Supplementary Information

Cytolytic circumsporozoite-specific memory CD4$^+$ T cell clones are expanded during *Plasmodium falciparum* infection

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Supplementary Figures and Data

Supplementary Figure 1, related to Figure 1.

Supplementary Figure 2, related to Figure 2.

Supplementary Figure 3, related to Figure 3 and 4.

Supplementary Figure 4, related to Figure 5.

Supplementary Figure 5, related to Figure 6.

Supplementary Figure 6, related to Figure 7.

Supplementary Data 1: List of IgG reactive *Pf* antigens.

Supplementary Data 2: List of *Pf* -reactive antigens between patient's groups.

Supplementary Data 3: List of IgG-specific *Pf* antigens with greater reactivity in protected compared to susceptible children.

Supplementary Data 4: List of antibodies for CyTOF, spectral flow cytometry and AIM assay.

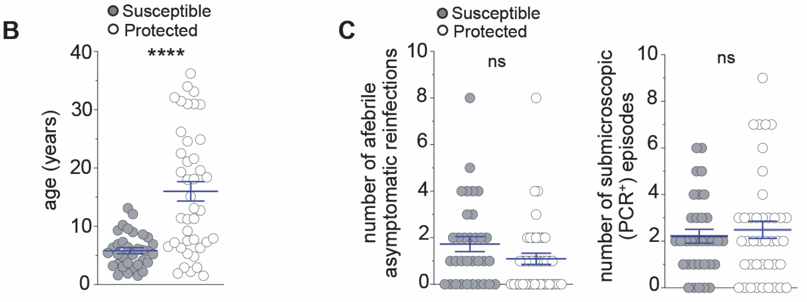
Supplementary Data 5: List of expressed genes in naive and memory CD4$^+$ T cell clusters defined by single cell transcriptomic.

A

Participant enrollment characteristics by age

Variable	1-5 yr (n=35)	6-12 yr (n=33)	13-50 yr (n=27)	*P* value (ANOVA)
Sex (% female)	40	61	70	<0.05†
Temperature (ºC)	38.0 (36.7-39.1)	38.1 (36.25-38.8)	36.4 (35.9-36.6)	<0.0001
Parasitemia (x10^3/µl)	38.2 (15.9-79.9)	18.86 (1.4-62.8)	0.88 (0.5-6.5)	<0.0001
Hemoglobin (g/dL)	10.3 (9.3-11.7)	11.5 (10.8-12.2)	12.6 (10.9-1.0)	<0.0001

*Median and IQR
† Chi-squared test

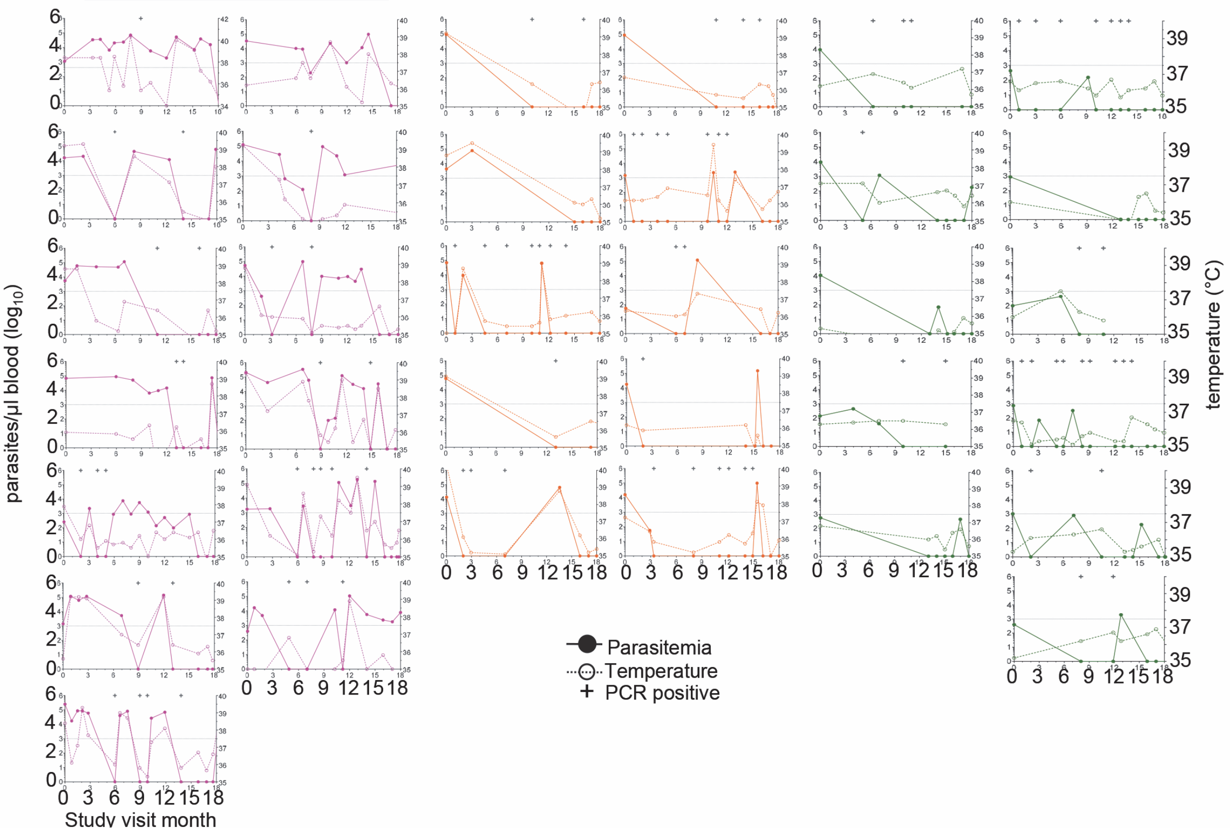


D

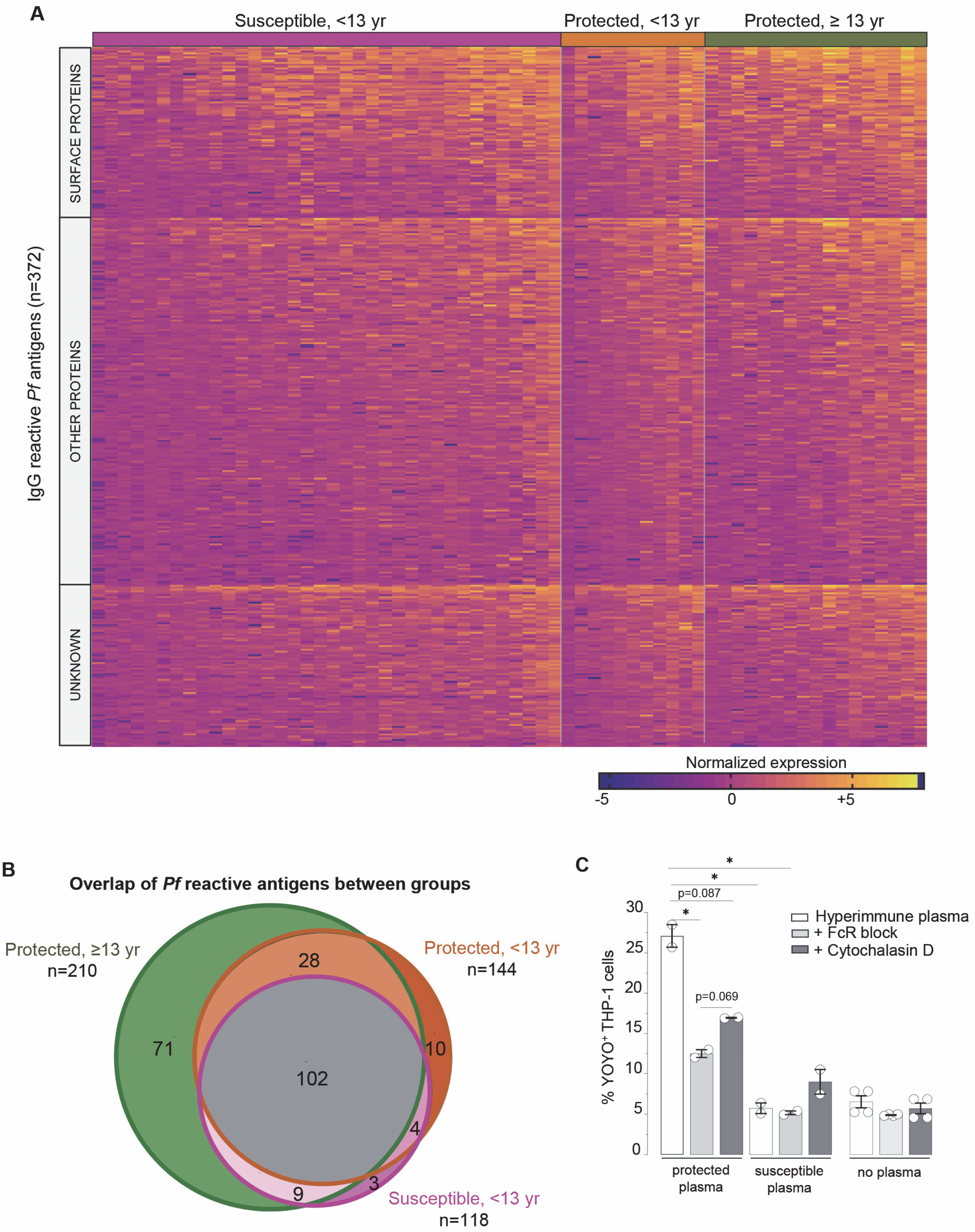
Participant enrollment characteristics by clinical immunity status and age

Variable	Susceptible, <13 yr (n=32)	Protected, <13 yr (n=20)	Protected, ≥13 yr (n=21)	*P* value (ANOVA)
Age, (yr)	5.2 (3.3-7.2)	6.9 (3.4-8.9)	24.6 (18.9-31.3)	NA
Sex (% female)	56	30	71	0.027†
Temperature (ºC)	38.4 (36.4-39.2)	38.0 (36.2-39.1)	36.3 (36.0-36.7)	0.002
Parasitemia (x10^3/µl)	32 (4.2-68)	21 (2.2-66)	0.7 (0.4-4.1)	0.003
Hemoglobin (g/dL)	10.7 (9.9-12.1)	11.4 (10.8-12.2)	12.8 (11.6-14.1)	0.001

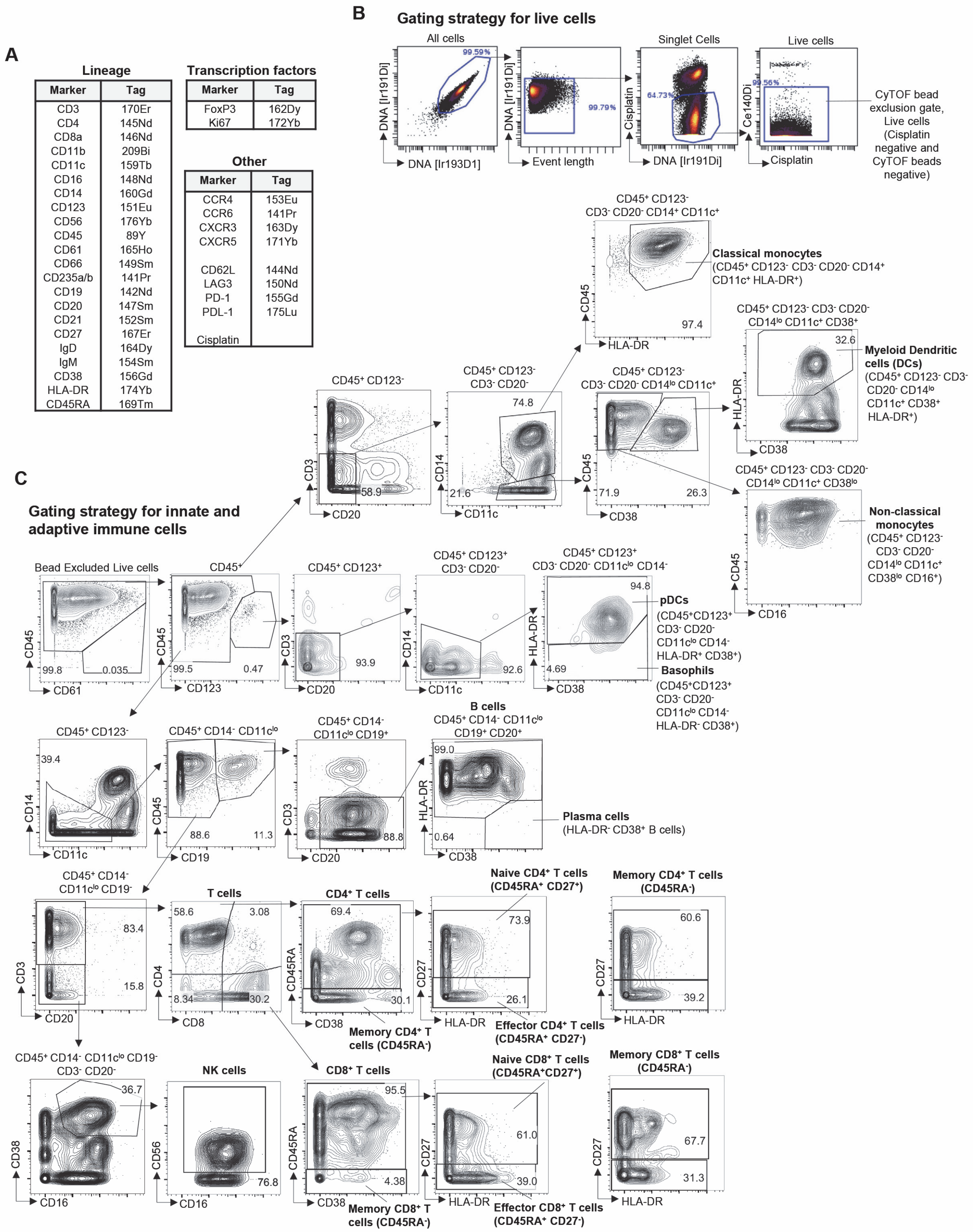
*Median and IQR
† Chi-squared test



Supplementary Figure 1. Clinical characteristics of the mild malaria cohort in the 18-month longitudinal study: (A) Table with participant characteristics and clinical parameters at enrollment in the study, categorized by participants' age. Median and IQR values, and p-value determined by two-way ANOVA statistical analysis across participant's groups is indicated. **(B)** Average number of days between clinical reinfection episodes (>2,500 parasites/µl blood) and age (years) of each participant that completed the study within the protected (≤1 clinical reinfection, n=41) and susceptible groups (≥3 clinical reinfections, n=33). **(C)** Number of afebrile asymptomatic reinfections (<2,500 parasites/µl blood, temperature <37.5°C) and submicroscopic episodes (*Pf* PCR^{+}) two sided Student's t-test was conducted between indicated groups, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **(D)** Table participant enrollment characteristics by clinical immunity status and age. **(E)** Parasitemia (left axis), temperature (right axis) and *Pf* PCR status of a subset of susceptible, <13 yr, protected, <13 yr and protected, ≥13 yr at each clinical visit over the 18-month longitudinal study. Data are presented as means ± SEM (B,C).



Supplementary Figure 2. *Pf* specific antibody responses in participants determined by *Pf* antigen array: (A) Heatmap of normalized intensity of IgG reactivity to 372 *Pf* antigens (rows) in plasma from individual participants (columns) within susceptible, <13 yr, protected, <13 yr and protected, ≥ 13 yr groups during malaria illness. Surface and non-surface *Pf* antigens are indicated. **(B)** Venn diagram indicating overlapping *Pf* IgG reactivity to 372 *Pf* Ags by susceptible n=32, protected age-matched (n=20) and protected > 13 yr groups (n=21). Total *Pf* Ags recognized by each group is indicated next to the group label. **(C)** *Pf* merozoite opsonization assay with YOYO1$^+$ *Pf* merozoites incubated with or without indicated plasma (1:9 dilution, duplicate wells) and co-cultured with THP1 cells in the presence or absence of FcR block or Cytochalasin D (n=2). Mean and SEM of the % YOYO1$^+$ THP-1 cells are shown. Statistics is two sided Welch's unpaired t-test between indicated groups *$p<0.05$. Data are presented as means ± SEM (C).



Lineage

Marker	Tag
CD3	170Er
CD4	145Nd
CD8a	146Nd
CD11b	209Bi
CD11c	159Tb
CD16	148Nd
CD14	160Gd
CD123	151Eu
CD56	176Yb
CD45	89Y
CD61	165Ho
CD66	149Sm
CD235a/b	141Pr
CD19	142Nd
CD20	147Sm
CD21	152Sm
CD27	167Er
IgD	164Dy
IgM	154Sm
CD38	156Gd
HLA-DR	174Yb
CD45RA	169Tm

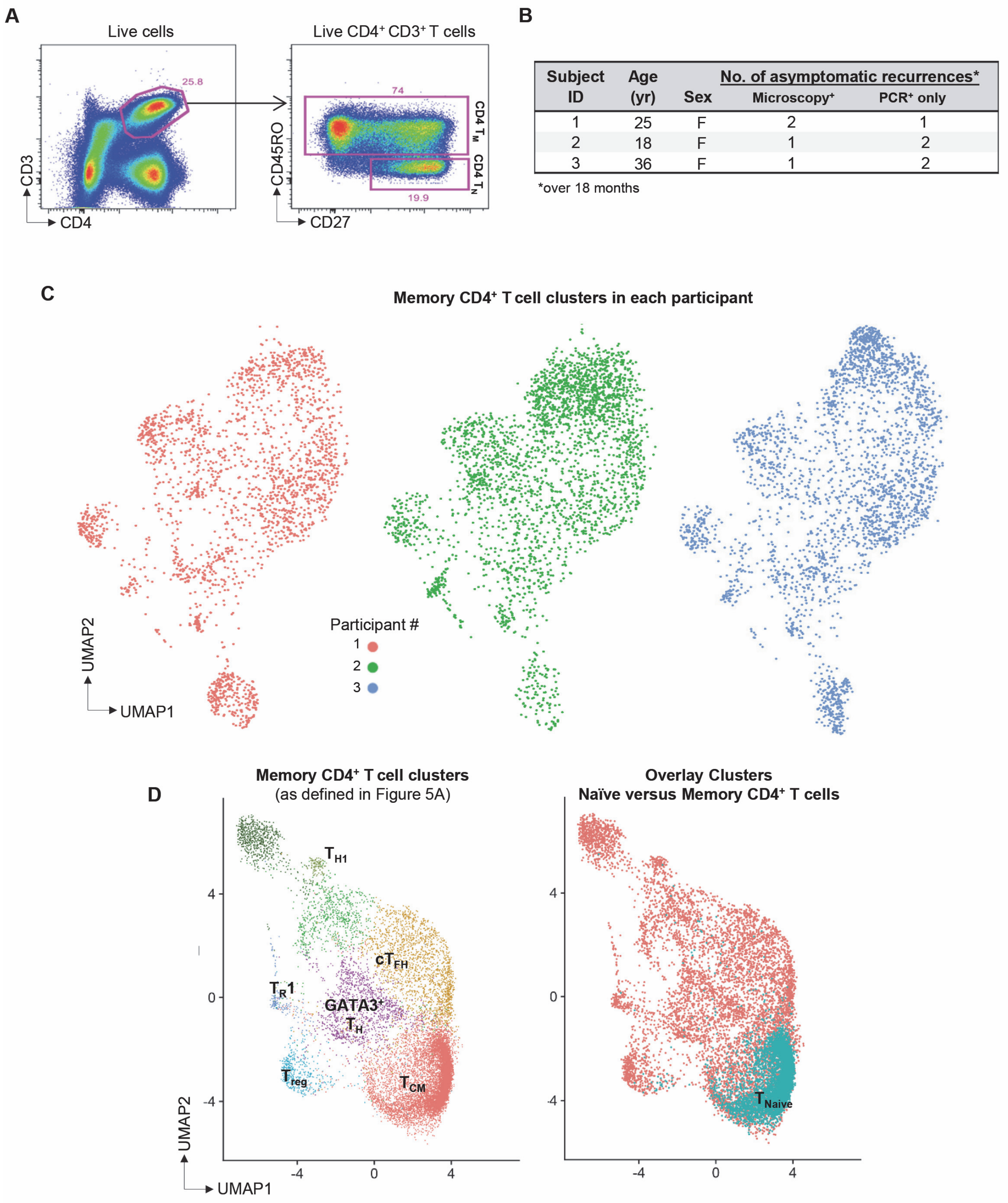
Transcription factors

Marker	Tag
FoxP3	162Dy
Ki67	172Yb

Other

Marker	Tag
CCR4	153Eu
CCR6	141Pr
CXCR3	163Dy
CXCR5	171Yb
CD62L	144Nd
LAG3	150Nd
PD-1	155Gd
PDL-1	175Lu
Cisplatin	

Supplementary Figure 3. CyTOF immunophenotyping panel and gating strategy of immune cell populations: (A) Participant PBMCs were immunophenotyped with a 33 marker mass cytometry antibody panel with indicated heavy-metal tags. **(B)** Gating strategy for live cells among PBMCs immunophenotyped by CyTOF. A density plot that is a representative sample during malaria illness is shown. **(C)** Contour plots with gating strategy for innate and adaptive immune cell populations among PBMCs stained with the antibody panel in (A).

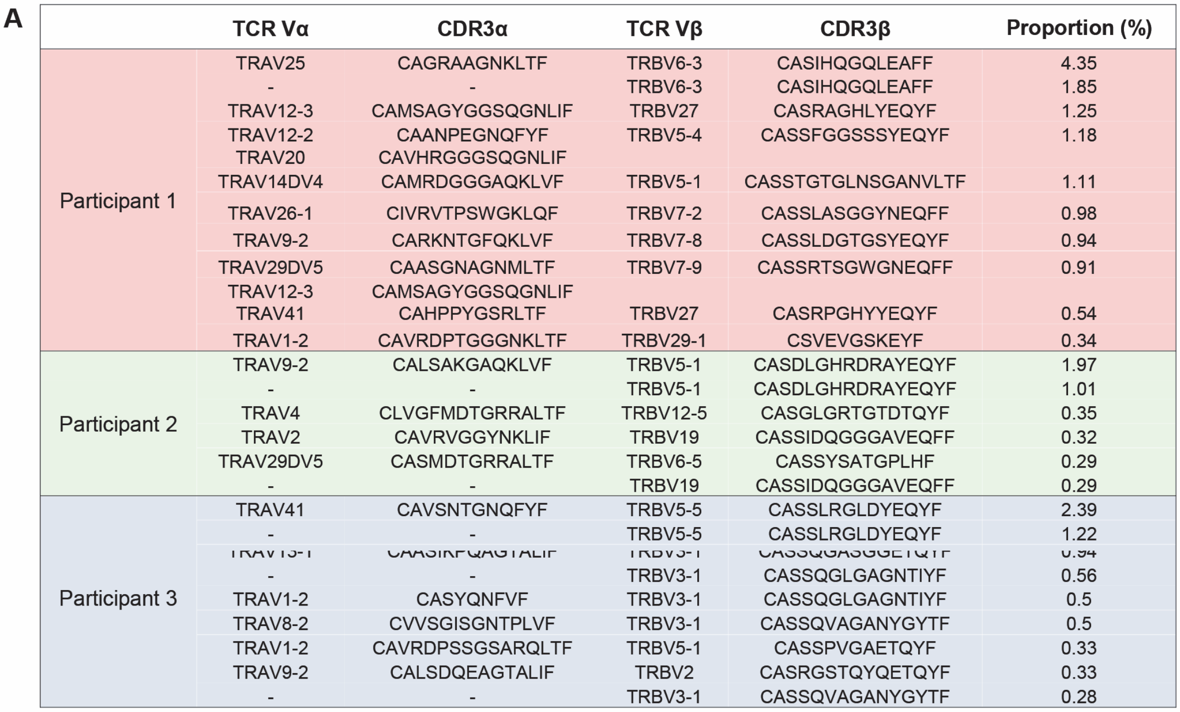


Subject ID	Age (yr)	Sex	No. of asymptomatic recurrences*	
			Microscopy$^+$	PCR$^+$ only
1	25	F	2	1
2	18	F	1	2
3	36	F	1	2

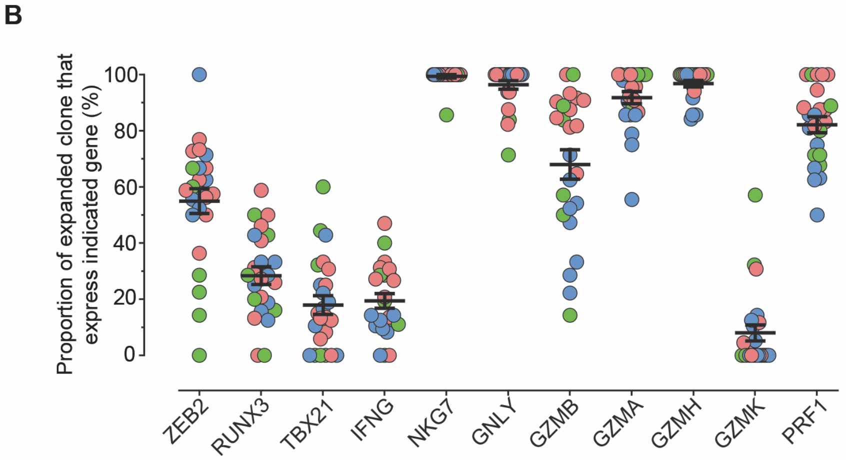
*over 18 months

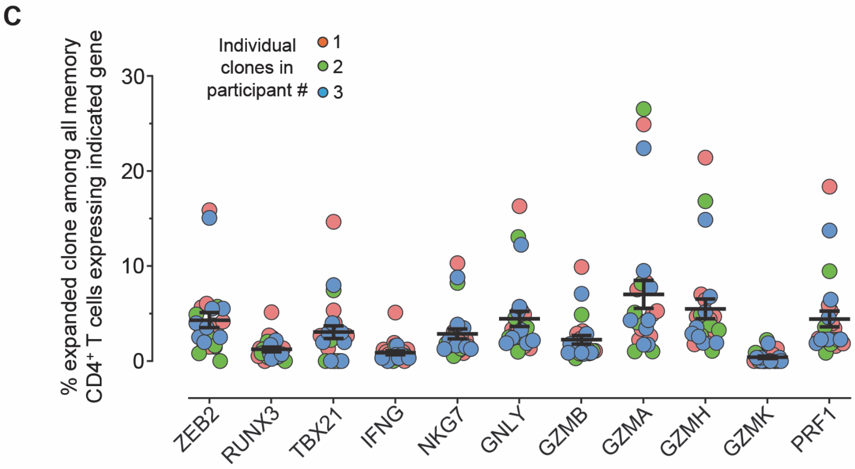
Supplementary Figure 4. Single-cell (sc) transcriptomic analysis of memory and naive CD4$^+$ T cells from protected participants during malaria illness: (A) Dot plots show the gating strategy for sorting live (L/D aqua negative), memory (CD45RO$^+$) and naïve (CD45RO$^-$

CD27$^+$) CD4$^+$ T cells on the BD Aria III for scRNA-seq analysis. **(B)** Table of the characteristics of the three protected participants from which PBMC were used for sc transcriptomic and TCR-seq. **(C)** UMAP visualization of scRNA-seq results on the memory CD4$^+$ T cells from each of the 3 protected participants during malaria infection. **(D)** UMAP visualization of scRNA-seq results on isolated naïve versus memory CD4$^+$ T cells from 3 protected participants during malaria infection. **(D)** UMAP visualizations of i) memory (CD45RO$^+$) clusters as defined in Figure 5A (left) and ii) overlay of naive and memory CD4$^+$ T cell distributions from the 3 protected participants during malaria infection (right).



	TCR Vα	CDR3α	TCR Vβ	CDR3β	Proportion (%)
Participant 1	TRAV25	CAGRAAGNKLTF	TRBV6-3	CASIHQGQLEAFF	4.35
	-	-	TRBV6-3	CASIHQGQLEAFF	1.85
	TRAV12-3	CAMSAGYGGSQGNLIF	TRBV27	CASRAGHLYEQYF	1.25
	TRAV12-2 TRAV20	CAANPEGNQFYF CAVHRGGGSQGNLIF	TRBV5-4	CASSFGGSSSYEQYF	1.18
	TRAV14DV4	CAMRDGGGAQKLVF	TRBV5-1	CASSTGTGLNSGANVLTF	1.11
	TRAV26-1	CIVRVTPSWGKLQF	TRBV7-2	CASSLASGGYNEQFF	0.98
	TRAV9-2	CARKNTGFQKLVF	TRBV7-8	CASSLDGTGSYEQYF	0.94
	TRAV29DV5	CAASGNAGNMLTF	TRBV7-9	CASSRTSGWGNEQFF	0.91
	TRAV12-3 TRAV41	CAMSAGYGGSQGNLIF CAHPPYGSRLTF	TRBV27	CASRPGHYYEQYF	0.54
	TRAV1-2	CAVRDPTGGGNKLTF	TRBV29-1	CSVEVGSKEYF	0.34
Participant 2	TRAV9-2	CALSAKGAQKLVF	TRBV5-1	CASDLGHRDRAYEQYF	1.97
	-	-	TRBV5-1	CASDLGHRDRAYEQYF	1.01
	TRAV4	CLVGFMDTGRRALTF	TRBV12-5	CASGLGRTGTDTQYF	0.35
	TRAV2	CAVRVGGYNKLIF	TRBV19	CASSIDQGGGAVEQFF	0.32
	TRAV29DV5	CASMDTGRRALTF	TRBV6-5	CASSYSATGPLHF	0.29
	-	-	TRBV19	CASSIDQGGGAVEQFF	0.29
Participant 3	TRAV41	CAVSNTGNQFYF	TRBV5-5	CASSLRGLDYEQYF	2.39
	-	-	TRBV5-5	CASSLRGLDYEQYF	1.22
	TRAV13-1	CAASIKPQAGTALIF	TRBV3-1	CASSQGASGGETQYF	0.94
	-	-	TRBV3-1	CASSQGLGAGNTIYF	0.56
	TRAV1-2	CASYQNFVF	TRBV3-1	CASSQGLGAGNTIYF	0.5
	TRAV8-2	CVVSGISGNTPLVF	TRBV3-1	CASSQVAGANYGYTF	0.5
	TRAV1-2	CAVRDPSSGSARQLTF	TRBV5-1	CASSPVGAETQYF	0.33
	TRAV9-2	CALSDQEAGTALIF	TRBV2	CASRGSTQYQETQYF	0.33
	-	-	TRBV3-1	CASSQVAGANYGYTF	0.28





Supplementary Figure 5. Additional characterization of expanded memory CD4$^+$ T cell clones of the 3 protected participants: (A) Variable region sequences of the TCRα and TCRβ chains in expanded memory CD4$^+$ T cell clones and their frequencies in protected malaria participants (n=3), defined by single-cell TCR-seq. **(B)** Relative expression of indicated gene in each individual expanded memory CD4$^+$ T cell clone. **(C)** Frequency of expanded memory CD4$^+$ T cell clone among all memory CD4$^+$ T cells that express the indicated gene in the 3 protected participants.

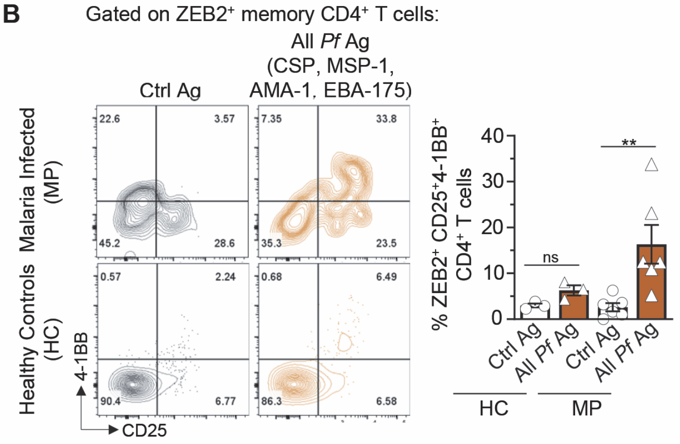
A

Characteristics of study subjects for AIM assay

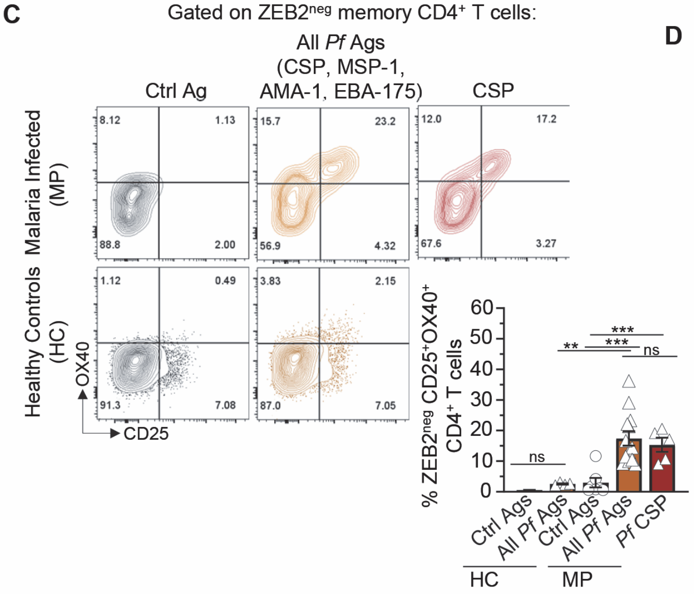
Variable	Median and IQR n=15
Age, (yr)	5.2 (2.9-9)
Sex (% female)	33
Number symptomatic events	1 (0-3)
Number all recurrent events	6 (3-9)

#Symptomatic malaria ≥ 2500 parasites/ul and ≥ 37.5°C

B



C



D

Lineage

Marker	Tag
CD3	AF532
CD4	Pacific Orange
CD14	Qdot-705
CD45	Qdot-800
CD19	BUV737
CD27	PerCP
CD45RO	BV570

Transcription factors

Marker	Tag
ZEB2	AF700

Other

Marker	Tag
CCR6	BV711
CX3CR1	PE Dazzle 594
CXCR3	BV650
CXCR5	Super Bright 436
CD25	PE-Cy5
CD39	APC Fire750
CD62L	BV480
CD127	BUV750
IgG	BUV395
Granzyme B	PE-Cy5.5
ICOS	AF488
LAG3	PE-Cy7
PD-1	BB515
OX40	BB700
TIGIT	BV605
Live/Dead	Zombie NIR

E

Characteristics of study subjects analyzed during repeated infection

Variable	Not gaining protection (n=6)	Gaining protection (n=5)	*P* value
Age, (yr)	2.7 (1.6-4.4)	6.2 (4.6-7.6)	0.026
Sex (% female)	50	50	>0.999†
Number symptomatic events	4.5 (2.5-5.3)	1.0 (1.0-1.3)	0.015
Number all recurrent events	11.5 (8.5-13.5)	8 (4.5-11)	0.093

*Median and IQR

† Fisher's exact test

#Symptomatic malaria ≥ 2500 parasites/ul and ≥ 37.5°C

Supplementary Figure 6. AIM assay on ZEB2$^+$ and ZEB2^{neg} memory CD4$^+$ T cells in participants during illness: (**A**) Table summarizing the characteristics of the study participants whose PBMC were used for the AIM assay. (**B**) Representative FACS dot plots of CD25 and 4-1BB expression by ZEB2$^+$ memory CD4$^+$ T cells in i) Healthy controls (HC) stimulated with control antigen (ctrl Ag) (n=3) or all *Pf* Ag (Circumsporozoite Protein (CSP), Merozoite Surface Protein-1 (MSP-1), Apical Membrane Antigen 1 (AMA-1), Erythrocyte Binding Antigen 175 (EBA-175) (n=3) and in ii) Malaria Patients (MP) stimulated with ctrl Ag (n=6) or all *Pf* Ag (n=6). (**C**) Representative FACS dot plots of CD25 and OX40 expression by ZEB2^{neg} memory CD4$^+$ T cells in i) HC stimulated with ctrl Ag (n=3) or all *Pf* Ag (n=4) and in ii) MP ctrl Ag (n=7), all *Pf* Ag (n=13) or *Pf* CSP (n=5). Bar graphs pool 3 independent experiments across distinct participants, each symbol is one participant. Two-sided Student's t-test was conducted between indicated groups ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. (**D**) Spectral flow cytometry panel (Aurora, Cytek) used for phenotypic characterization of PBMCs in Figures 7 and 8. **(E)** Table summarizing the characteristics of the study participants who gain or do not gain clinical immunity during the study.