Figure S1. Single and dual lysin kill switches. (a) TetON single lysin kill switch schematic. Lysin expression is repressed by tetracycline repressor (TetR) and induced by anhydrotetracycline (atc) or doxycycline (doxy) which bind TetR and prevent it from binding DNA.

(b) Impact of atc on growth of BCG-TetON single lysin strains. The paper disc contains 1 μg of atc.

(c) TetOFF dual lysin kill switch schematic. Reverse TetR (Rev TetR) binds to DNA in complex with atc or doxy to repress D29L. TetR represses PipR and in the presence of atc/doxy PipR is expressed and represses L5L.

(d) Impact of atc on growth of BCG-TetOFF-DL. The paper disc contains 1 μg of atc.

Figure S2. In vitro characterization of BCG kill switch strains.

(a) Growth of BCG TetON single and dual lysin kill switch strains with and without atc. OD_{580} data are means from duplicate cultures.

(b) Western blot analysis of culture filtrates from BCG-TetON-DL and BCG-TetOFF-DL strains grown in the absence of detergent. A whole cell lysate of WT BCG serves as control. Eno and PrcB were enriched in culture filtrate of BCG-TetON-DL after 6 and 9 days of growth in the presence of atc and in culture filtrate of BCG-TetOFF-DL after 6 and 9 days of growth in the absence of atc indicating cell lysis.

(c) Expression of two lysins reduces the fraction of escape mutants compared to expression of single lysins. Cultures were grown in the presence of atc. Symbols represent data from 3-6 individual cultures and means ± SD are depicted. Significance was determined by one-way analysis of variance (ANOVA) with Tukey's adjusted p-values shown; **** $P < 0.0001$.

(d) Growth of BCG TetOFF single and dual lysin kill switch strains with and without atc. Data are means ± SD from triplicate cultures. Error bars are too small to be seen.

(e) Expression of two lysins reduces the fraction of escape mutants compared to expression of single lysins. Cultures were grown in the absence of atc. Symbols represent data from 6 individual cultures and means ± SD are depicted. Statistical significance was assessed by one-way ANOVA with Tukey's adjusted p-values shown; **** $P < 0.0001$, ***, $p < 0.001$.

Figure S3. Survival of BCG, BCG-TetOFF-DL and BCG-TetON-DL following intravenous vaccination.

(a) CFU from lungs and spleens of mice infected with BCG and BCG-TetOFF-DL not receiving doxy.

(b) CFU quantification from lungs and spleens of mice infected with BCG and BCG-TetON-DL treated with doxy.

Data are means ± SD from 4-5 mice per group and time point.

(c) CFU quantification from lungs and spleens of BCG-TetOFF-DL infected SCID mice treated or not with doxy for the indicated times. Data are means ± SD from 4 mice per group and time point.

(a,b,c) Multiple unpaired t-tests run on log_{10}-transformed data at each time point with Holm-Šídák adjusted p-values shown. **** $p < 0.0001$, ***, $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, # $0.05 < p < 0.10$, ns $p > 0.10$.

(d,e) Lung sections stained with hematoxylin and eosin from SCID mice infected with BCG-TetOFF-DL for 84 days. (d) Mice were treated with doxy from day 1-14. (e) Mice were treated with doxy from day 1- 84. Each section is from an individual mouse and is representative of each lung.

Figure S4. BCG-TetON-DL and wt BCG provide similar protection against Mtb infection in mice. Mice were i.v. vaccinated with BCG or BCG-TetON-DL or received PBS.

(a,b) Quantification of effector memory CD4 and CD8 T cells in mouse lungs from mice vaccinated with BCG and BCG-TetON-DL. Symbols are data from individual mice with lines indicating mean ± SD. Two-way ANOVA was performed with Tukey's adjusted p-values shown for each time point. **** $p < 0.0001$, ***, $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns $p > 0.10$.

(c) Quantification of cytokine producing antigen specific CD4 T cells from mice vaccinated with BCG and BCG-TetON-DL on day 30 post vaccination. Lung cells were restimulated ex vivo with PPD prior to intracellular

cytokine staining. Two-way ANOVA with Tukey's adjusted p-values shown for each cytokine producing population. For most of the cytokine producing cells, there were no statistically significant differences among the treatment groups. **** $p < 0.0001$, ***, $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns $p > 0.10$.

(d) Bacterial burden in lungs and spleens of vaccinated and PBS treated mice. Mice were infected with Mtb H37Rv by aerosol 90 days post vaccination. Symbols represent data from individual mice with lines indicating mean ± SEM. Two-way ANOVA performed on log_{10}-transformed data with Tukey's adjusted p-values shown at each time point. **** $p < 0.0001$, ***, $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns $p > 0.10$.

Figure S5 Histologic analysis indicates microgranulomas in spleen, liver and lymph nodes 8 weeks post- BCG-TetOFF-DL. H&E staining of fixed spleen (a), lymph node (b) and liver (c) tissue. (d, e) Lymphocyte composition of lung tissue (d, n=4) and thoracic lymph nodes (e, n=3) from NHP in persistence study at necropsy.

Figure S6 T cell responses in airways are similar between BCG-TetOFF-DL and WT BCG after vaccination. Total number of cells, CD4+, CD8+, effector memory CD4+ and CD8+ cells during the vaccination phase with BCG-TetOFF-DL, WT BCG or unvaccinated macaques. BAL samples were obtained pre-vaccination and 4, 12, 20 weeks post vaccination and stimulated with Mtb WCL. Flow cytometry was performed with intracellular staining for effector molecules IFN-γ, TNF, IL-17, IL-2, GzmB, GzmK, granulysin or perforin. Multiple unpaired t-tests were used to compare groups at each time point with Holm-Šídák multiple comparison adjusted p-values (# $0.05 < p < 0.10$). Median and IQR shown.

Figure S7 Individual effector molecules produced by T cells in lungs of vaccinated and challenged NHP. Total number of CD4+, CD8+, effector memory CD4+ and CD8αβ+ cells in the lung at necropsy (n=8). Cells producing either cytokines or cytotoxic molecules (IFN-γ, TNF, IL-17, IL-2, GzmB, GzmK, granulysin or perforin) were analyzed. Mann-Whitney p-values reported. Median shown.

Figure S8 Splenomegaly reduces over time post IV-BCG vaccination. Spleen size at necropsy for macaques in persistence study and macaques vaccinated and challenged with Mtb. Dashed lines represent normal spleen size range of adult male MCMs.

Figure S9 Gating strategy

Table S1: Flow cytometry panel for NHP samples

Table S2 Full details on macaques used in this study

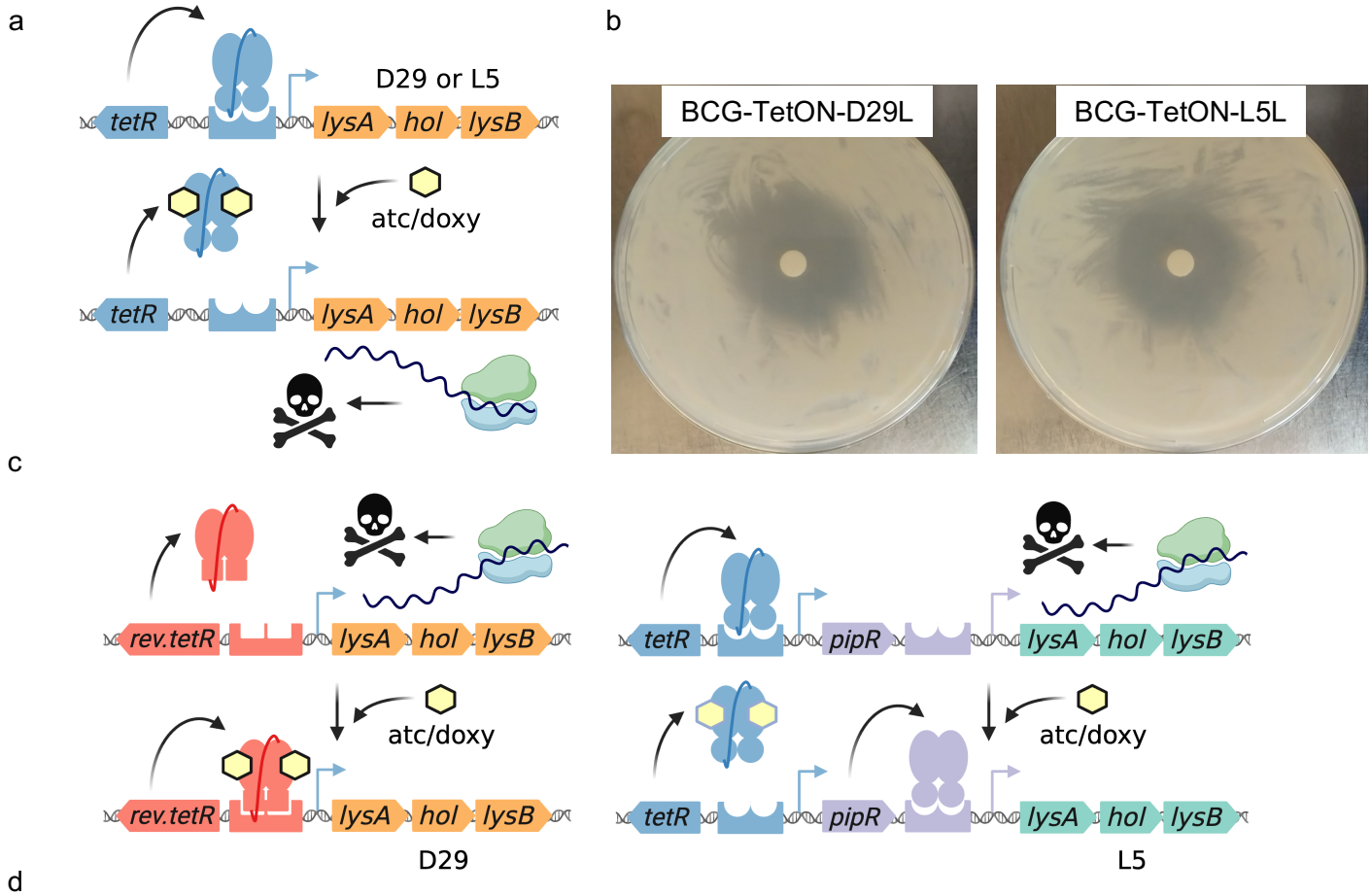
a
D29 or L5
tetR
lysA
hol
lysB
atc/doxy
b
BCG-TetON-D29L
BCG-TetON-L5L
c
rev.tetR
D29
tetR
pipR
L5
d
BCG-TetOFF-DL

Figure S1

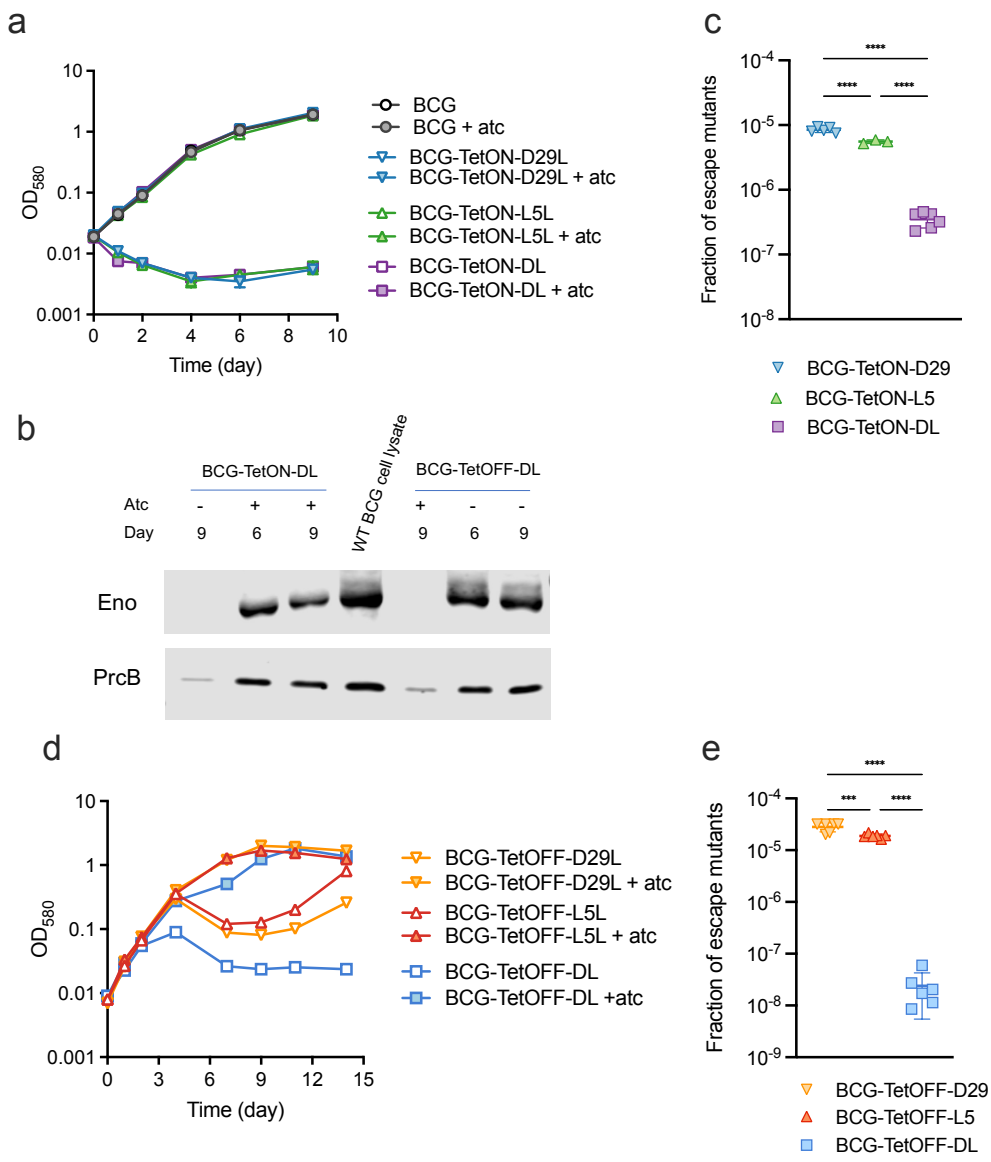
a
OD580
Time (day)
BCG
BCG + atc
BCG-TetON-D29L
BCG-TetON-D29L + atc
BCG-TetON-L5L
BCG-TetON-L5L + atc
BCG-TetON-DL
BCG-TetON-DL + atc
c
Fraction of escape mutants
BCG-TetON-D29
BCG-TetON-L5
BCG-TetON-DL
b
BCG-TetON-DL
BCG-TetOFF-DL
Atc
Day
WT BCG cell lysate
Eno
PrcB
d
OD580
Time (day)
BCG-TetOFF-D29L
BCG-TetOFF-D29L + atc
BCG-TetOFF-L5L
BCG-TetOFF-L5L + atc
BCG-TetOFF-DL
BCG-TetOFF-DL +atc
e
Fraction of escape mutants
BCG-TetOFF-D29
BCG-TetOFF-L5
BCG-TetOFF-DL

Figure S2

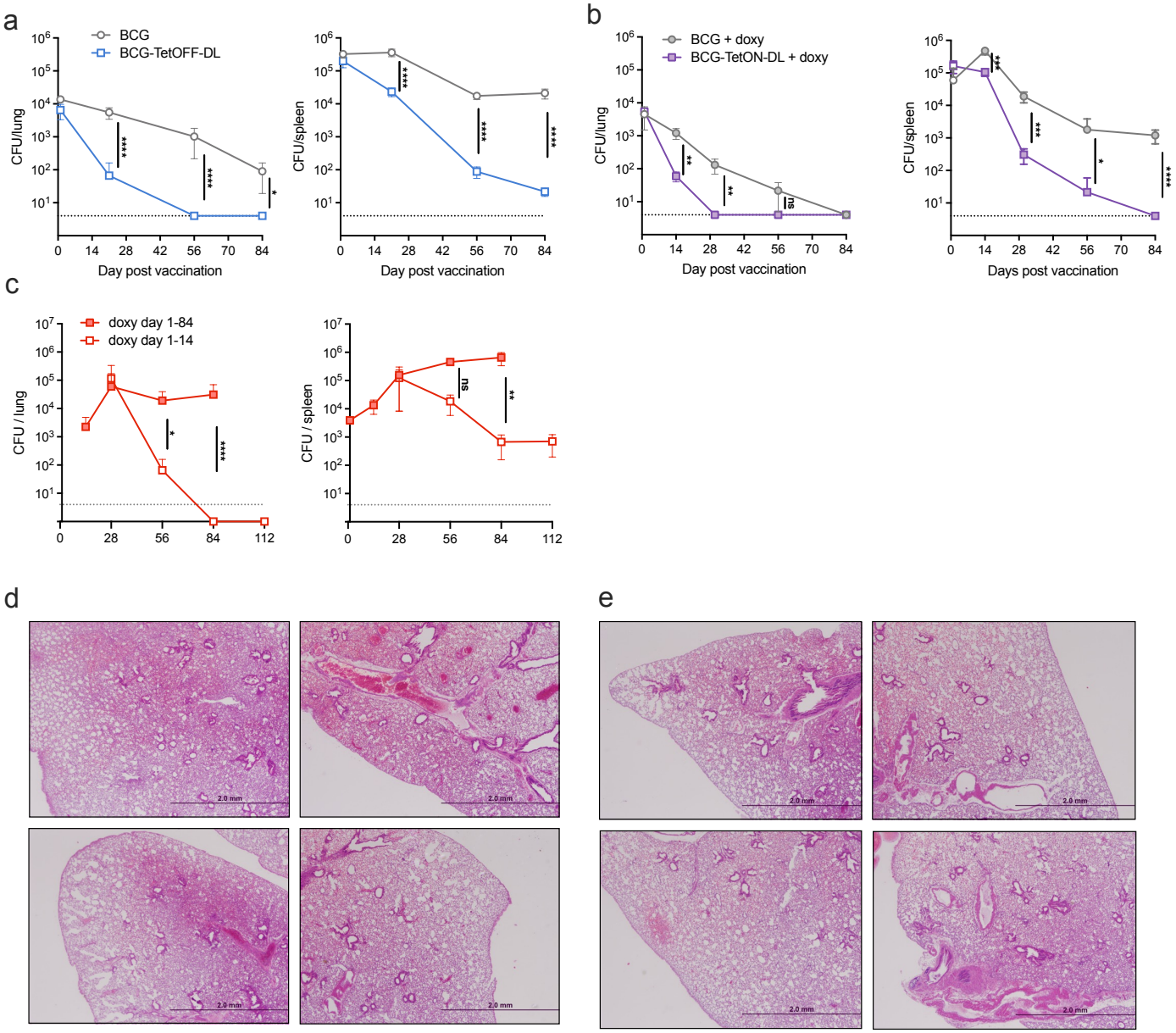
a
BCG
BCG-TetOFF-DL
CFU/lung
CFU/spleen
Day post vaccination
b
BCG + doxy
BCG-TetON-DL + doxy
CFU/lung
CFU/spleen
Day post vaccination
Days post vaccination
c
doxy day 1-84
doxy day 1-14
CFU / lung
CFU / spleen
d
e

Figure S3

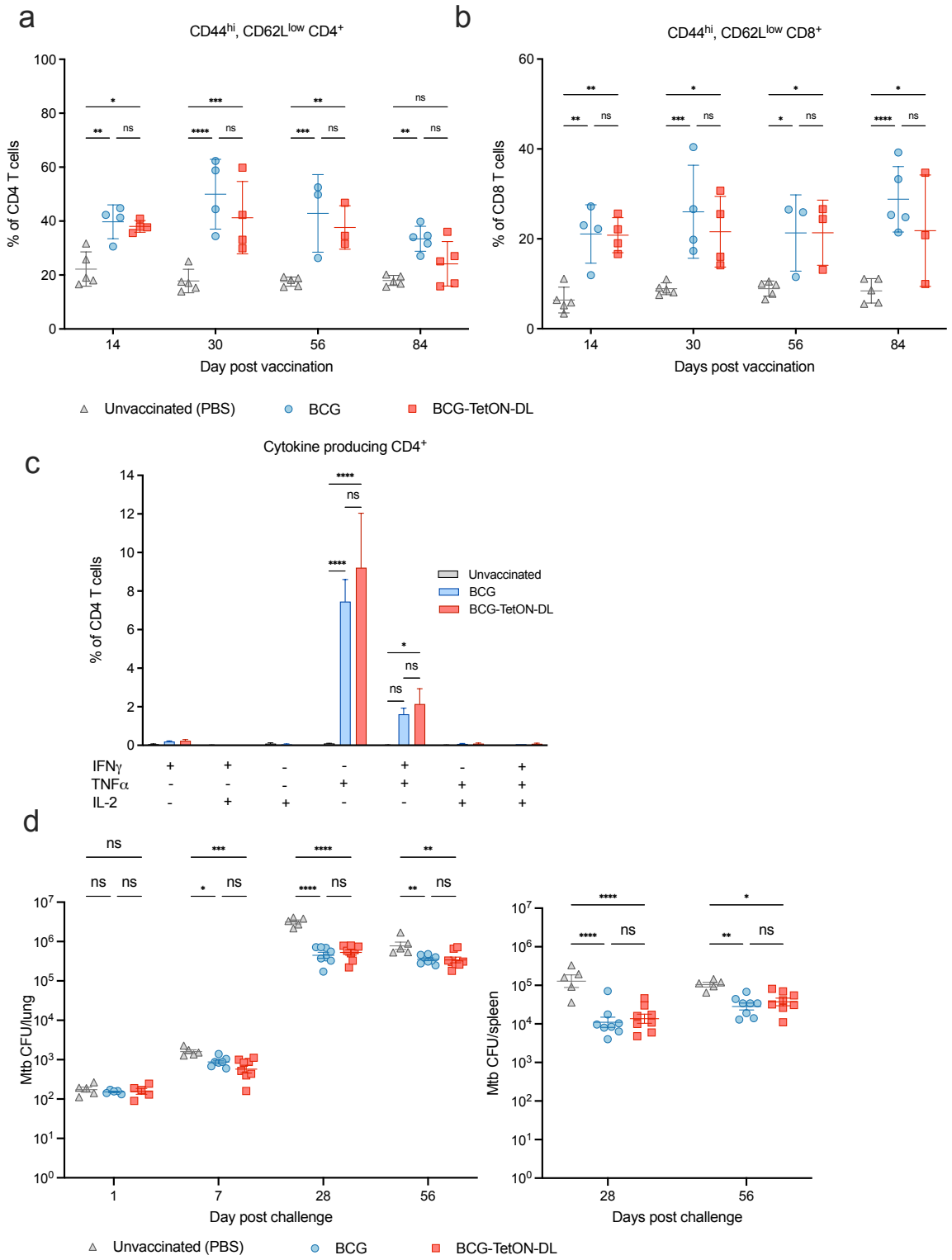
a
CD44hi, CD62Llow CD4+
% of CD4 T cells
Day post vaccination
b
CD44hi, CD62Llow CD8+
% of CD8 T cells
Days post vaccination
Unvaccinated (PBS)
BCG
BCG-TetON-DL
c
Cytokine producing CD4+
% of CD4 T cells
Unvaccinated
BCG
BCG-TetON-DL
IFNγ
TNFα
IL-2
d
Mtb CFU/lung
Day post challenge
Mtb CFU/spleen
Days post challenge
Unvaccinated (PBS)
BCG
BCG-TetON-DL

Figure S4

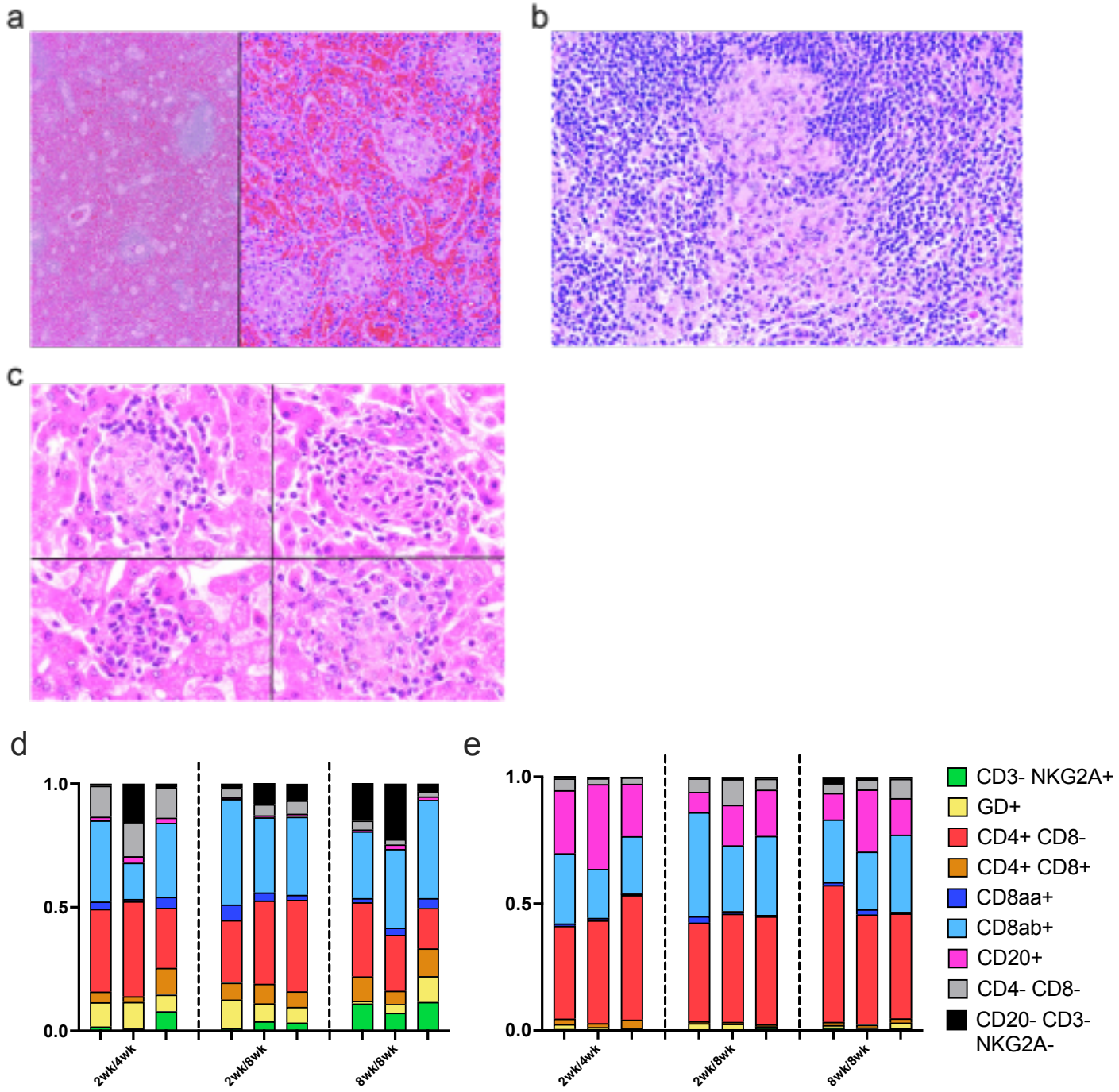
a
b
c
d
e
1.0
0.5
0.0
2wk/4wk
2wk/8wk
8wk/8wk
CD3- NKG2A+
GD+
CD4+ CD8-
CD4+ CD8+
CD8aa+
CD8ab+
CD20+
CD4- CD8-
CD20- CD3- NKG2A-

Figure S5

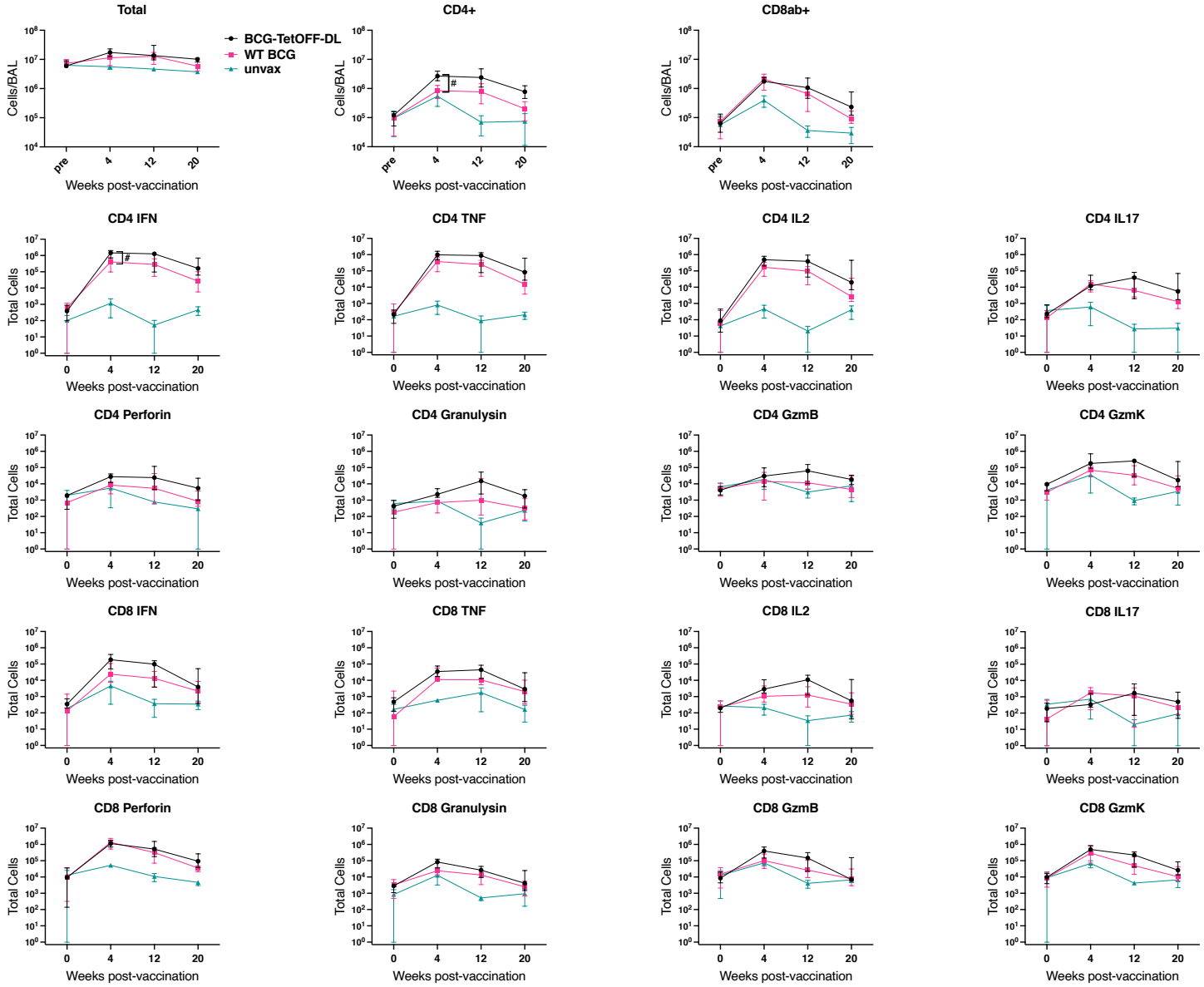
Total
CD4+
CD8ab+
BCG-TetOFF-DL
WT BCG
unvax
Cells/BAL
Weeks post-vaccination
pre
4
12
20
CD4 IFN
CD4 TNF
CD4 IL2
CD4 IL17
CD4 Perforin
CD4 Granulysin
CD4 GzmB
CD4 GzmK
CD8 IFN
CD8 TNF
CD8 IL2
CD8 IL17
CD8 Perforin
CD8 Granulysin
CD8 GzmB
CD8 GzmK
Total Cells
0
#

Figure S6

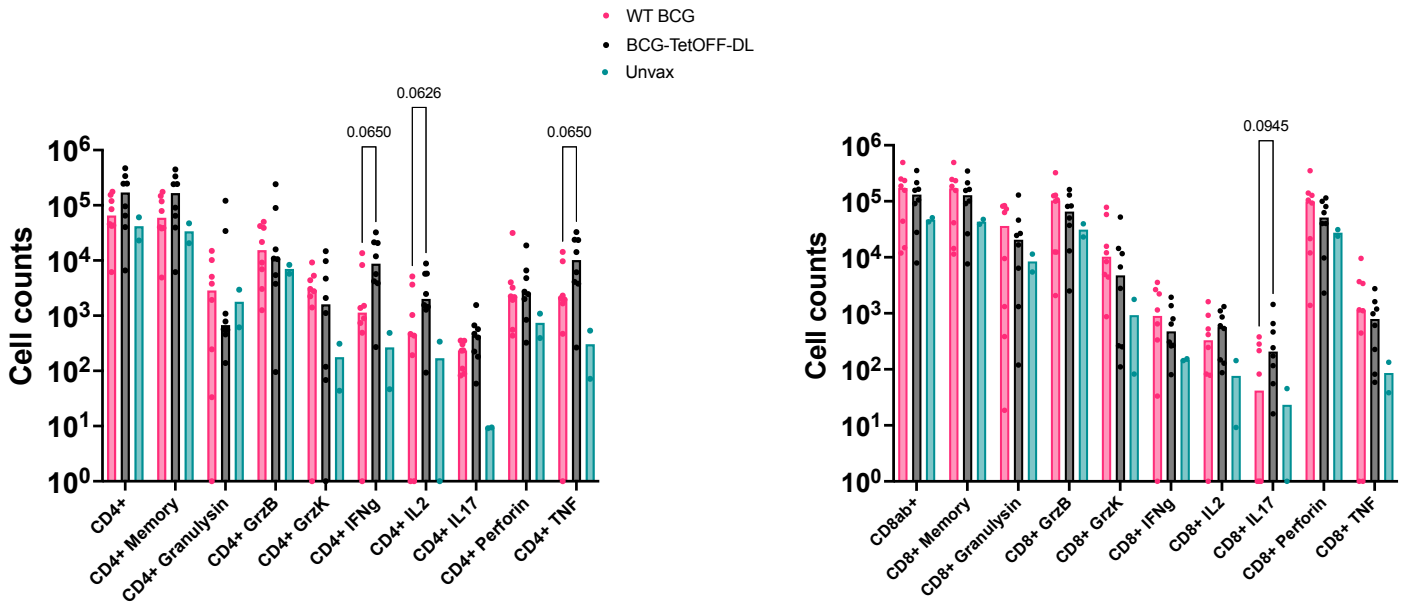
WT BCG
BCG-TetOFF-DL
Unvax
Cell counts
10^0
10^1
10^2
10^3
10^4
10^5
10^6
0.0650
0.0626
0.0650
0.0945
CD4+
CD4+ Memory
CD4+ Granulysin
CD4+ GrzB
CD4+ GrzK
CD4+ IFNg
CD4+ IL2
CD4+ IL17
CD4+ Perforin
CD4+ TNF
CD8ab+
CD8+ Memory
CD8+ Granulysin
CD8+ GrzB
CD8+ GrzK
CD8+ IFNg
CD8+ IL2
CD8+ IL17
CD8+ Perforin
CD8+ TNF

Figure S7

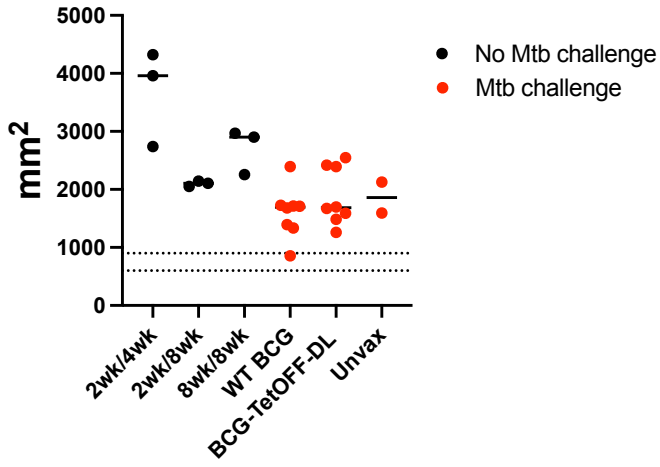
5000
4000
3000
2000
1000
0
mm2
No Mtb challenge
Mtb challenge
2wk/4wk
2wk/8wk
8wk/8wk
WT BCG
BCG-TetOFF-DL
Unvax

Figure S8

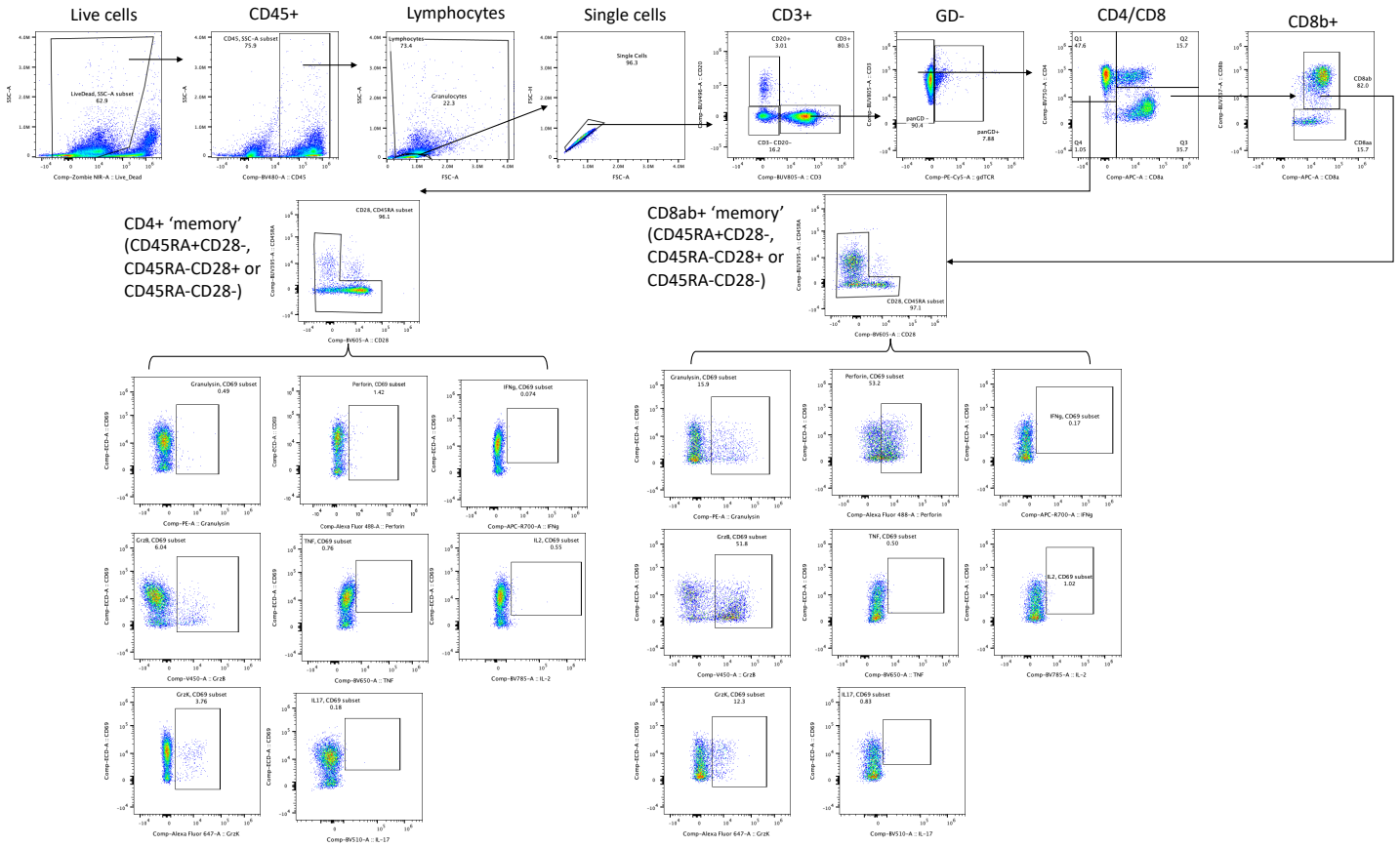
Live cells
CD45+
Lymphocytes
Single cells
CD3+
GD-
CD4/CD8
CD8b+
CD4+ 'memory' (CD45RA+CD28-, CD45RA-CD28+ or CD45RA-CD28-)
CD8ab+ 'memory' (CD45RA+CD28-, CD45RA-CD28+ or CD45RA-CD28-)
Cytokine/cytotoxic molecule (granulysin, perforin, IFNg, GrzB, TNF, IL2, GrzK, IL17)
Cytokine/cytotoxic molecule (granulysin, perforin, IFNg, GrzB, TNF, IL2, GrzK, IL17)

Figure S9

	Target	Clone	Fluor	Product #	Vendor
Surface	CD3	SP34-2	APC-Cy7	624072	BD Biosciences
	CD4	L200	BV750	747202	BD Biosciences
	CD8a	DK25	FITC	FCMAB176F	EMD Millipore
	CD8b	2ST8.5H7	BUV737	748324	BD Biosciences
	Pan gd	5A6.E9	PE	MHGD04	Thermo Fischer Scientific
	CD16	3G8	BV570	302036	Biolegend
	CD159a	Z199	PE-Cy7	B10246	Beckman Coulter
	CD45	D058-1283	BV480	566145	BD Biosciences
	CD28	CD28.2	BV605	302968	BD Biosciences
	CD45RA	5H9	BUV395	740315	BD Biosciences
	CD20	2H7	PE-CY5	302308	Biolegend
	CD11b	ICRF44	BUV563	741357	BD Biosciences
	CD11c	3.9	BV421	301628	Biolegend
ICS	CD69	TP1.55.3	ECD	6607110	Beckman Coulter
	Granzyme B	GB11	V450	561151	BD Biosciences
	Granzyme K	G3H69	AF647	566655	BD Biosciences
	IL-17	BL168	BV510	512330	Biolegend
	TNF	MAb11	BV650	502938	Biolegend
	IFNg	B27	APC	506510	Biolegend
	IL-2	MQ1-17H12	BV785	500348	Biolegend
L/D	Zombie		NIR	423106	Biolegend

Table S1

NHP	Vendor	Origin	Gender	DOB	BCG Strain	BCG Vax Date	BCG Dose	Doxy Start	Doxy End	Mtb Infectio	Mtb Dose	Necropsy Date	Weeks PI	Grans4wks	PET4wks	Grans8wks	PET8wks	PET12Weeks	NecropsyScore	GransNX	TotalCFU	LungCFU	LNCFU	BodyCFU
10022	Bioculture-Mauritius	Mauritius	Male	7/26/18	BCG-TetOFFDL	8/2/22	5x10^7	8/1/22	8/15/22	1/4/23	9	3/27/23	11.71429	0	0	0	0	0	8	0	0	0	0	
10122	Bioculture-Mauritius	Mauritius	Male	4/25/18	BCG-TetOFFDL	8/2/22	5x10^7	8/1/22	8/15/22	1/4/23	9	4/5/23	13	0	0	0	0	0	7	0	0	0	0	
10622	Bioculture-Mauritius	Mauritius	Male	5/23/14	WT	8/2/22	5x10^7	8/1/22	8/15/22	1/4/23	9	4/5/23	13	5	178.119	8	1214.96702	1660.237383	17	13	8550	6550	2000	
10722	Bioculture-Mauritius	Mauritius	Male	8/6/12	WT	8/2/22	5x10^7	8/1/22	8/15/22	1/4/23	9	3/29/23	12	0	0	1	13.4643538	0	8	0	0	0	0	
10822	Bioculture-Mauritius	Mauritius	Male	10/1/16	BCG-TetOFFDL	8/2/22	5x10^7	8/1/22	8/15/22	1/4/23	9	4/3/23	12.71429	1	0	1	0	0	9	0	0	0	0	
10922	Bioculture-Mauritius	Mauritius	Male	3/1/16	BCG-TetOFFDL	8/2/22	5x10^7	8/1/22	8/15/22	1/4/23	9	3/29/23	12	38	12.72893	38	0	0	23	14	0	0	0	
11022	Bioculture-Mauritius	Mauritius	Male	2/12/18	WT	8/2/22	5x10^7	8/1/22	8/15/22	1/4/23	9	4/3/23	12.71429	5	0	6	6717.6468	65104.86686	19	tntc	2048450	2047850	600	
11122	Bioculture-Mauritius	Mauritius	Male	1/6/18	WT	8/2/22	5x10^7	8/1/22	8/15/22	1/4/23	9	4/10/23	13.71429	1	0	1	0	0	9	3	10038	10038	0	
11222	Bioculture-Mauritius	Mauritius	Male	4/26/18	BCG-TetOFFDL	9/1/22	4.9x10^7	8/31/22	9/13/22	1/31/23	16	4/24/23	11.85714	1	0	1	0	0	8	0	0	0	0	
11322	Bioculture-Mauritius	Mauritius	Male	8/25/18	BCG-TetOFFDL	9/1/22	4.9x10^7	8/31/22	9/13/22	1/31/23	16	4/26/23	12.14286	0	15.56539	0	0	0	11	0	1075	0	1075	
11422	Bioculture-Mauritius	Mauritius	Male	4/3/18	WT	9/1/22	4.48x10^7	8/31/22	9/13/22	1/31/23	16	4/26/23	12.14286	6	0	6	0	0	13	10	5800	5400	400	
11522	Bioculture-Mauritius	Mauritius	Male	7/1/18	WT	9/1/22	4.48x10^7	8/31/22	9/13/22	1/31/23	16	5/10/23	14.14286	3	0	3	11.8317564	0	11	3	705	685	20	
11822	Bioculture-Mauritius	Mauritius	Male	11/23/16	BCG-TetOFFDL	9/1/22	4.9x10^7	8/31/22	9/13/22	1/31/23	16	5/2/23	13	2	0	3	0	0	9	1	30	30	0	
11922	Bioculture-Mauritius	Mauritius	Male	12/22/16	BCG-TetOFFDL	9/1/22	4.9x10^7	8/31/22	9/13/22	1/31/23	16	5/2/23	13	0	0	0	0	0	8	0	0	0	0	
12022	Bioculture-Mauritius	Mauritius	Male	9/2/18	WT	9/1/22	4.48x10^7	8/31/22	9/13/22	1/31/23	16	5/8/23	13.85714	0	0	0	0	0	12	1	0	0	0	
12122	Bioculture-Mauritius	Mauritius	Male	9/26/18	WT	9/1/22	4.48x10^7	8/31/22	9/13/22	1/31/23	16	5/8/23	13.85714	4	0	4	0	0	11	5	270	250	20	
12722	Bioculture-Mauritius	Mauritius	Male	2/23/16	none					1/4/23	9	3/27/23	11.71429	40	39853.77	tntc	287211.79	321798.3491	88	tntc	1503960	1332110	136000	
12822	Bioculture-Mauritius	Mauritius	Male	3/14/14	none					1/31/23	16	4/24/23	11.85714	6	835.3731	10	24.7	0	22	12	20293	12533	7760	
15719	Bioculture-Mauritius	Mauritius	Male	5/25/14	none historical					12/17/19	21	03/09/20	11.85714	19	12247.09	tntc	110612.275	119644.7832	75	tntc	1359498.29	1196783.29	162715	
15819	Bioculture-Mauritius	Mauritius	Male	11/25/13	none historical					12/17/19	21	03/11/20	12.14286	8	5393.503	23	94418.4916	203119.0381	56	tntc	552445.159	502985.159	49460	
15919	Bioculture-Mauritius	Mauritius	Male	1/25/12	none historical					12/17/19	21	03/17/20	13	12	1312.402	12	776.293776	888.2410389	23	18	17740	11950	5790	
16019	Bioculture-Mauritius	Mauritius	Male	11/25/13	none historical					12/17/19	21	03/19/20	13.28571	19	9132.703	19	14718.1518	10045.66079	37	32	27504.9491	16574.9491	10930	
16119	Bioculture-Mauritius	Mauritius	Male	1/25/11	none historical					01/14/20	6	04/14/20	13	6	978.5377	tntc	75438.3824	170898.7206	70	tntc	672398.189	637783.189	34615	
16419	Bioculture-Mauritius	Mauritius	Male	1/25/13	none historical					01/14/20	6	04/16/20	13.28571	8	10040.75	tntc	68661.0003	217106.4084	76	tntc	199383.539	31101.0387	168282.5	
16519	Bioculture-Mauritius	Mauritius	Male	1/25/12	none historical					02/14/20	4	05/12/20	12.57143	6	486.4546			62842.36694	64	tntc	7743349.94	7648649.94	93875	
16719	Bioculture-Mauritius	Mauritius	Male	10/25/14	none historical					02/14/20	4	05/14/20	12.85714	1	127.9454	3	536.135708	522.973755	27	13	11330	2580	8750	
23621	Bioculture-Mauritius	Mauritius	Male	12/29/13	BCG-TetOFFDL	1/12/22	3.74x10^7	1/11/22	1/26/22			2/9/22							11		160	0	120	160
23721	Bioculture-Mauritius	Mauritius	Male	12/11/15	BCG-TetOFFDL	1/12/22	3.74x10^7	1/11/22	1/26/22			2/9/22							7		945	615	330	1041
23821	Bioculture-Mauritius	Mauritius	Male	11/16/12	BCG-TetOFFDL	1/12/22	3.74x10^7	1/11/22	1/26/22			2/7/22							23		235	175	60	235
23921	Bioculture-Mauritius	Mauritius	Male	6/19/14	BCG-TetOFFDL	1/12/22	3.74x10^7	1/11/22	1/26/22			3/9/22							7		0	0	0	50
24021	Bioculture-Mauritius	Mauritius	Male	6/18/14	BCG-TetOFFDL	1/12/22	3.74x10^7	1/11/22	1/26/22			3/9/22							9		0	0	0	0
24121	Bioculture-Mauritius	Mauritius	Male	10/20/14	BCG-TetOFFDL	1/12/22	3.74x10^7	1/11/22	3/13/22			3/14/22							6		195	145	0	195
24221	Bioculture-Mauritius	Mauritius	Male	2/5/15	BCG-TetOFFDL	1/12/22	3.74x10^7	1/11/22	3/6/22			3/7/22							7		25	0	0	25
24321	Bioculture-Mauritius	Mauritius	Male	6/26/14	BCG-TetOFFDL	1/12/22	3.74x10^7	1/11/22	3/8/22			3/9/22							9		85	85	0	85
24421	Bioculture-Mauritius	Mauritius	Male	6/8/13	BCG-TetOFFDL	1/12/22	3.74x10^7	1/11/22	1/26/22			3/14/22							24		15	15	0	15

Variable	Description
NHP	Non-Human Primate ID
Vendor	Animal Vendor
Origin	Animal Origin
Gender	Gender
DOB	Date of Birth
BCG Strain	BCG Strain: WT= wild type BCG Pasteur, BCG-TetOFFDL = BCG Dual lysin Pasteur kill switch strain, none = Unvaccinated, none historical = Unvaccinated control from previous study
BCG Vax Date	Date of BCG Vaccination
BCG Dose	Estimated BCG Dose
Doxy Start	Doxycycline Start Date
Doxy End	Doxycycline End Date
Mtb Infection	Date of Mtb Infection
Mtb Dose	Estimated Mtb Dose
Necropsy Date	Date of Necropsy
Weeks PI	Weeks Post Mtb Infection
Grans4wks	Number of Granulomas at 4 Weeks Post-Infection
PET4wks	Total Lung FDG Activity at 4 Weeks Post-Infection
Grans8wks	Number of Granulomas at 8 Weeks Post-Infection (tntc = too numerous to count or TB Pneumonia)
PET8wks	Total Lung FDG Activity at 8 Weeks Post-Infection
PET12Weeks	Total Lung FDG Activity at 12 Weeks Post-Infection
NecropsyScore	Gross Pathology Score
GransNX	Number of Granulomas Found at Necropsy (tntc = too numerous to count or TB Pneumonia)
TotalCFU	Total CFU
LungCFU	Lung CFU
LNCFU	Thoracic Lymph Node CFU
BodyCFU	Estimated Total Body CFU

Table S2