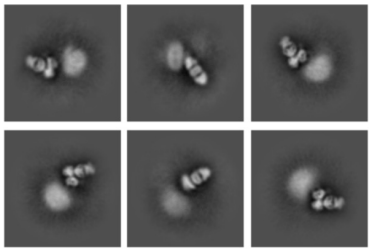
a

8,599 Movies Collected
8,521 Movies Kept (CTF<3.5Å)



10.0 M 2D template-based picks

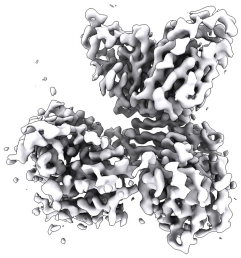
Multiple rounds 2D classification



~250K ptcls from 2D class averages

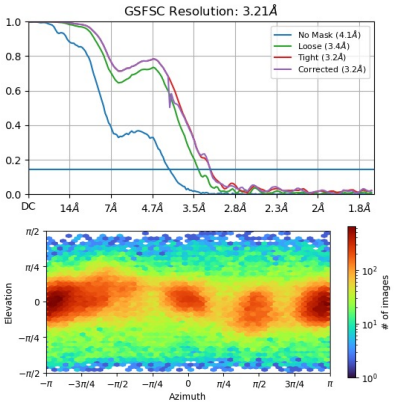
Ab initio (2 classes)

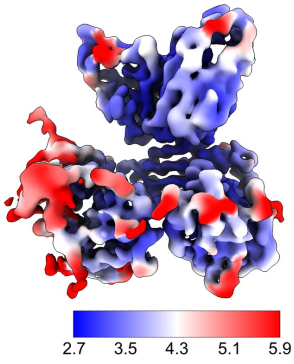
Local refinement
Applying mask
around γ9δ2 ECD
and Fab V domain



G115 ECD + Fab1 complex
156K particles
3.21 Å resolution

b



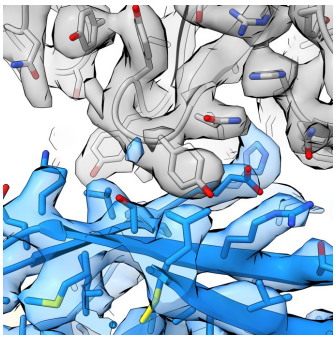


Local resolution (Å)

c

Fab 1

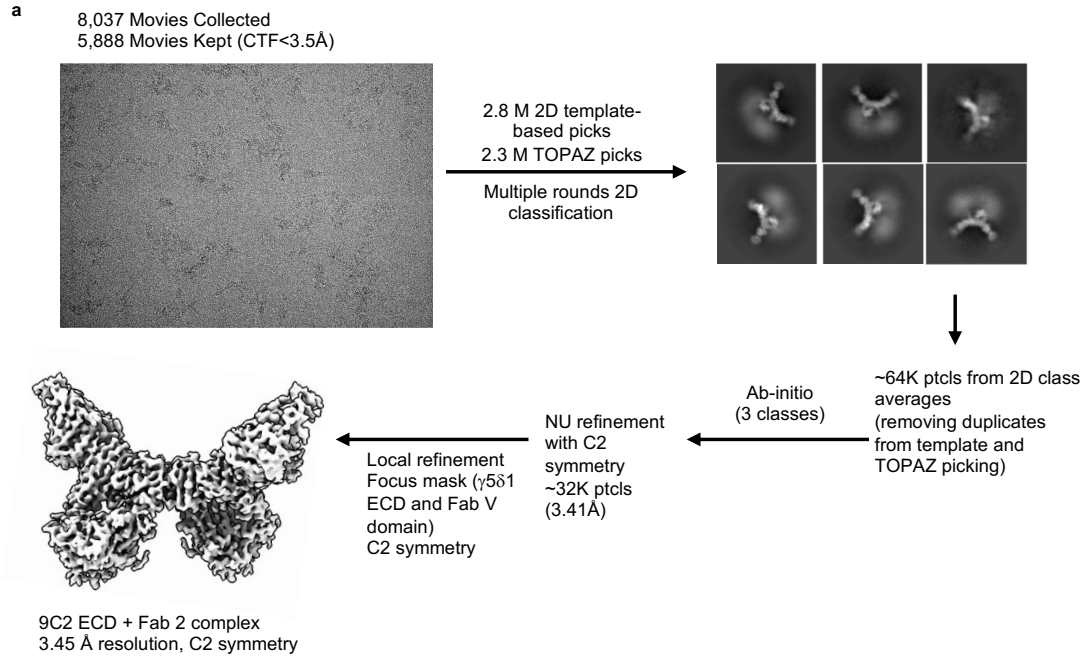
TCRδ1



Fab 1/TCRδ1 interface

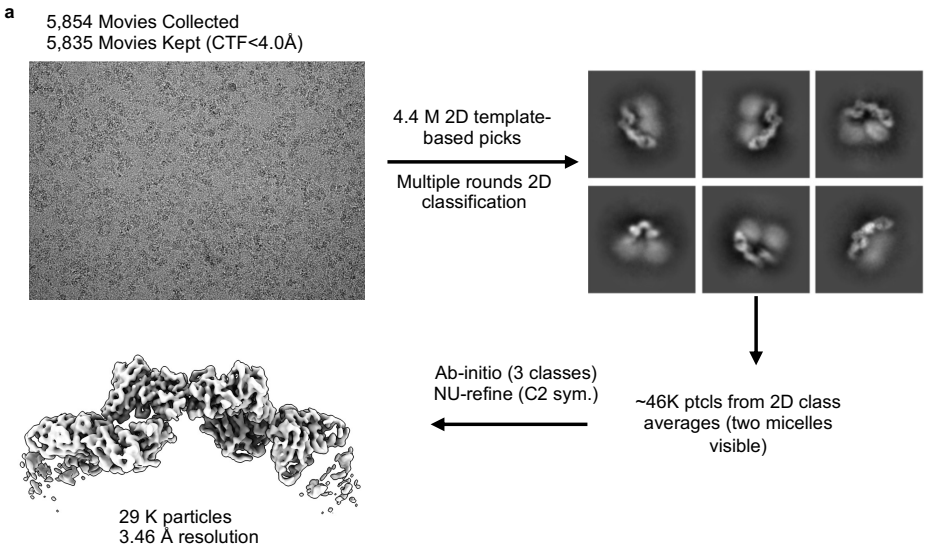
Supplementary Figure 5. CryoEM structure of Fab 1-bound G115 TCR ECD.

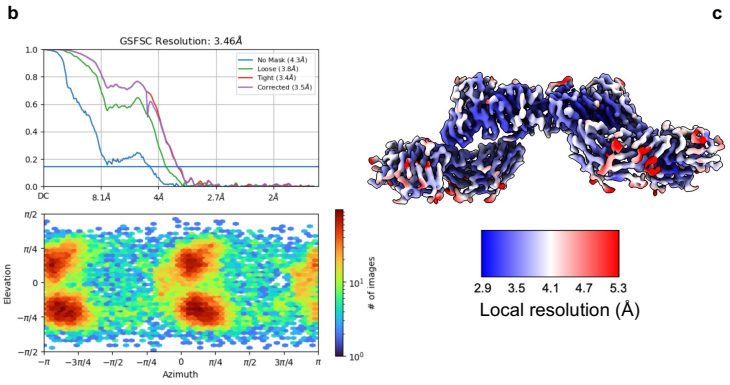
a) Data processing pipeline. Only representative 2D class averages are shown. **b**) CryoEM map filtered and colored according to local resolution, gold standard FSC curve, and particle orientation distribution as produced by CryoSPARC. **c**) Expanded view showing fit of model to density at the Fab 1-Vδ2 interface.

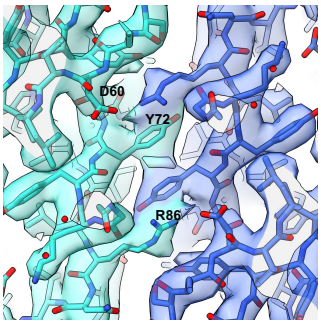

a
8,037 Movies Collected
5,888 Movies Kept (CTF<3.5Å)
2.8 M 2D template-based picks
2.3 M TOPAZ picks
Multiple rounds 2D classification
~64K ptcls from 2D class averages (removing duplicates from template and TOPAZ picking)
Ab-initio (3 classes)
NU refinement with C2 symmetry ~32K ptcls (3.41Å)
Local refinement Focus mask (γ5δ1 ECD and Fab V domain) C2 symmetry
9C2 ECD + Fab 2 complex
3.45 Å resolution, C2 symmetry
b
GSFSC Resolution: 3.45Å
No Mask (6.4Å)
Loose (3.9Å)
Tight (3.5Å)
Corrected (3.4Å)
Elevation
Azimuth
# of images
Local resolution (Å)
2.3 3.3 4.3 5.3 6.3
c
D60
Y72
R86
Vγ5/ Vγ5 interface

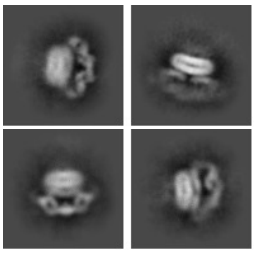
Supplementary Figure 6. CryoEM structure of Fab 2-bound 9C2 TCR ECD.

a) Data processing pipeline. Only representative 2D class averages are shown. **b**) CryoEM map filtered and colored according to local resolution, gold standard FSC curve, and particle orientation distribution as produced by CryoSPARC. **c**) Expanded view showing fit of model to density at the Vγ5/Vγ5 interface.


a
5,854 Movies Collected
5,835 Movies Kept (CTF<4.0Å)
4.4 M 2D template-based picks
Multiple rounds 2D classification
~46K ptcls from 2D class averages (two micelles visible)
Ab-initio (3 classes)
NU-refine (C2 sym.)
29 K particles
3.46 Å resolution

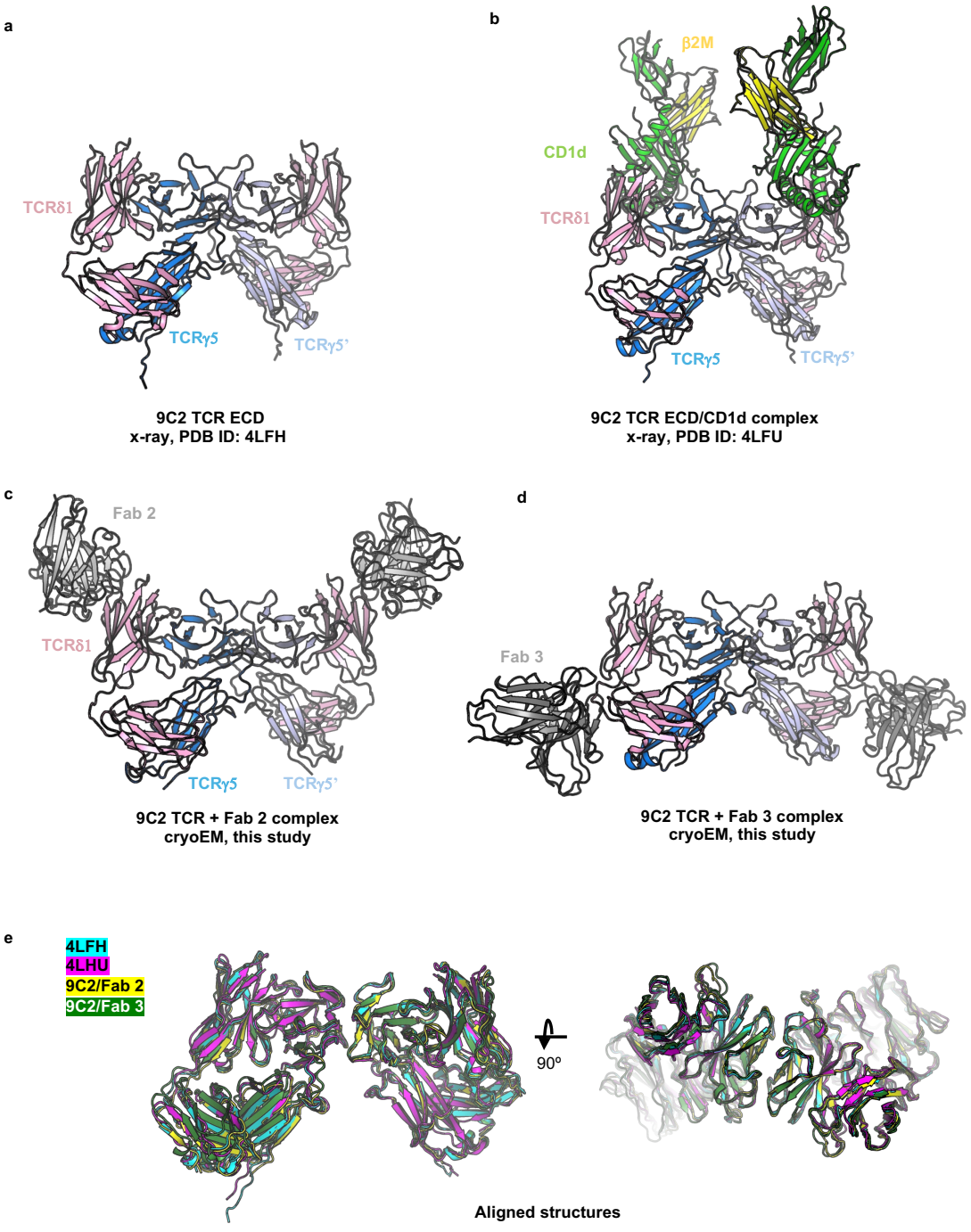

b
GSFSC Resolution: 3.46Å
No Mask (4.3Å)
Loose (3.8Å)
Tight (3.4Å)
Corrected (3.5Å)
DC
8.1Å
4Å
2.7Å
2Å
Elevation
Azimuth
# of images
2.9 3.5 4.1 4.7 5.3
Local resolution (Å)


c
D60
Y72
R86
Vγ5/ Vγ5 interface


d
~10K ptcls
(no further processing)

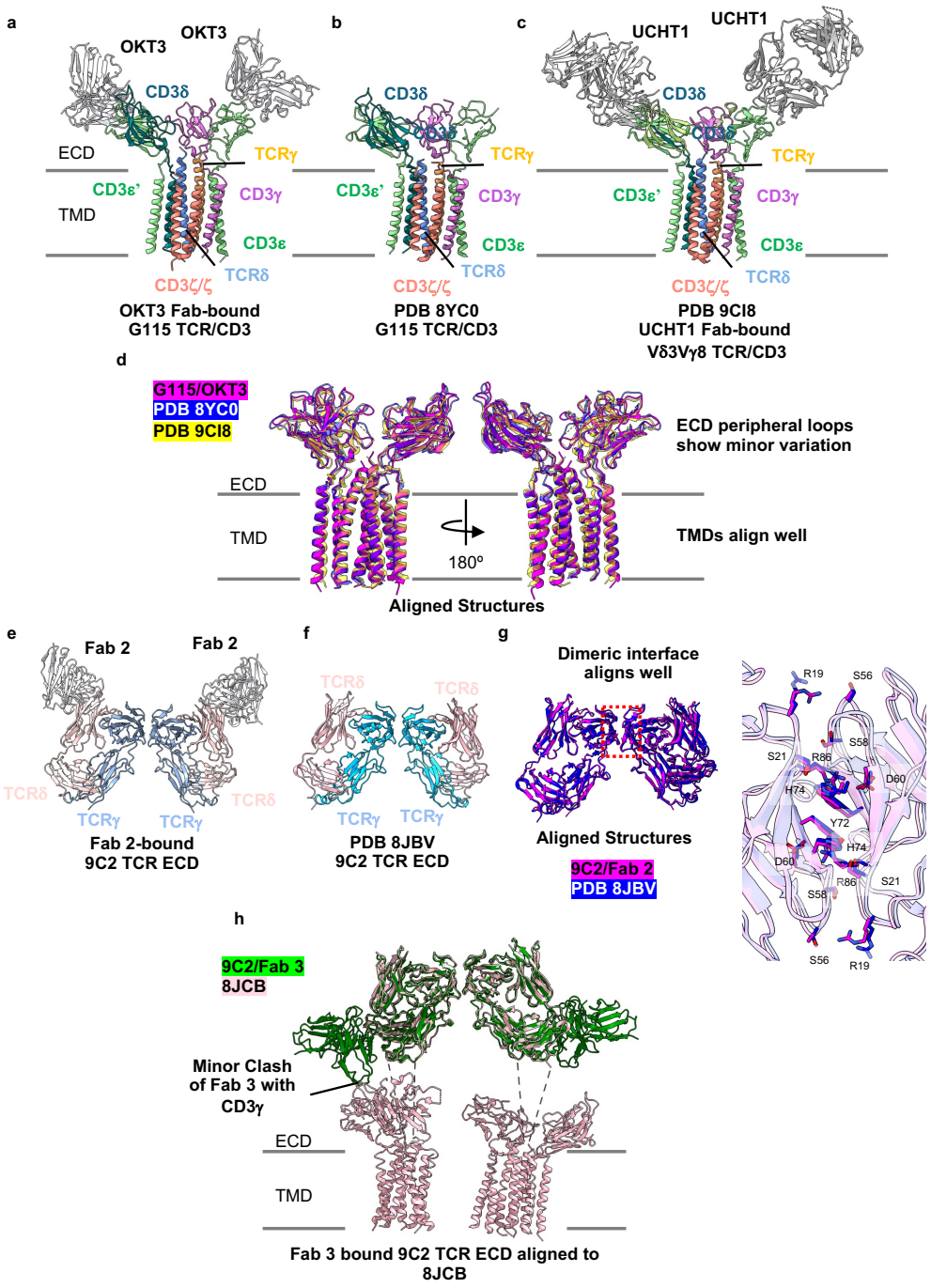
Supplementary Figure 7. CryoEM structure of Fab 3-bound 9C2 TCR ECD.

a) Data processing pipeline. Only representative 2D class averages are shown. **b**) CryoEM map filtered and colored according to local resolution, gold standard FSC curve, and particle orientation distribution as produced by CryoSPARC. **c**) Expanded view showing fit of model to density at the Vγ5/Vγ5 interface. **d**) 2D class averages of a minor subset of particles showing dimeric 9C2 TCR ECDs contained within one micellar density. 3D reconstruction was not successful for these particles.


a
TCRδ1
TCRγ5
TCRγ5'
9C2 TCR ECD
x-ray, PDB ID: 4LFH
b
β2M
CD1d
TCRδ1
TCRγ5
TCRγ5'
9C2 TCR ECD/CD1d complex
x-ray, PDB ID: 4LFU
c
Fab 2
TCRδ1
TCRγ5
TCRγ5'
9C2 TCR + Fab 2 complex
cryoEM, this study
d
Fab 3
9C2 TCR + Fab 3 complex
cryoEM, this study
e
4LFH
4LHU
9C2/Fab 2
9C2/Fab 3
90°
Aligned structures

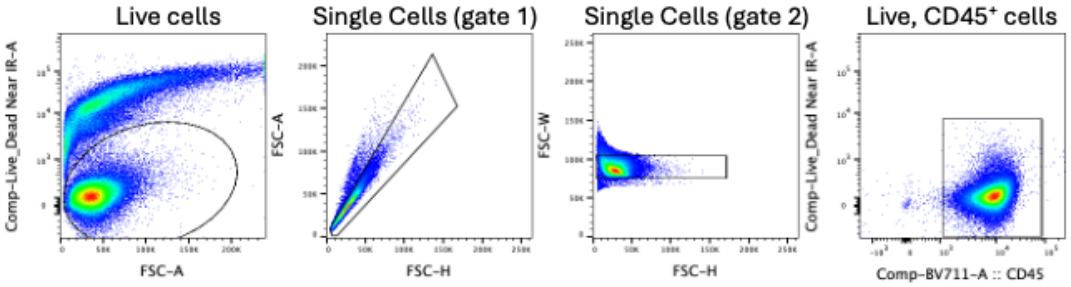
Supplementary Figure 8. Vγ5-mediated dimerization in 9C2 TCR ECD x-ray structures

a, b) Neighboring asymmetric units in 9C2 TCR ECD (**a**) and 9C2 TCR ECD in complex with CD1d (**b**) display a Vγ5-Vγ5 interaction. **c, d**) cryoEM structures of 9C2 TCR bound by Fab 2 (**c**) and Fab 3 (**d**) shown from the same viewing angle as **a** and **b**. **e**) Aligned structures (using TCR γ chain) from **a-b** with binding partners hidden for clarity. Each of the structures shows the same Vγ5-mediated dimerization mode.


a
OKT3
OKT3
CD3δ
ECD
TCRγ
CD3ε'
CD3γ
TMD
CD3ε
TCRδ
CD3ζ/ζ
OKT3 Fab-bound
G115 TCR/CD3
b
CD3δ
TCRγ
CD3ε'
CD3γ
CD3ε
TCRδ
CD3ζ/ζ
PDB 8YC0
G115 TCR/CD3
c
UCHT1
UCHT1
CD3δ
TCRγ
CD3ε'
CD3γ
CD3ε
TCRδ
CD3ζ/ζ
PDB 9CI8
UCHT1 Fab-bound
Vδ3Vγ8 TCR/CD3
d
G115/OKT3
PDB 8YC0
PDB 9CI8
ECD peripheral loops
show minor variation
ECD
TMD
180°
TMDs align well
Aligned Structures
e
Fab 2
Fab 2
TCRδ
TCRδ
TCRγ
TCRγ
Fab 2-bound
9C2 TCR ECD
f
TCRδ
TCRδ
TCRγ
TCRγ
PDB 8JBV
9C2 TCR ECD
g
Dimeric interface
aligns well
Aligned Structures
9C2/Fab 2
PDB 8JBV
R19
S56
S58
S21
R86
H74
D60
Y72
H74
D60
R86
S21
S58
S56
R19
h
9C2/Fab 3
8JCB
Minor Clash
of Fab 3 with
CD3γ
ECD
TMD
Fab 3 bound 9C2 TCR ECD aligned to
8JCB

Supplementary Figure 9. Comparison of cryoEM structures from this study to published γδ TCR structures

a) Structure of OKT3 Fab-bound G115 TCR/CD3 complex, with the TCR and CD3 chains colored as indicated. **b**) PDB: 8YC0, apo-G115 TCR/CD3 complex structure. **c**) PDB: 9CI8, UCHT1 Fab-bound Vδ3Vγ8 TCR/CD3 complex structure. **d**) Structural alignment of the three γδ TCR/CD3 structures shown in **a-c**. Complexes are colored as indicated. **e**) Structure of Fab 2-bound 9C2 γδ TCR ECD. **f**) PDB 8JVB, dimeric γδ 9C2 TCR ECD. **g**) Alignment of Fab 2 bound 9C2 TCR ECD to 8JBV. Boxed region in left panel is enlarged in right panel. Dimeric interface residues are shown as sticks and are colored based on the corresponding model. **h**) Alignment of Fab 3-bound 9C2 TCR ECD to published medium resolution structure of full-length 9C2 γδ TCR/CD3 complex (PDB 8JCB). Note: a minor clash is observed between Fab 3 and CD3γ of one asymmetric unit of 8JCB, which likely would not prevent binding of Fab 3 due to the flexibility of the γδ TCR ECD.


Live cells
Comp-Live_Dead Near IR-A
FSC-A
Single Cells (gate 1)
FSC-A
FSC-H
Single Cells (gate 2)
FSC-W
FSC-H
Live, CD45⁺ cells
Comp-Live_Dead Near IR-A
Comp-BV711-A :: CD45

Supplementary Figure 10. Gating strategy for flow cytometry experiment shown in Fig. 5C.