Immune-surveillance over EBV and EBV-associated Burkitt lymphoma

How does malaria alter immune-surveillance and how might this impact immunotherapeutic efficacy?

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Department of Medicine

Outline

Part I: EBV and Cancers

Part II: Translational Science

Epidemiology of endemic Burkitt lymphoma

What is the role of T cells?

What is the role of NK cells?

Implications for immunotherapy?

I have no conflicts of interest to declare

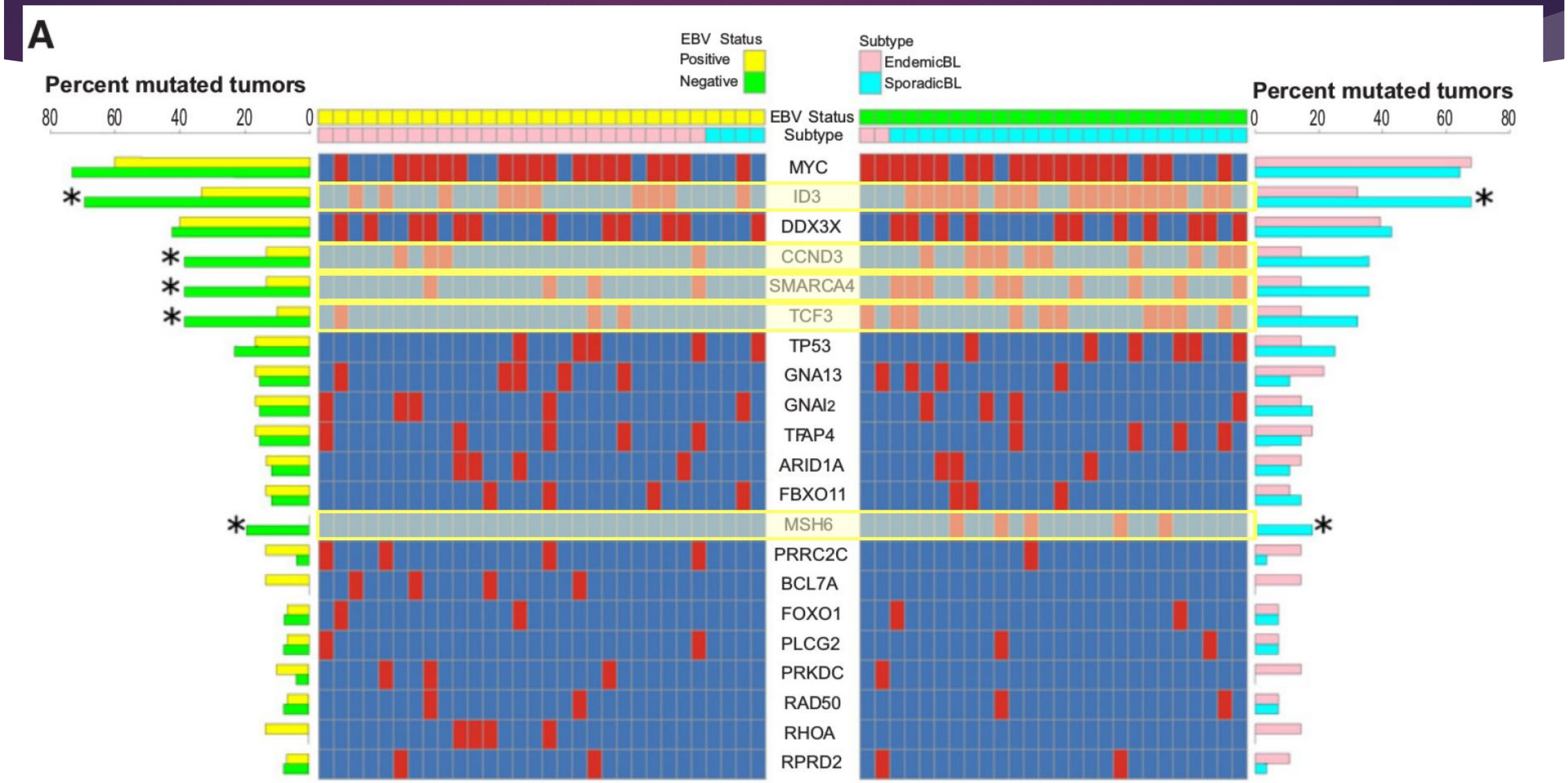
EBV (HHV-4)

- EBV was the first virus to be directly implicated in oncogenesis (endemic Burkitt lymphoma)
- >90% of adults worldwide are EBV infected (life-long herpes virus)
- 20% of human cancers are caused by an infectious agent and 80% of those are viral
- EBV is associated with 1-2% of all cancers

List of EBV-associated malignancies (not always B cells)

- Burkitt lymphoma (BL)
- non-Hodgkin's lymphomas (NHL)
- Hodgkin lymphoma (HL)
- Nasopharyngeal carcinoma (NPC)
- Gastric adenocarcinoma (GC)
- Post-transplant lymphoproliferative disorder (PTLD)
- Immuno-deficiency related cancers (IDL)
- T/NK cell lymphomas

Is EBV important or incidental?



EBV important beyond translocation

Kaymaz *et al*, Mol Cancer Res, 2017

EBV is not always present

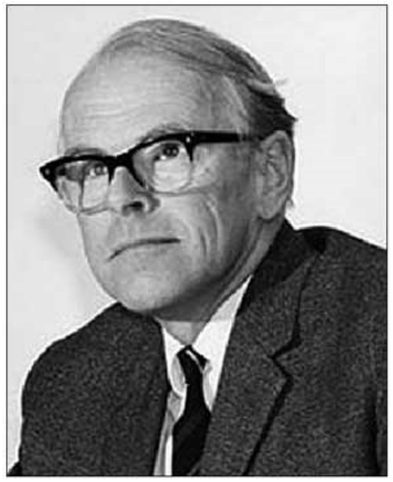
Table 4 Characterization of EBV-associated malignancies[a]

Malignancy	Subtype	EBV gene expression pattern	% EBV positivity
Burkitt's lymphoma	Endemic	Latency I	>95%
	Nonendemic		15–30%
Hodgkin's disease	MC	Latency II	70%
	LD		>95%
	NS		10–40%
	LP		<5%
Non-Hodgkin's lymphoma	Nasal T/NK	Latency II	>90%
	Angioimmunoblastic Lymphadenopathy	Latency II	Unknown
Nasopharyngeal carcinoma	Anaplastic	Latency II	>95%
Breast Cancer	Medullary carcinoma	Not clear	0–51%
	Adenocarcinoma		
Gastric Cancer	Lymphoepithelioma-like	Controversial novel LMP-1 negative	>90%
	Adenocarcinoma	Latency III	5–25%
Posttransplant lymphoproliferative disorders		Latency III	>90%
AIDS-associated lymphomas	IP-CNS	Latency III	>95%
	Other		30–50%
Leiomyosarcomas in immunosuppressed individuals	Leiomyosarcomas varies	Unclear	Frequent

Thompson *AACR* 2004

Part II: endemic Burkitt lymphoma
A tale of two infections

First described by Denis Burkitt in 1958



D. B. Burkitt

A COMBINED MEDICAL AND SURGICAL STAFF MEETING

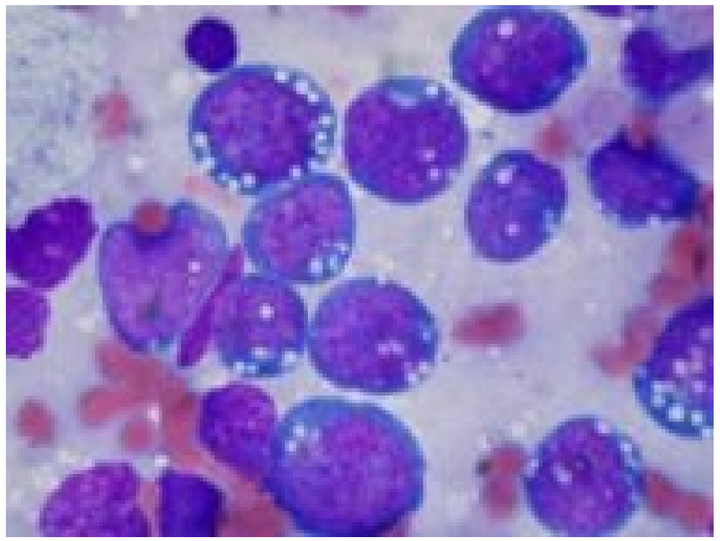
will be held

on Wednesday, 22nd March, 1961 at 5:15 p.m.

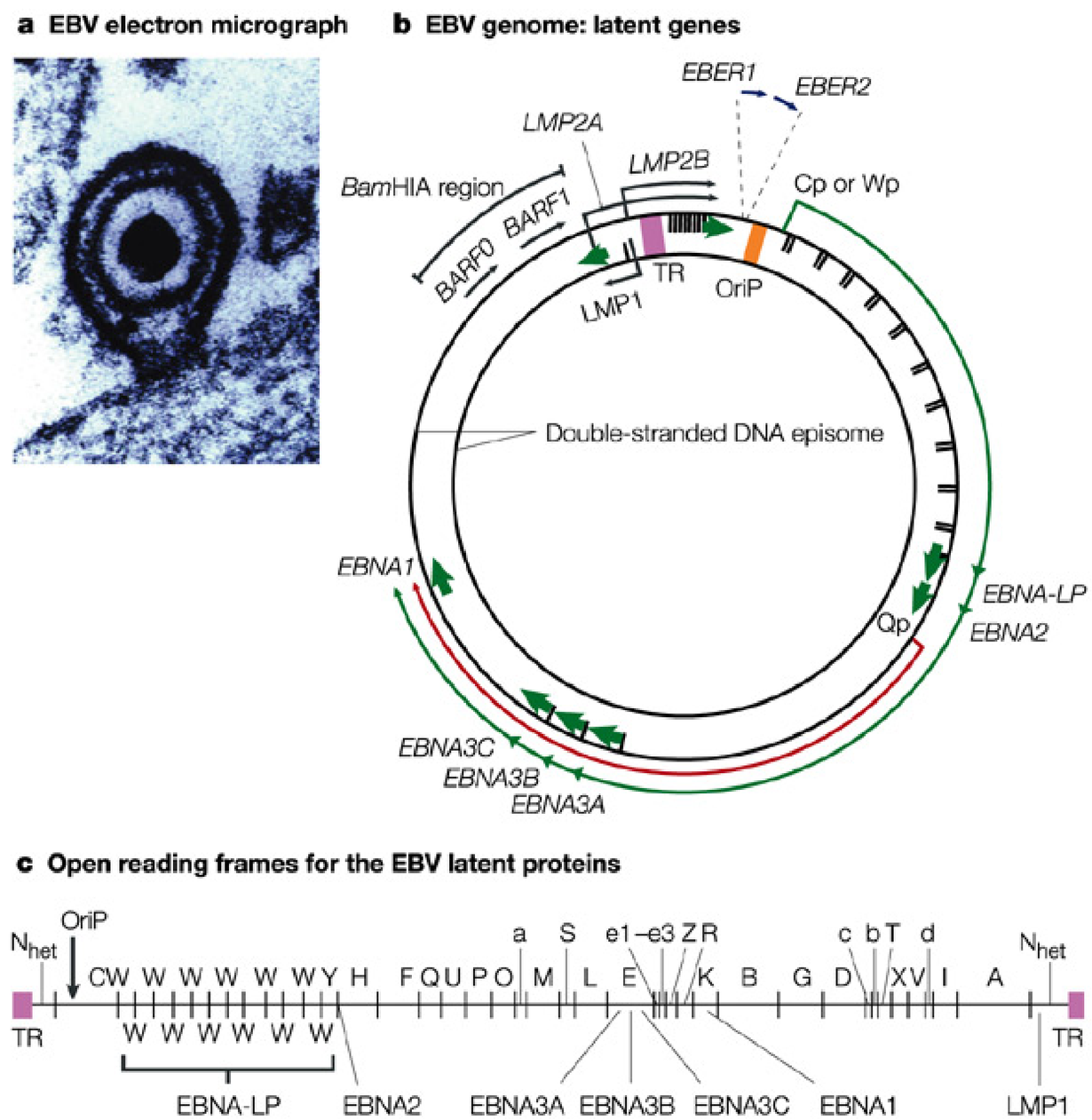
IN THE COURTAULD LECTURE THEATRE.

Mr. D.P. Burkitt from Makerere College, Uganda will talk on "The Commonest Children's Cancer in Tropical Africa. A Hitherto Unrecognised Syndrome".

Virus isolated from BL tumor in 1964 by Tony Epstein, Yvonne Barr and Bert Achong



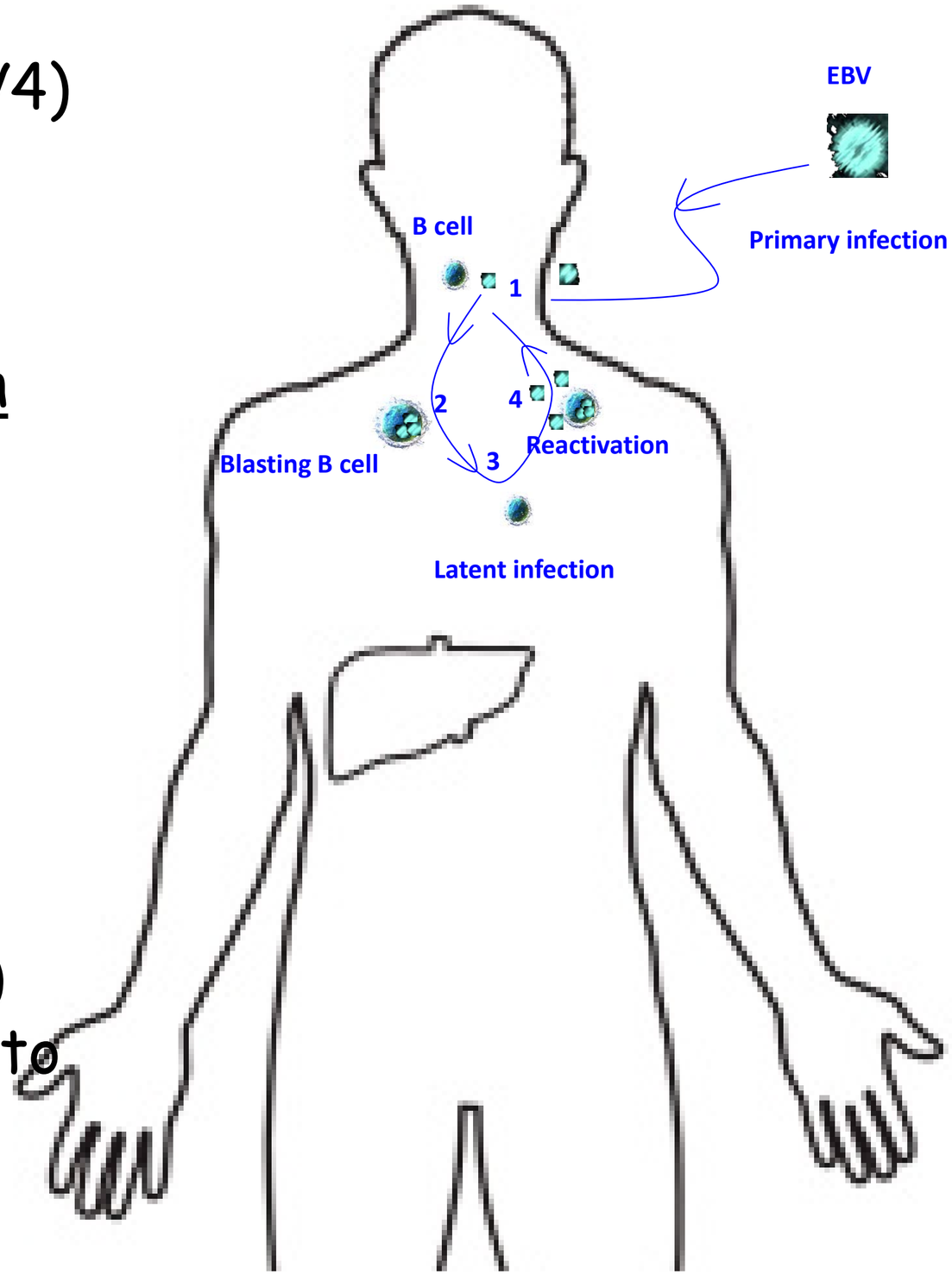
Human Herpes Virus 4 (HHV4)



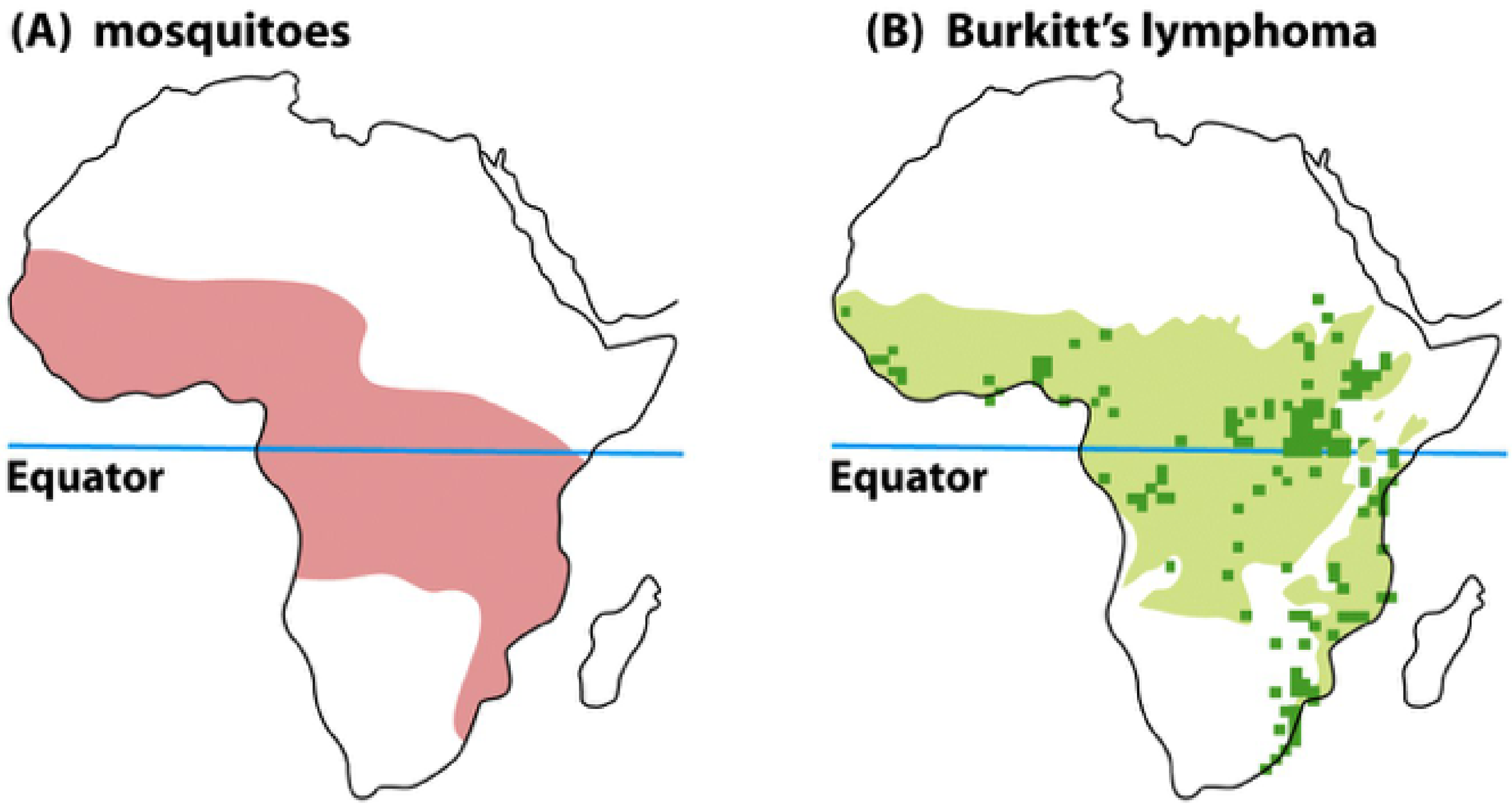
Nature Reviews | Cancer

Epstein Barr virus (EBV)

- Herpesvirus (*Herpesviridae* HHV4) Double strain DNA virus
- Latent proteins (5 EBNAs & 2 LMPs)
- EBV type 1 and type 2 common in African populations (EBNA-3 variants)
- Transmitted in saliva
- Asymptomatic infection young children but causes Acute Infectious Mononucleosis in adolescents
- Lytic reactivation (~44 proteins)
- EBV used to transform B cells into lymphoblastoid cell line (LCL)
- ***Life long latent infection in B cells (excellent immune evasion strategies)***

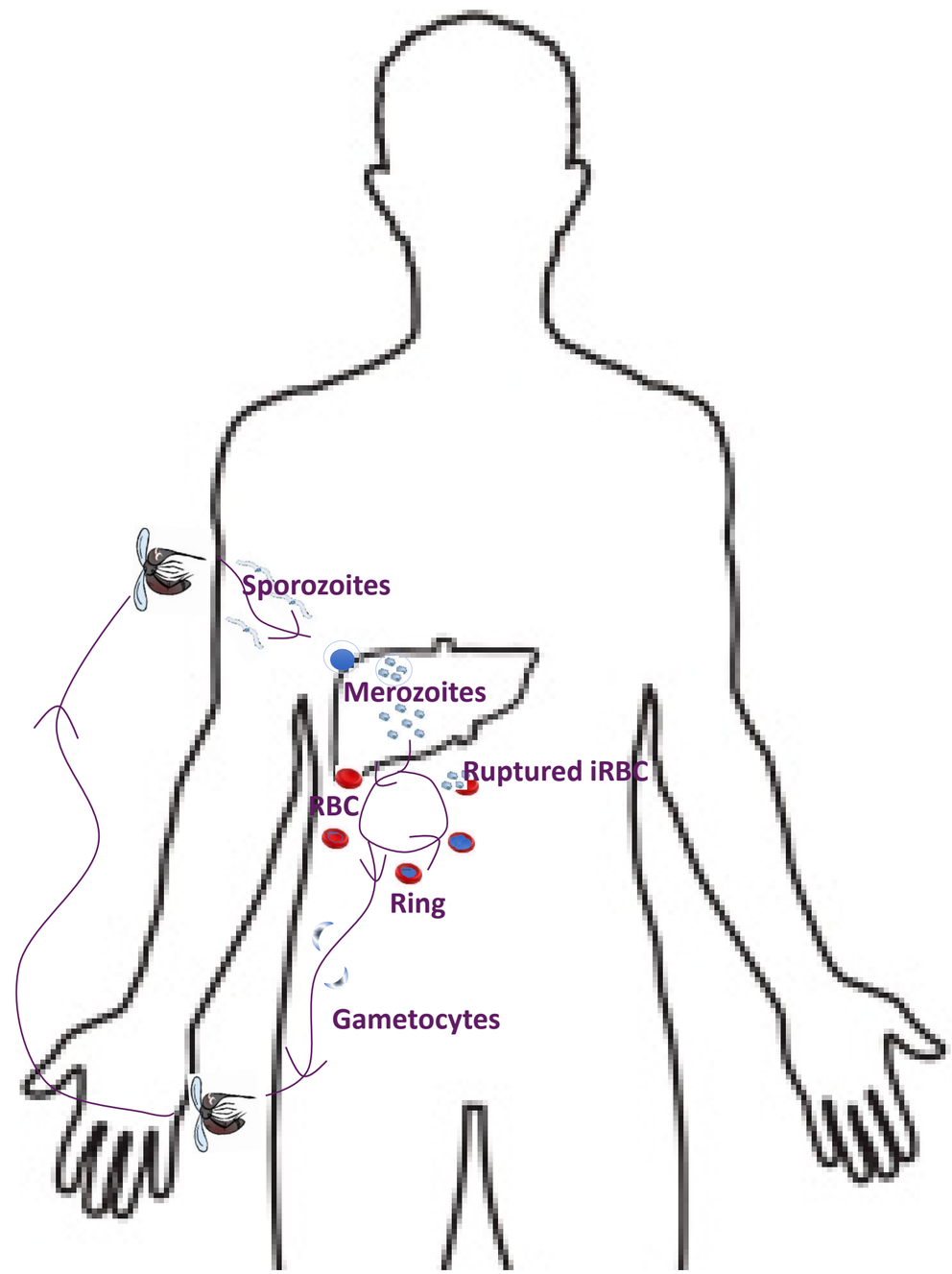


Geographic overlap with malaria: first cancer to be mapped

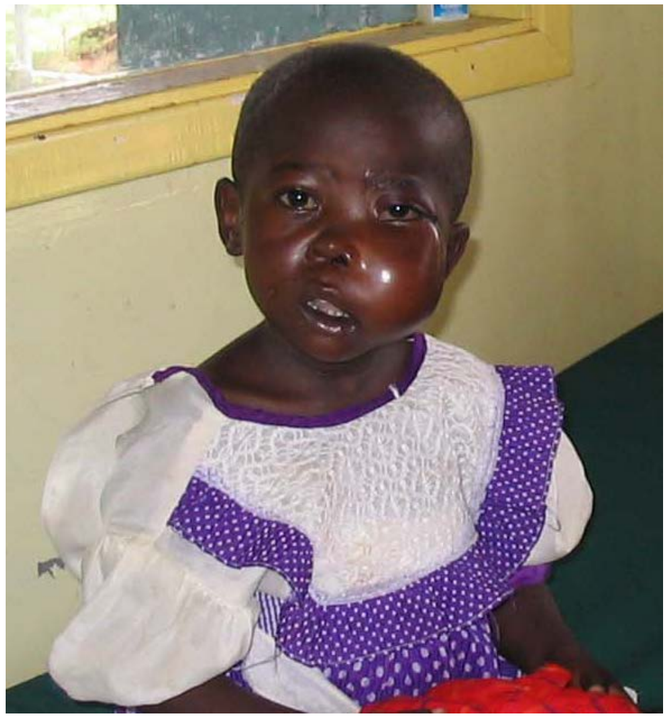


Plasmodium falciparum malaria

- Complex life cycle – infects liver cells and red blood cells (RBC)
- 22.8 Mb genome, 14 chromosomes & 2,400 proteins (allelic variants)
- Common parasitic infection for children living in Africa
- Number one cause of death for children under 5 years of age
- Semi-protective immunity develops after repeated infections which allows an acute infection to become a chronic infection of mature RBC (no APC)



The face of Burkitt lymphoma today



Rochford, Cannon, Moormann
NatRevMicro 2005

- Most common pediatric cancer in equatorial Africa
- Annual incidence 2-5 per 100,000 children
- Peak incident age 5-9 years
- Extranodal monoclonal B cell tumor
- High tumor proliferation index
- Tumor presentation in abdomen as well as jaw
- Conventional chemotherapy used without the need for surgery or radiotherapy
- Endemic BL in Africa > 90% EBV-associated tumor
- Curious note: **~10% of eBL are EBV-negative tumors,** if you only counted the incidence of non-EBV BL in Africa it would be the same as sporadic BL in the US.

Buckle *IJC* 2017

Old Literature in support of malaria-induced 'immuno-suppression' model

Gambian patients with acute malaria were unable to control outgrowth of EBV-transformed cells measured by an *in vitro* regression assay *(*Whittle *Nature 1984)*.

Healthy adults living in malaria holoendemic regions of Papua New Guinea had impaired EBV-specific T cells responses measured by regression assay (Moss *Int J Canc 1983*).

Both were cross-sectional studies.

Focus on testing the immune “suppression” hypothesis

Children who have had chronic malaria infections develop qualitative and quantitative differences in their immune control over EBV compared to those who have not been exposed to malaria.

My immunology studies started in 2002
~ 20 years since last publications on this topic

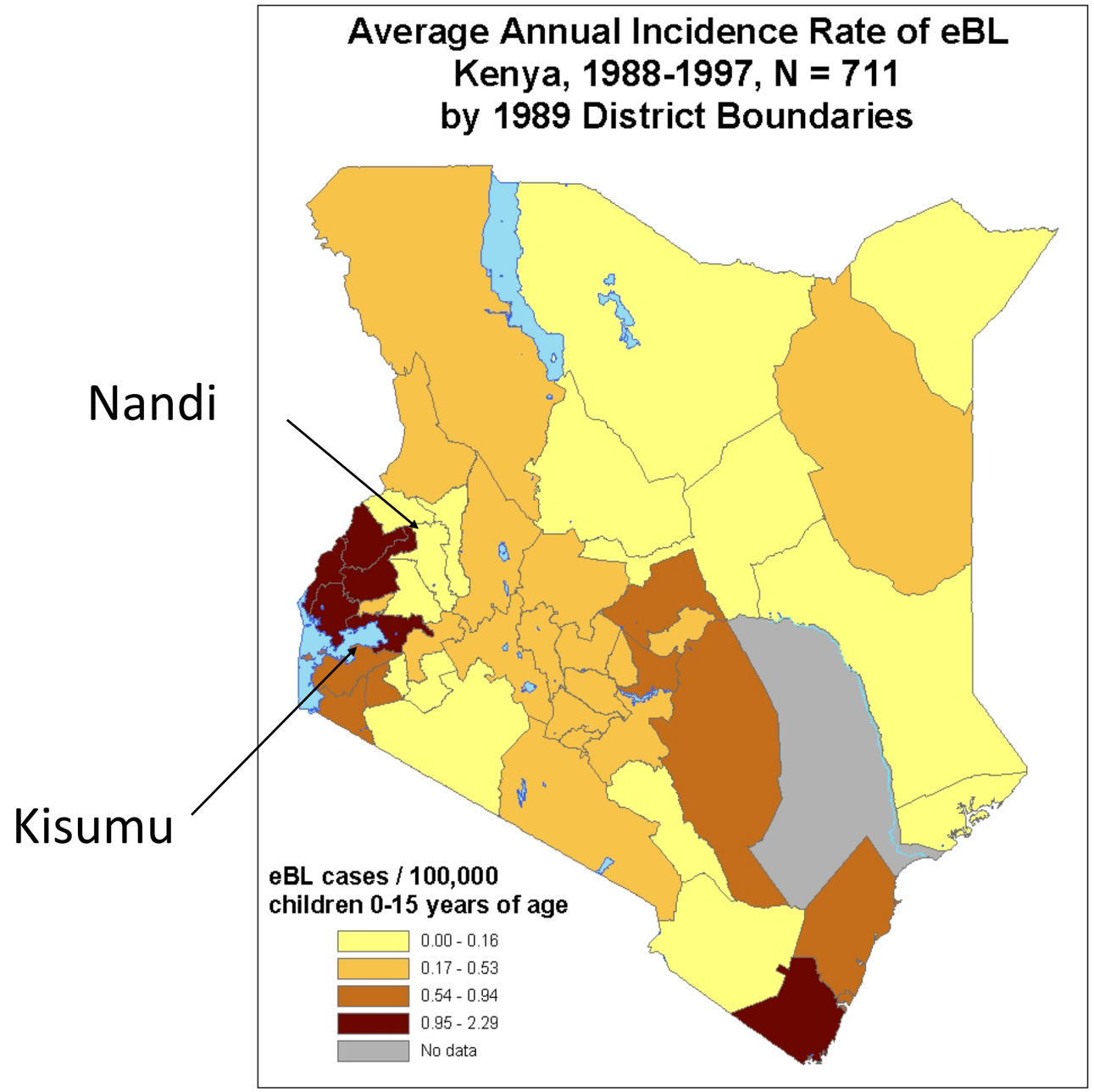
Study design: Healthy children with and without malaria exposure

Nandi County

- Hypoendemic malaria transmission
- Low incidence of eBL
- Sample size = 130 children ages 1-14 years

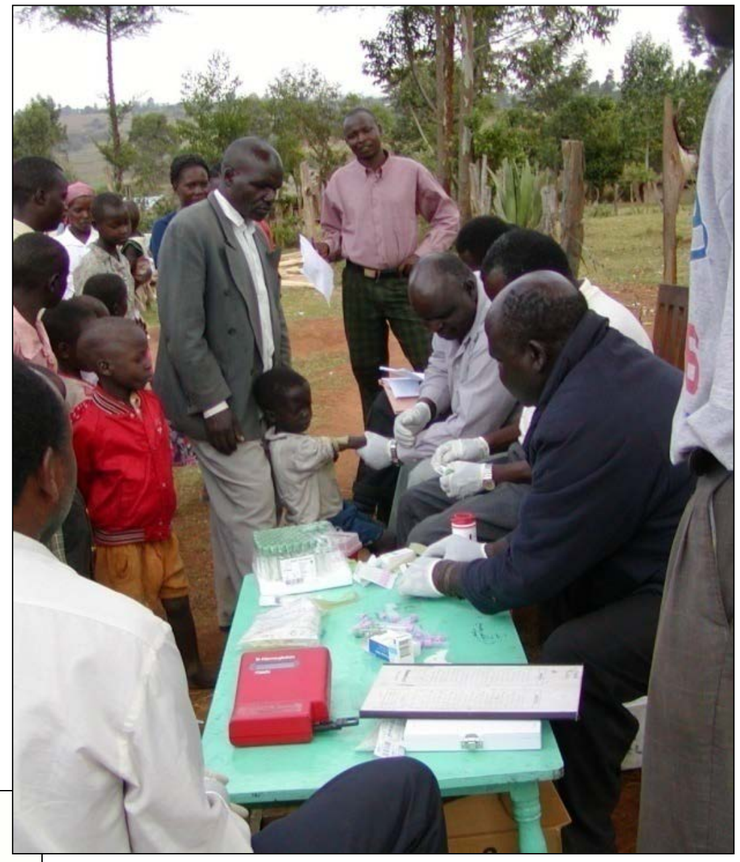
Kisumu County

- Holoendemic malaria
- High incidence eBL
  Sample size = 106 children ages 1-14 years



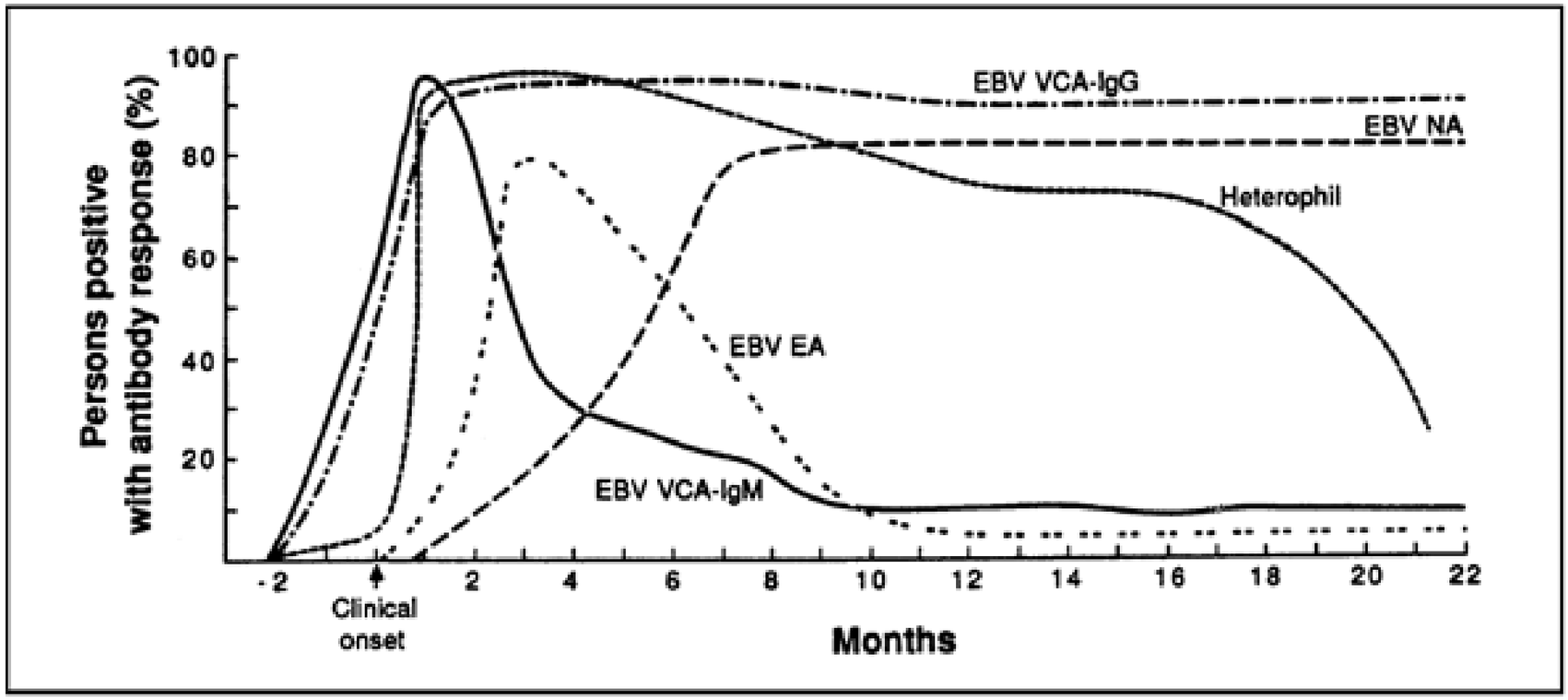
Rainey *TMIH* 2007

Village-based, age-structured cross-sectional studies



UMMS-KEMRI lab in Kisumu

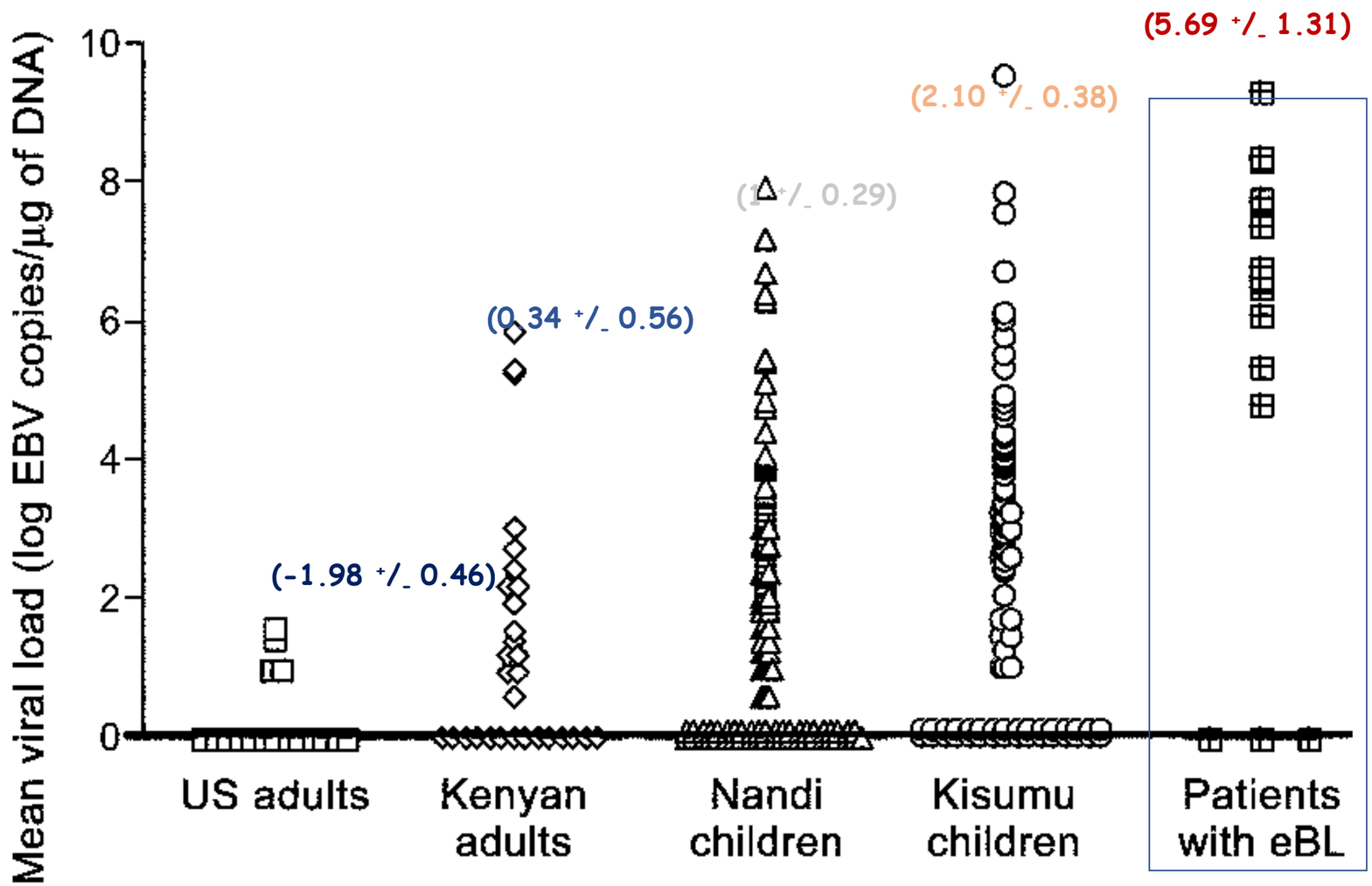
How do you measure EBV infections?



IgM and IgG antibody titers to a panel of EBV antigens

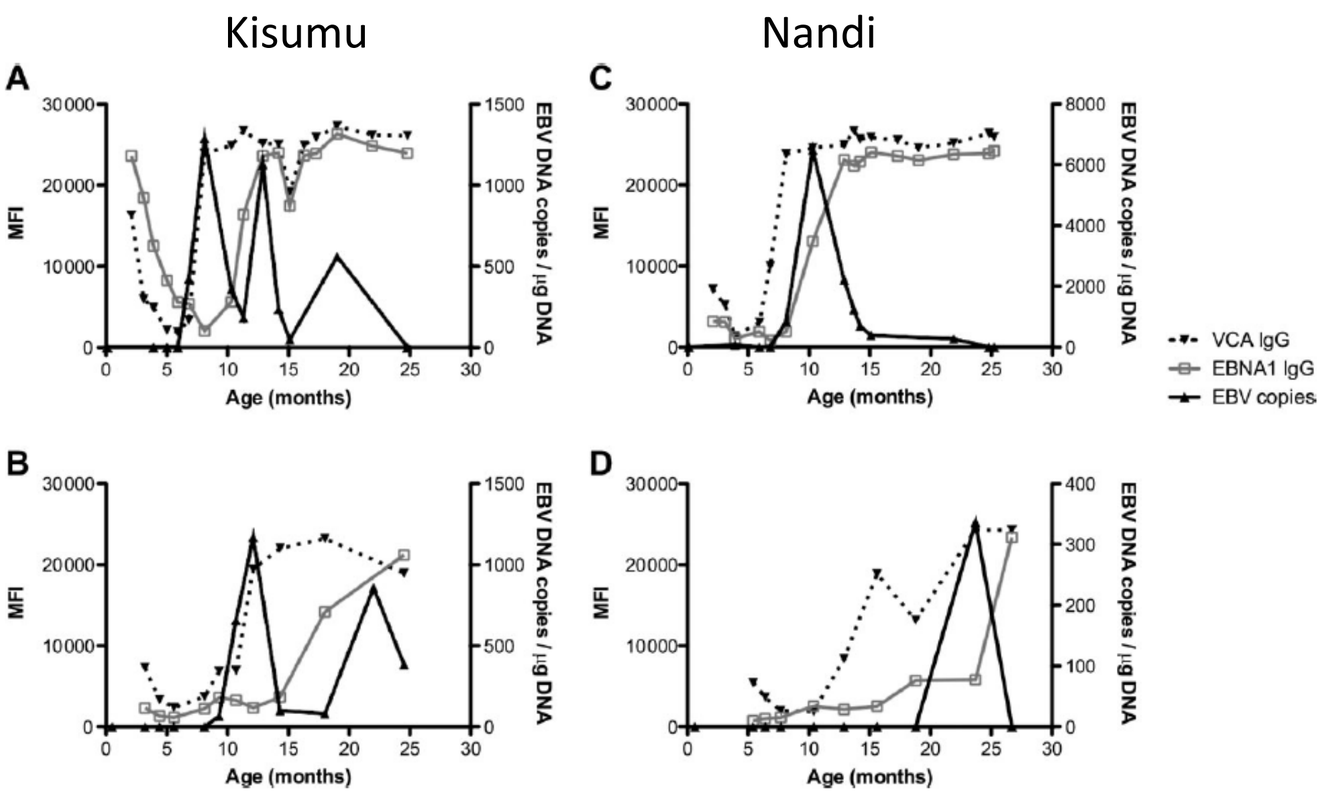
1. Viral Capsid Antigen (VCA)
2. Early Antigen (EA)
3. EBV Nuclear Antigen 1 (EBNA1)
4. Zta Reactive Antigen (ZEBRA)

Higher EBV loads in Kisumu and eBL children by qPCR: surrogate for immune control

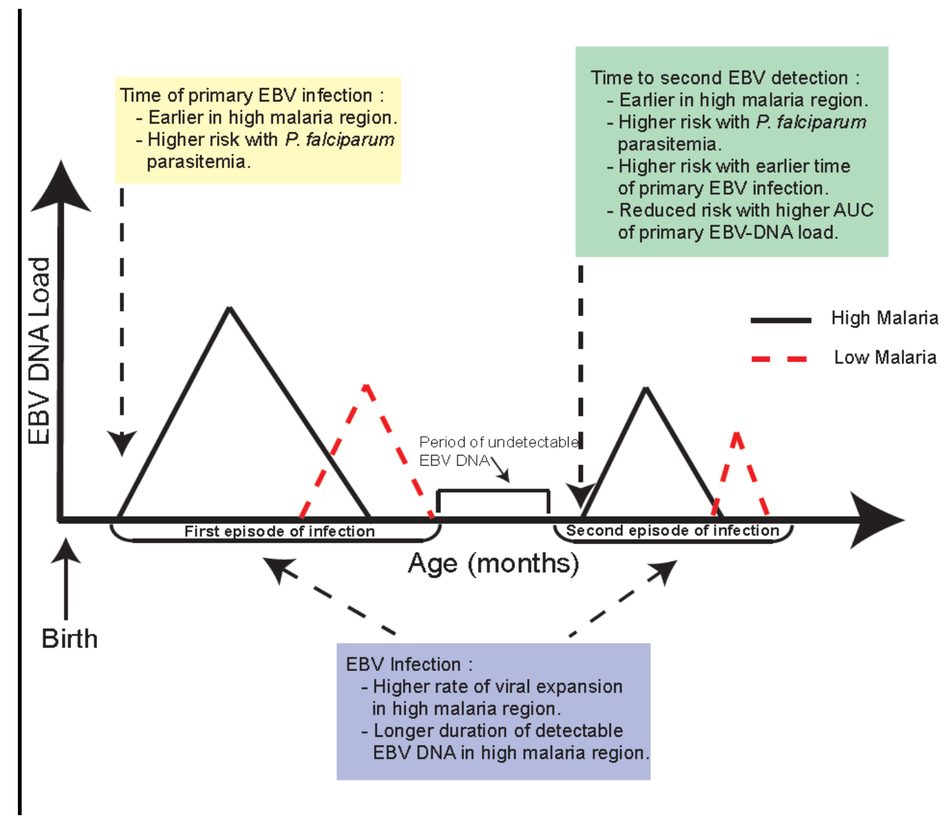


Moormann, JID, 2005

Longitudinal studies reveal higher cumulative EBV burden in children with chronic malaria

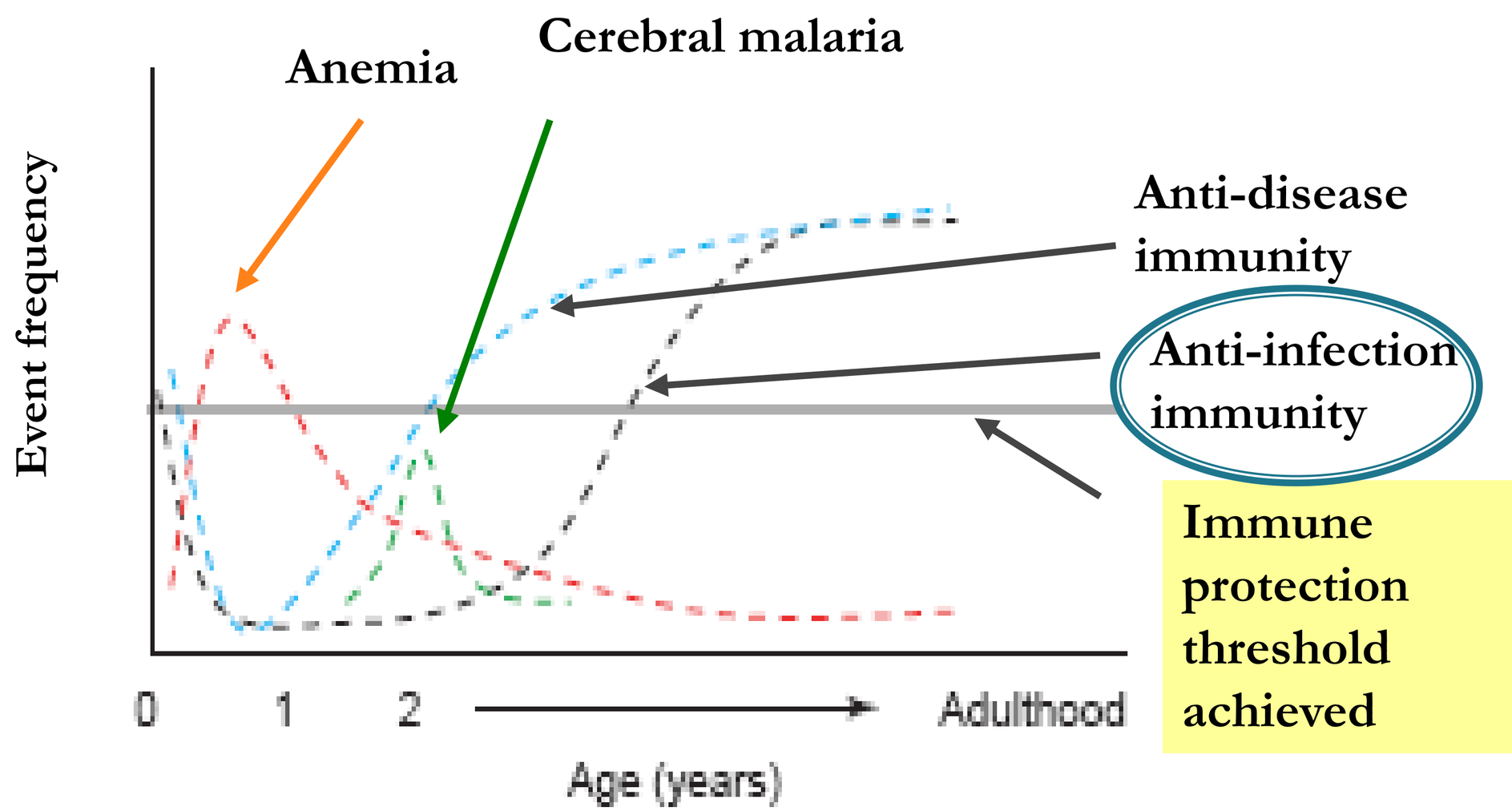


Piriou *et al* JID 2012



Reynaldi *et al* JID 2016

How do you measure malaria infections in children?

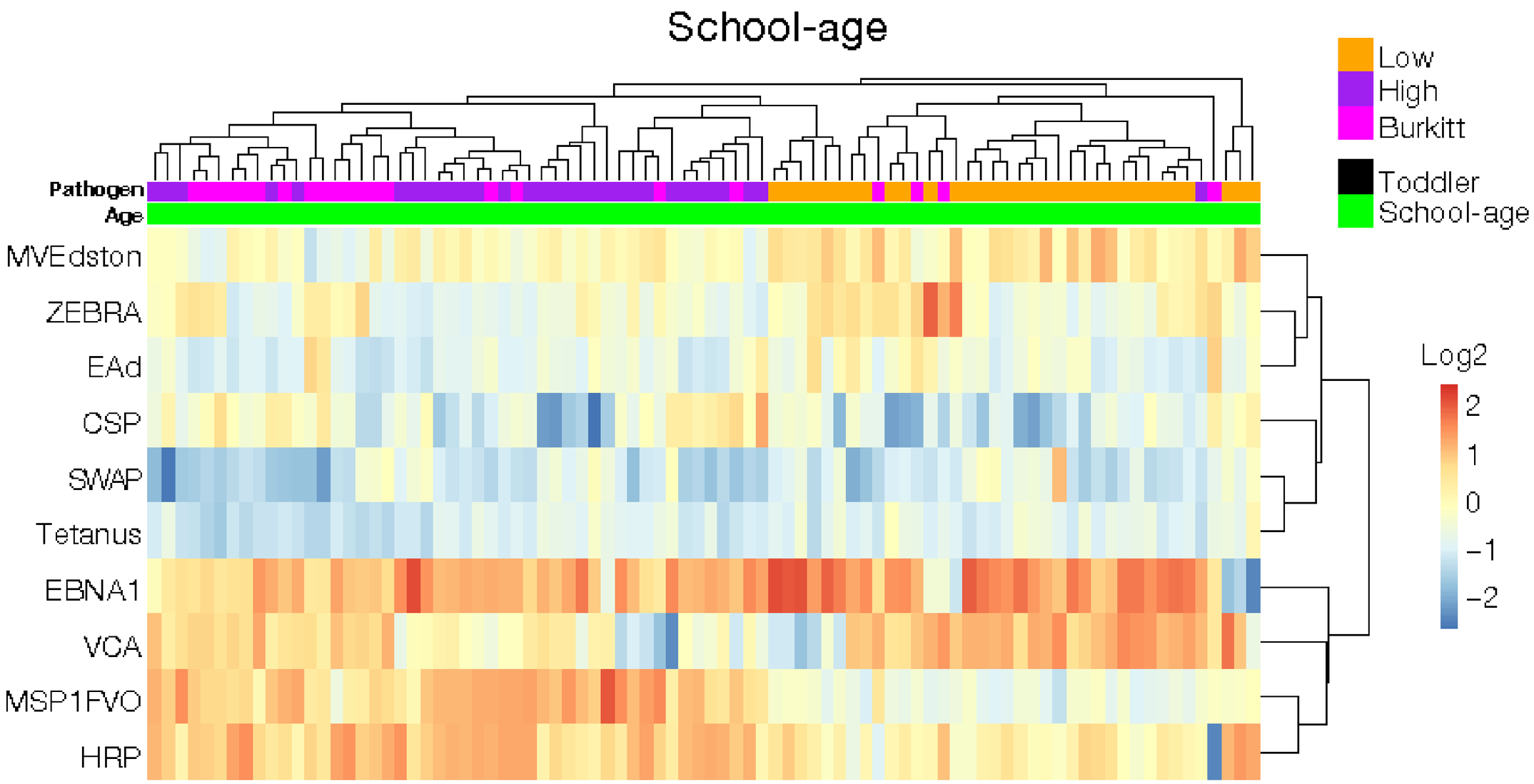


Malaria morbidity and mortality highest < 5 years

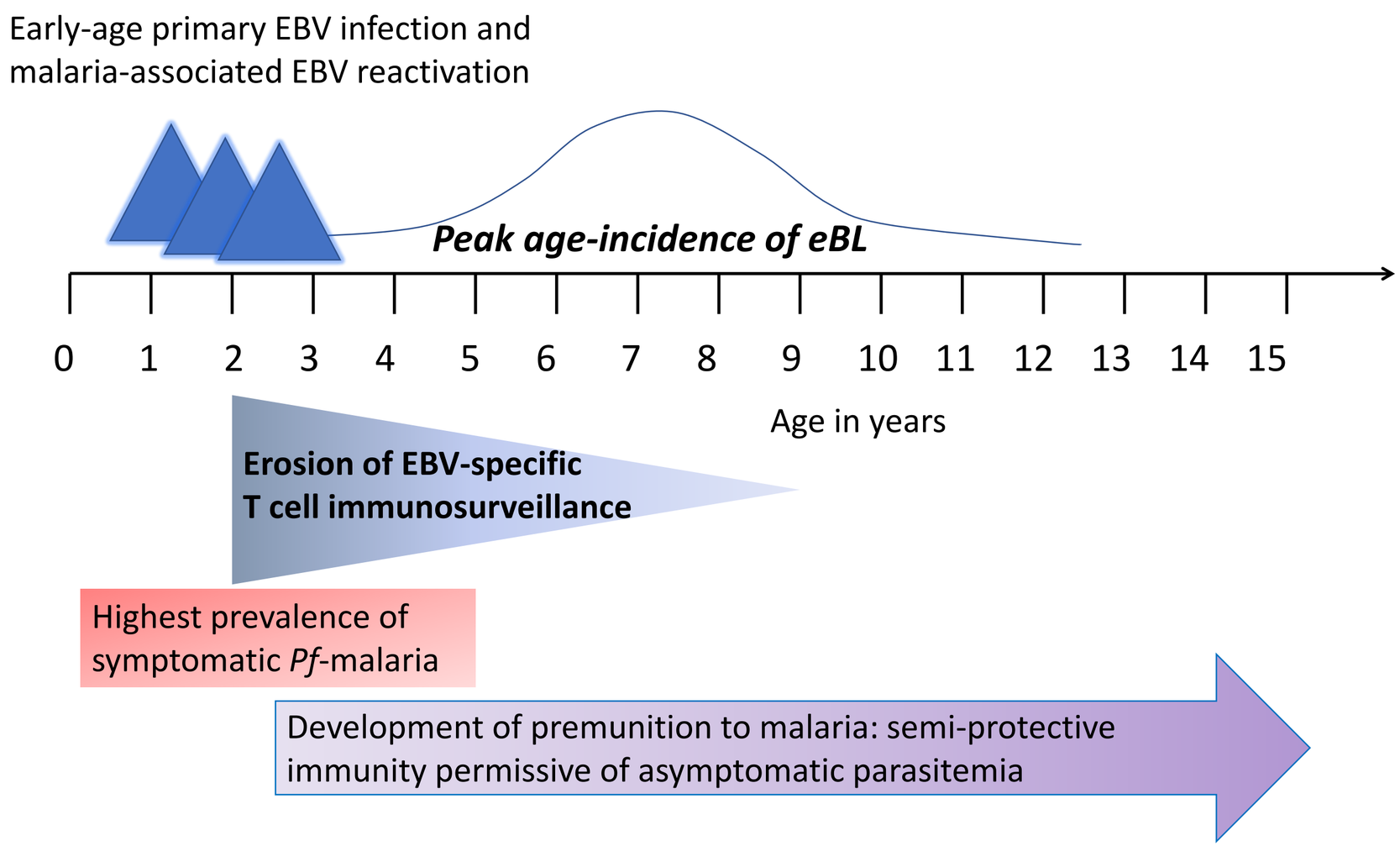
BL incidence highest in children 5-9 years old

Modified from Stoute Trends Parastiology 2005

Luminex serology profiles unsupervised hierarchical clustering



What is the cumulative impact of *Plasmodium falciparum* malaria co-infections on immunity to Epstein Barr virus?



Rochforld and Moormann Curr Trop Microbiol Immunol 2015

How is EBV-specific T cell immunity influenced by malaria exposure?

Characteristics of T cell mediated EBV immunosurveillance learned from adult studies

- Immunodominant epitopes to EBV lytic and latent antigens
- HLA Class I restricted IFN-γ responses mediated by CD8+ T cells
- Stable IFN-γ responses in healthy adults who are EBV seropositive with low to no detectable virus in peripheral circulation.
- EBNA1 induces IFN-γ responses primarily from CD4+ T cells but also cross-priming for CD8 + T cells

T cell immunity to EBV antigens

- Latent genes:
  - 2 non-translated small RNAs (EBER-1 and -2),
  - 6 nuclear proteins (EBNA-1, EBNA-2, EBNA-3A, -3B, -3C and EBNA-LP).
  - 3 latent membrane proteins (LMP-1, -2A and -2B)
- Lytic genes ~70: (Bam H1 fragments) BZLF1, BMLF1, BMRF1, BRLF1, BARF0, BHRF1, gp85, gp110, gp350.

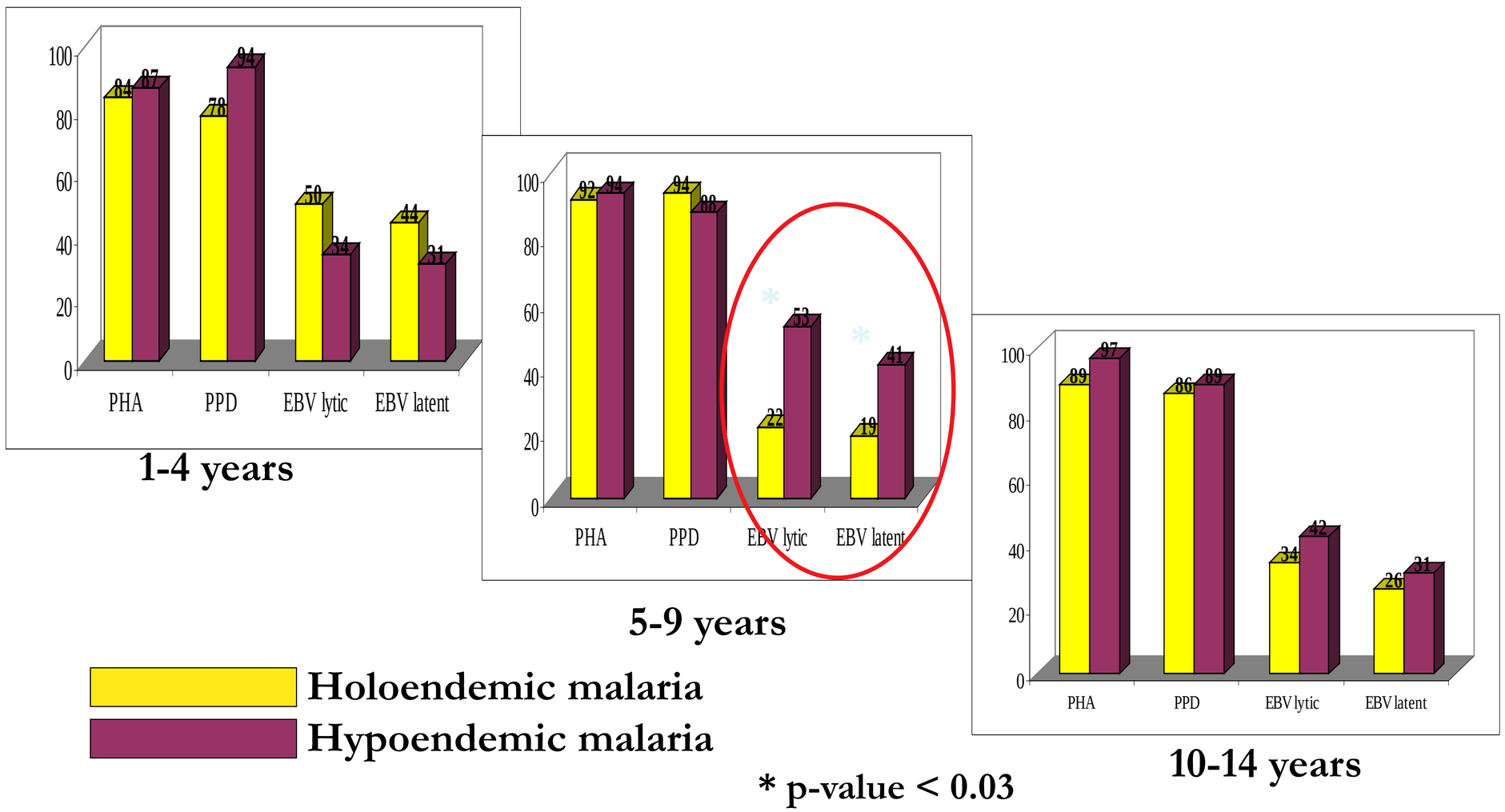
Demographics of Kenyan study population

		Age Group (years)			
		1-4	**5-9**	**10-14**	**All ages**
		n (%)	n (%)	n (%)	n (%)
Kisumu					
	No. of subjects	34 (32)	37 (35)	35 (33)	106 (100)
	EBV seropositive	32 (94)	37 (100)	35 (100)	104 (98)
	Mean hemoglobin (g/dl)	9.72	12.25	12.51	11.53
	Subjects with P.f. positive smear	26 (77)	27 (73)	29 (83)	82 (77)
	Mean body temperature (^{o}C)	36.80	36.76	36.89	36.82
Nandi					
	No. of subjects	39 (30)	50 (38)	41 (32)	130 (100)
	EBV seropositive	36 (92)	50 (100)	41 (100)	127 (98)
	Mean hemoglobin (g/dl)	12.18	12.82	13.29	12.77
	Subjects with P.f. positive smear	3 (8)	7 (14)	11 (26)	21 (16)
	Mean body temperature (^{o}C)	37.21	37.18	37.09	37.16

HLA Class I-restricted EBV peptide selection for IFN-γ ELISPOT assays

EBV protein	Cycle	Amino Acids	Peptide Sequence	HLA-restriction
BZLF1	Lytic	190-197	RAK FKQ LL	HLA B8
BMLF1	Lytic	280-288	GLC TLV AML	HLA A2
BRLF1	Lytic	148-156	RVR AYT YSK	HLA A3
BRLF1	Lytic	28-37	DYC NVL NKE F	HLA A24
EBNA 3A	Latent	379-387	RPP IFI RRL	HLA B7
EBNA 3C	Latent	258-266	RRI YDL IEL	HLA B27
EBNA 3B	Latent	217-225	TYS AGI VQI	HLA A24
EBNA 3A	Latent	325-333	FLR GRA YGL	HLA B8
EBNA 3A	Latent	596-604	SVR DRL ARL	HLA A2
EBNA 3A	Latent	603-611	RLR AEA QVK	HLA A3
EBNA 3B	Latent	657-666	VEI TPY KPT W	HLA B44

EBV-specific IFN-γ ELISPOT responses deficient in children 5-9 yrs old from malaria holoendemic area



Moormann *et al* JID 2007

HLA Class I Tetramer specificity of T cells

Tube 1: FLY (LMP2), CMV (pp65), YLL (LMP1), CLG (LMP2)

Tube 2: FLY (LMP2), YVL (BRFL1), GLC (BMLF1), LLD (EBNA3C)

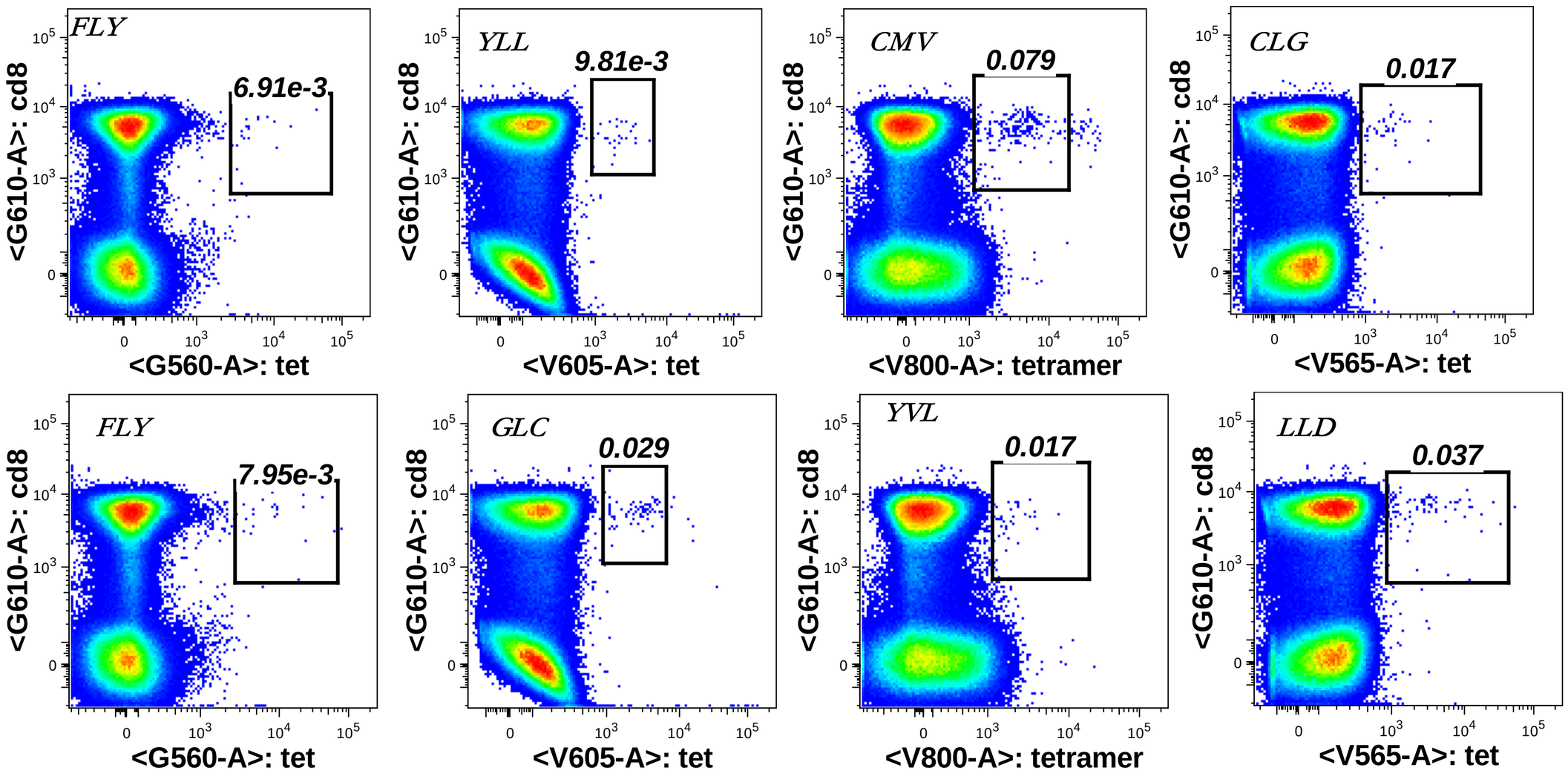
Or another way to think about the HLA-A2 panel:

Latent antigen peptides to LMP1, LMP2 x 2 and EBNA3C

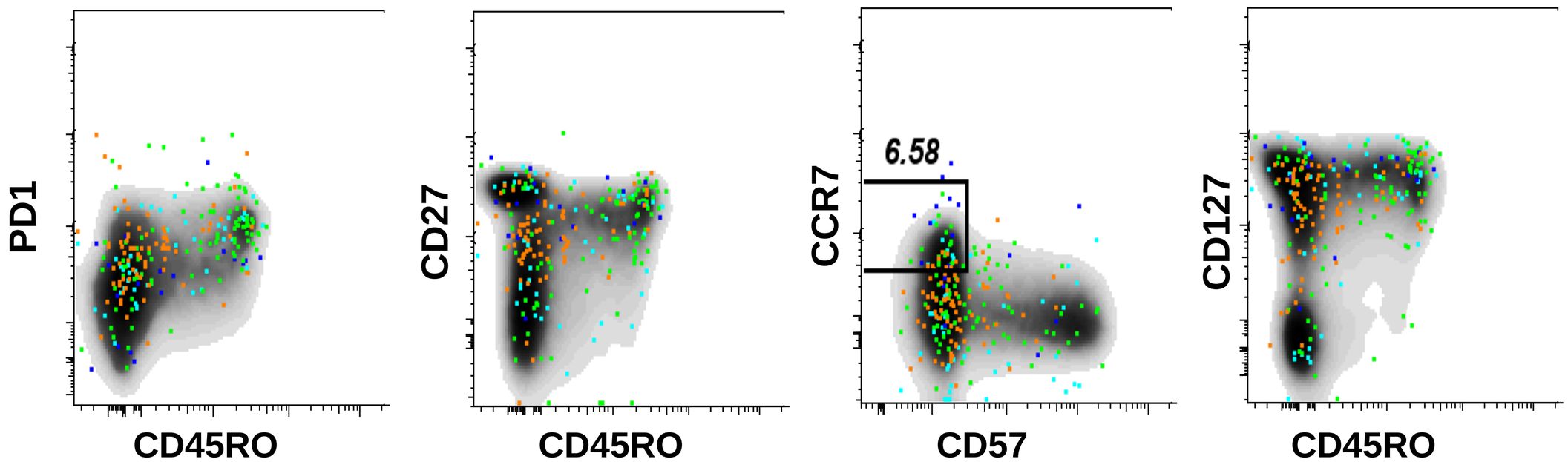
Lytic antigen peptides to BRLF1 and BMFL1

Collaboration with Pratip Chattopadhyay and David Price

Example of EBV-specificity and frequency of CD8 T cells by tetramer staining for an individual

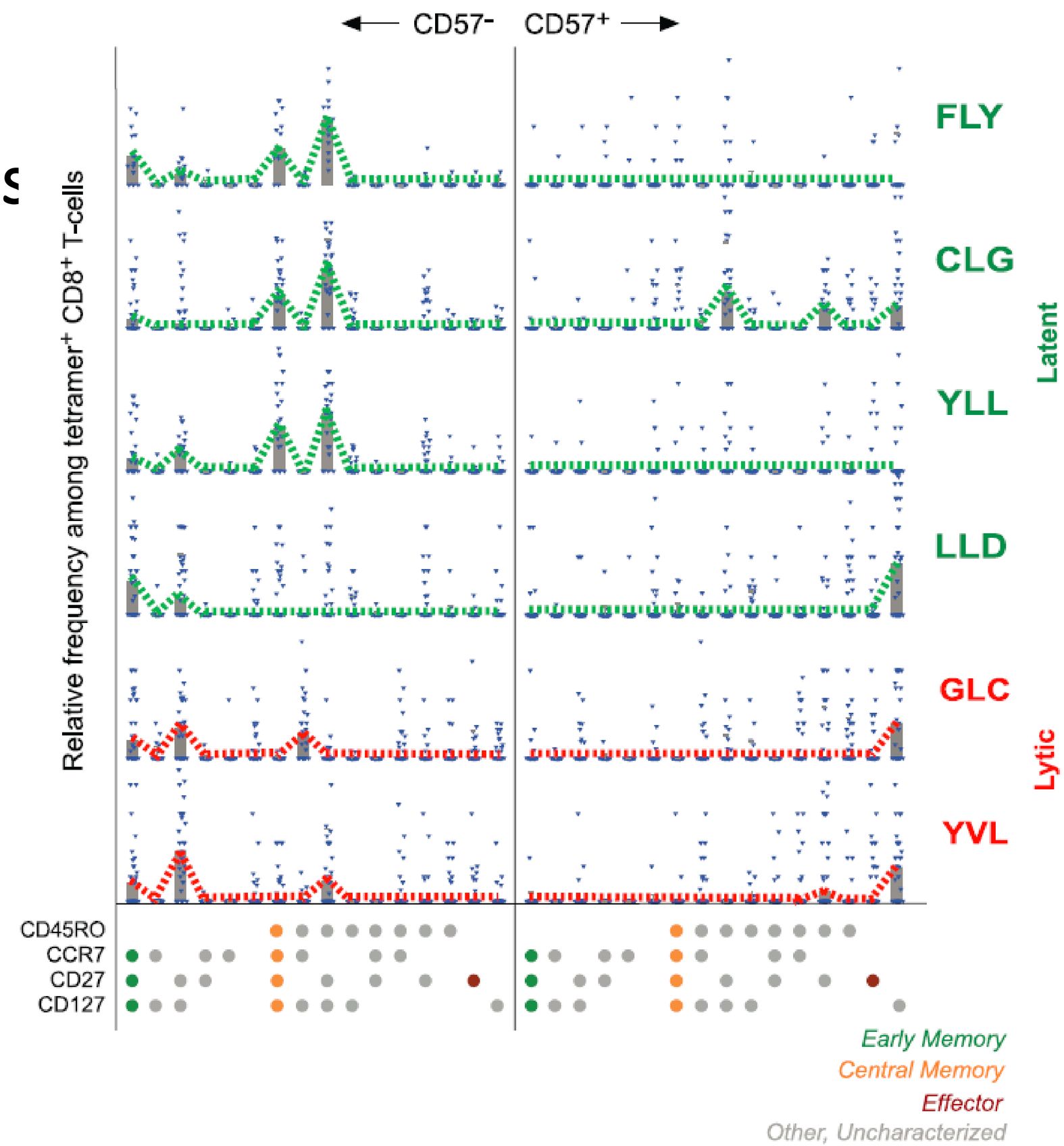


Overlay of EBV-specific CD8 T cells by phenotype



Each color dot represents a CD8 T cell specific for an EBV-peptide HLA-A2 Tetramer

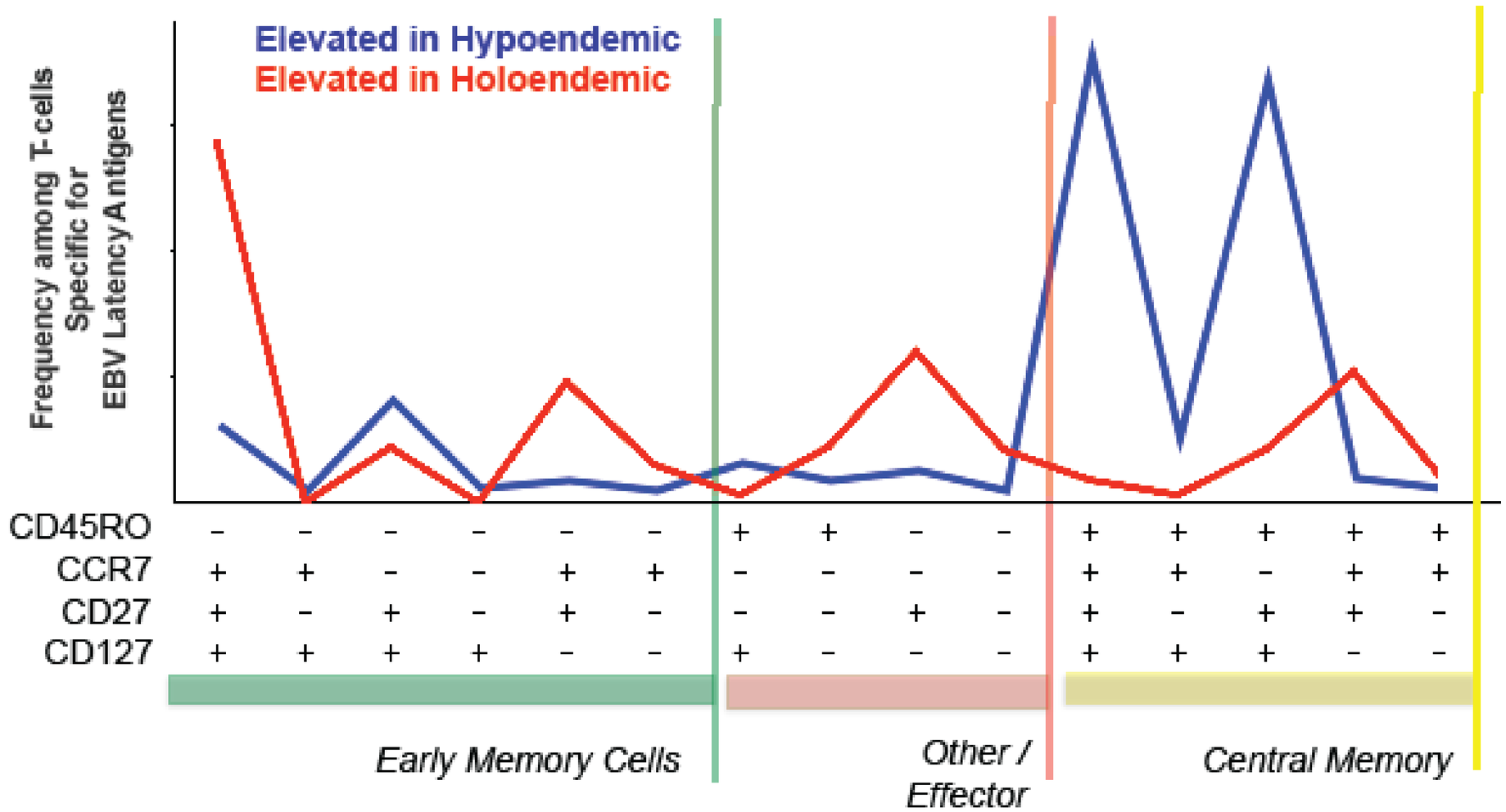
Qualitative differences exists between EBV-specific lytic and latent T cell subsets



Chattopadhyay JV 2013

Qualitative Differences in CD8$^+$ T-cells subsets for EBV latent antigens associated with malaria endemicity

Chattopadhyay JV 2013

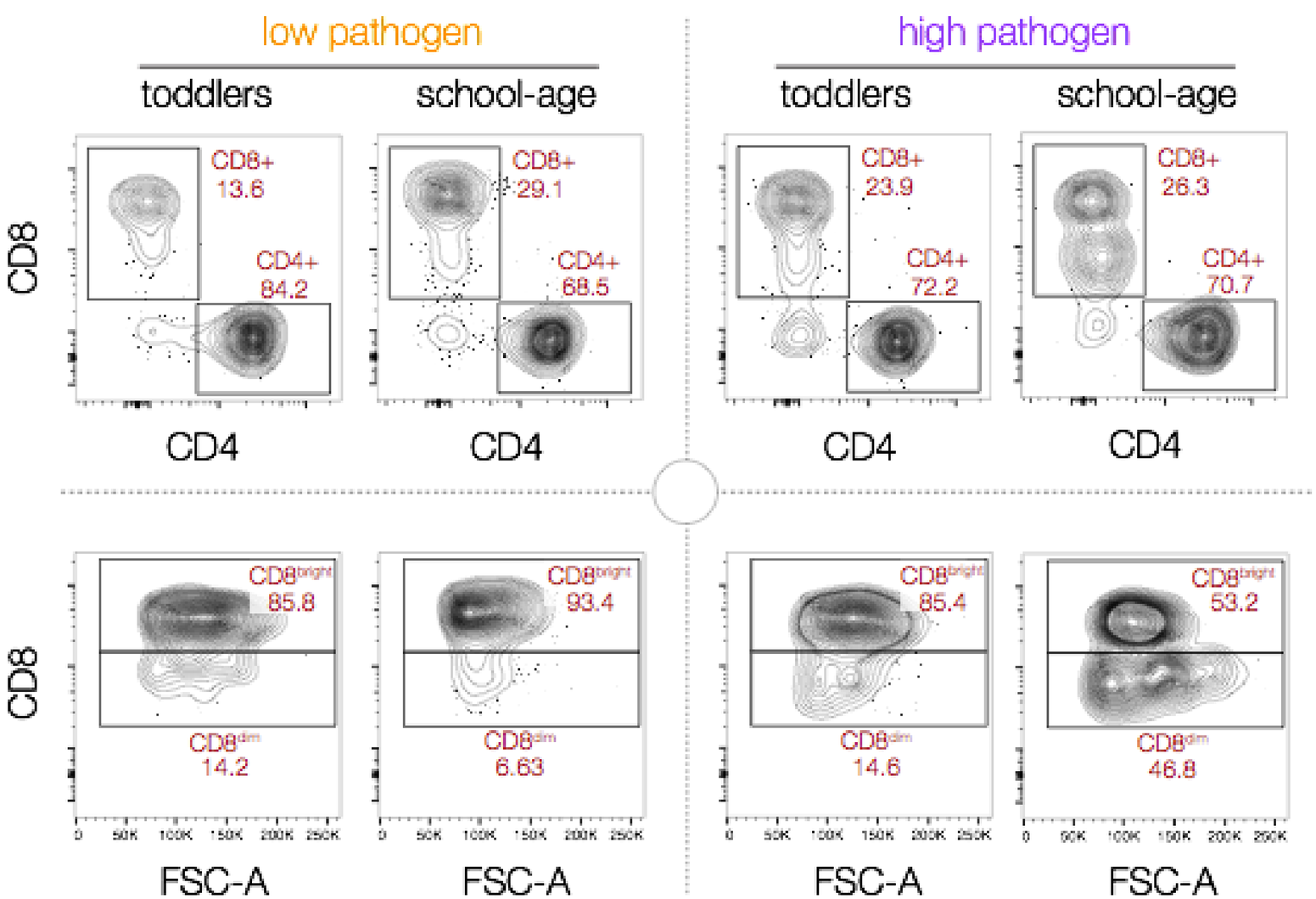


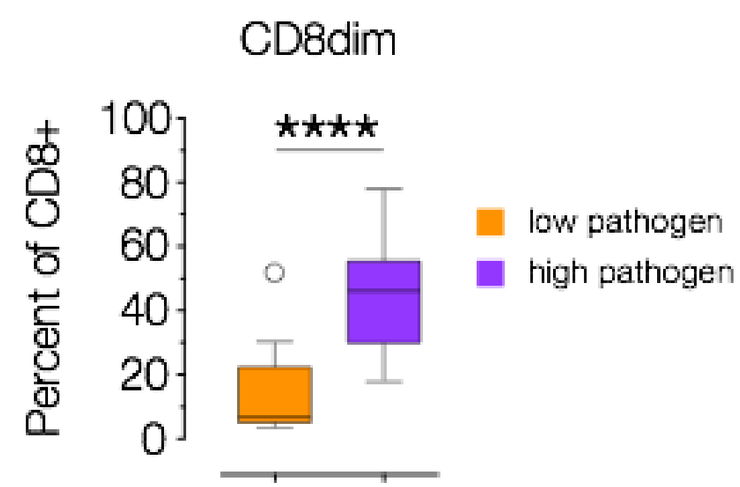
Conclusion from tetramer studies

- Children exposed to chronic malaria infections had fewer memory EBV-specific T-cells that were more differentiated and less capable of homeostatic proliferation compared to those from the hypoendemic malaria area.

- Malaria has a specific effect on EBV-T cells in contrast to CMV or bulk T cells which did not differ between groups (data not shown).

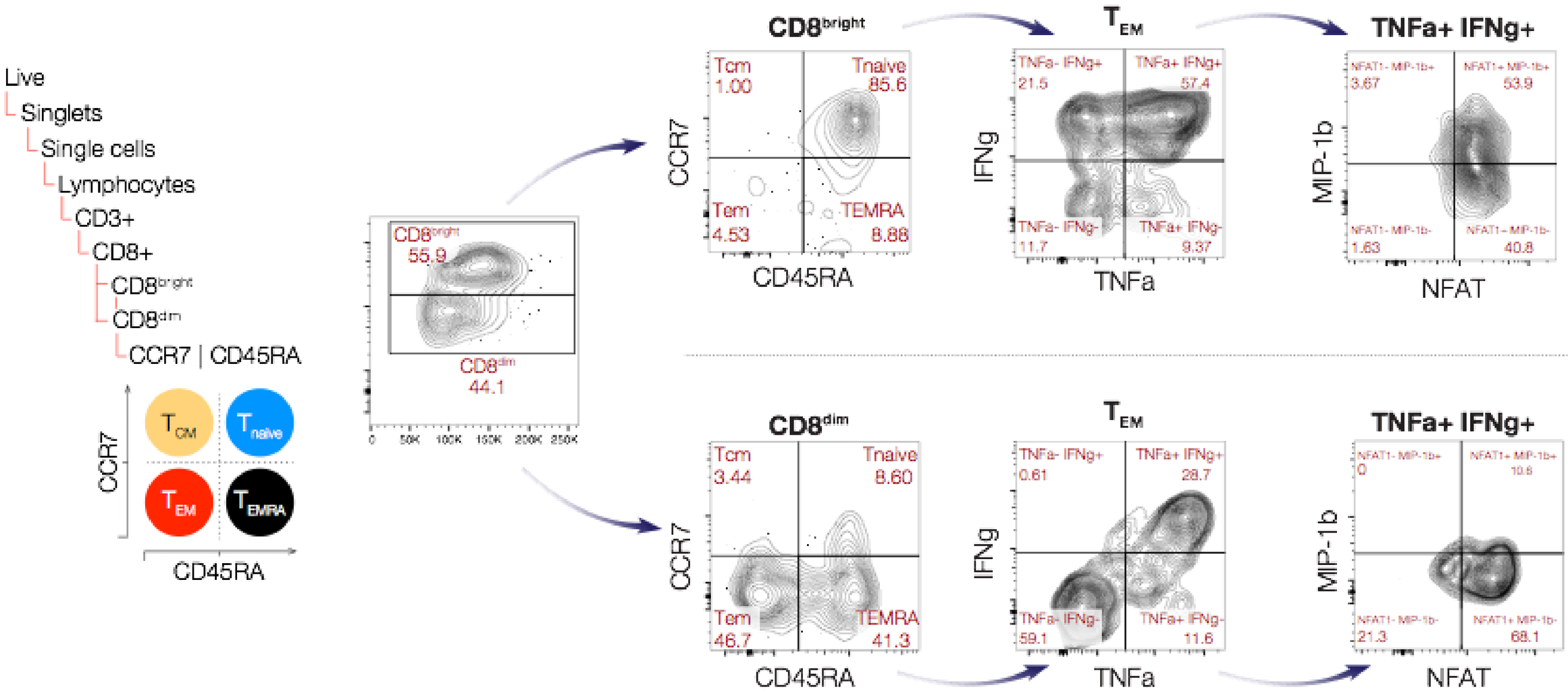
Persistent malaria exposure is associated with the generation of a distinct population of CD8^{dim} T cells



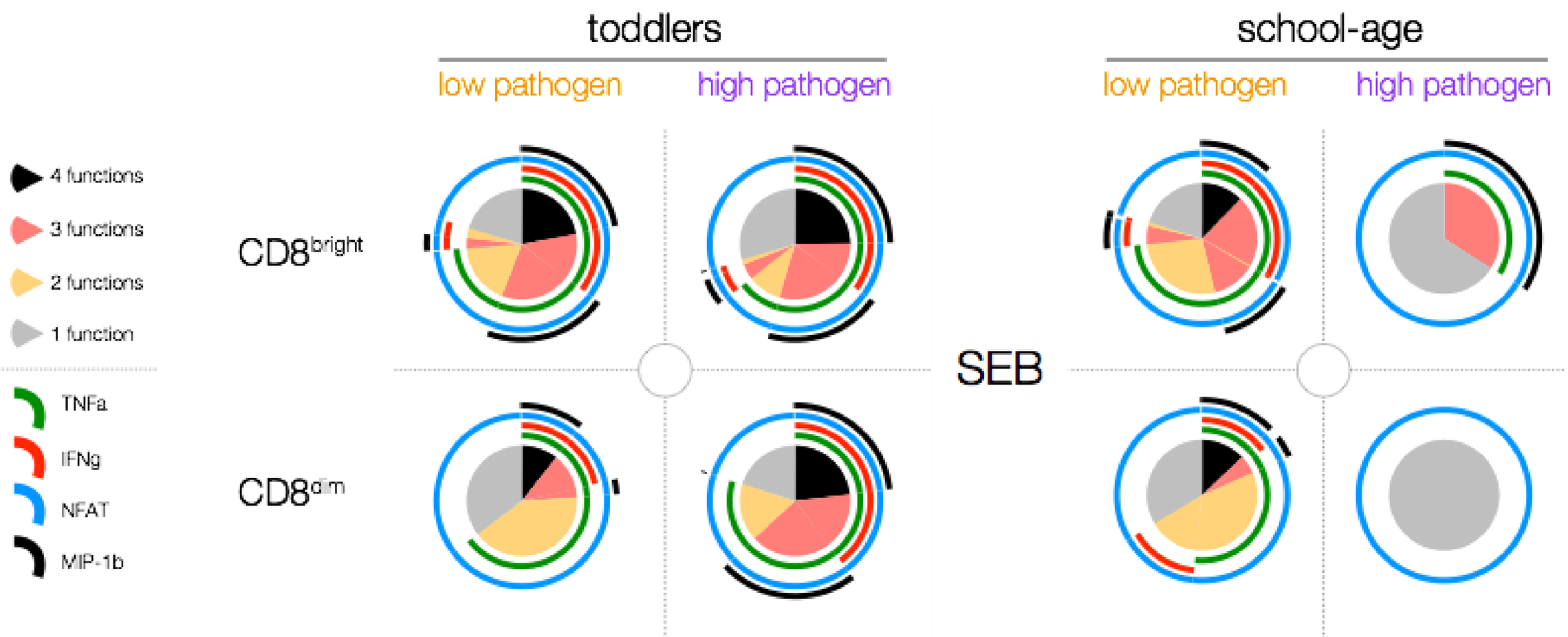


Yves Falanga
Falanga *JCI Insight* 2018

What is the impact of chronic malaria exposure on CD8 T cell function?



CD8 T cell effector function is significantly diminished in children after prolonged exposure to malaria



Summary of T cell studies, to date

- The phenotype and function of CD8 T cells <u>change over time</u> in malaria exposed children compared to non-malaria exposed children.
- CD4 T cells seem to be influenced by age but not by malaria exposure

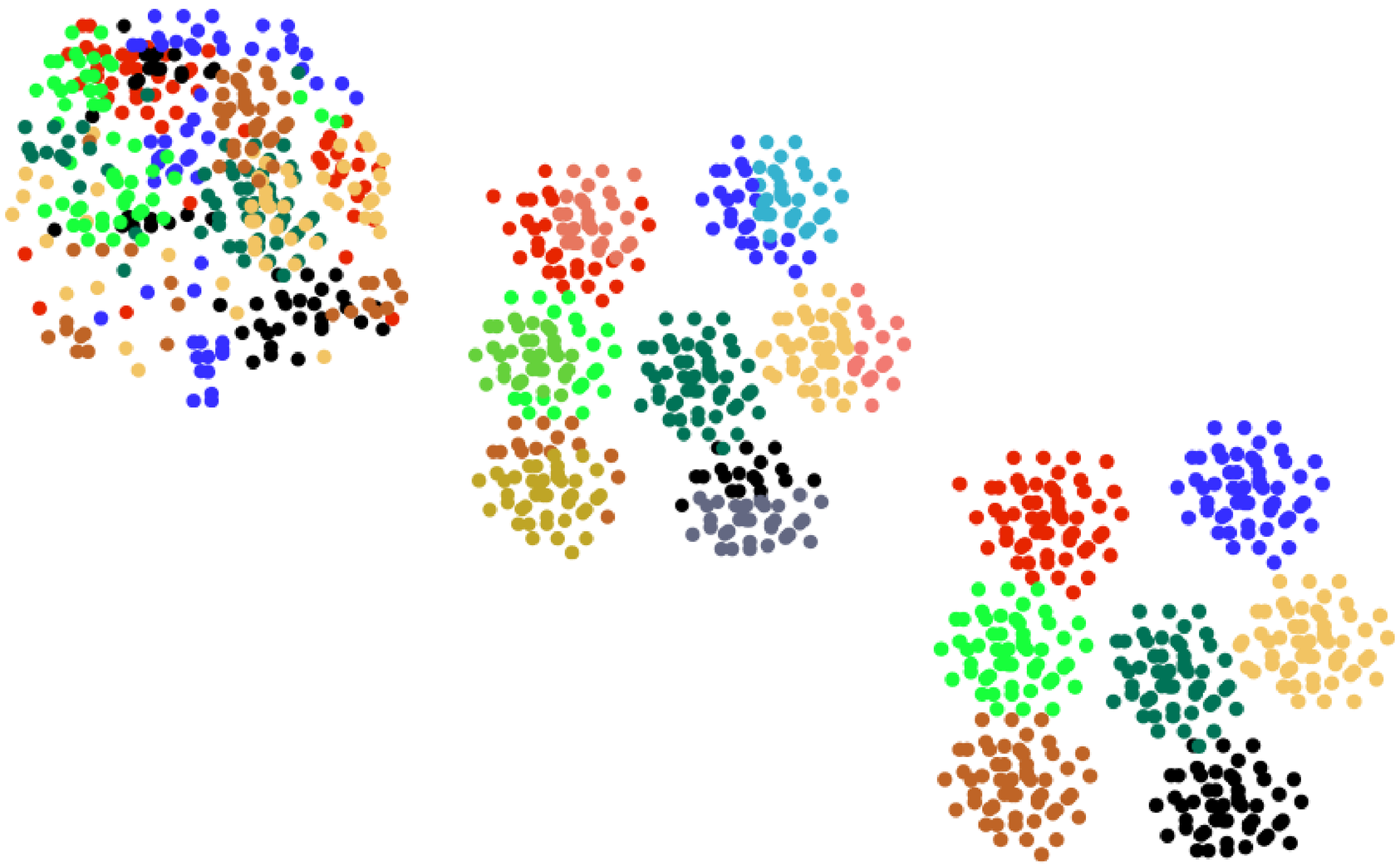
Next steps:

- Functional implications for EBV immune surveillance?
- Are they present as Tumor infiltrating lymphocytes in eBL patients?

Which cells are making IFN-γ to EBV in young children if not T cells?

Dimensionality reduction clustering t-SNE visualization

each color represents a marker



Step 1: ACCENSE – multidimensional principal components analysis (PCA) that clustering cells by expression of surface and intracellular markers.

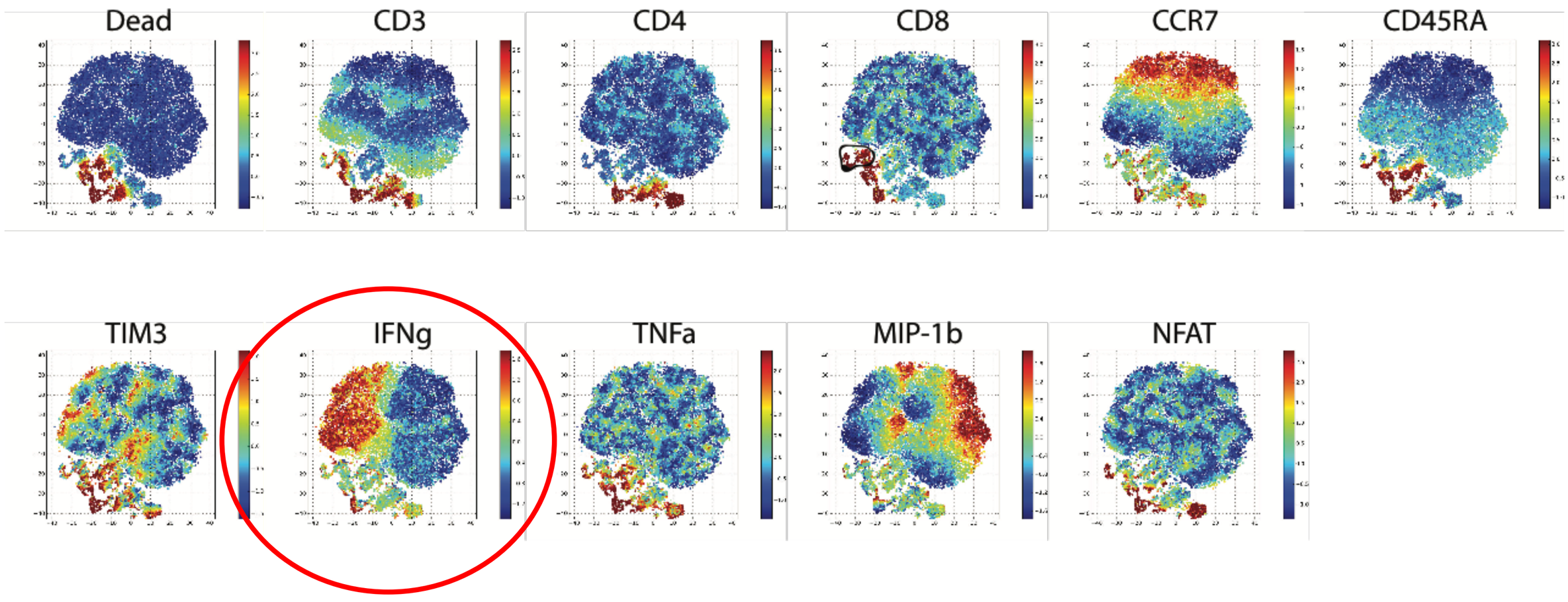
Step 2: Interrogate phenotype of cell clusters by multiparameter flow cytometry.

Step 3: Isolate populations of interest for single cell RNAseq.

Step 4: Link immune cell type and function with clinical status and epidemiology. Track changes in immune profile as child ages or during course of chemotherapy.

each shade represents the intensity of the marker

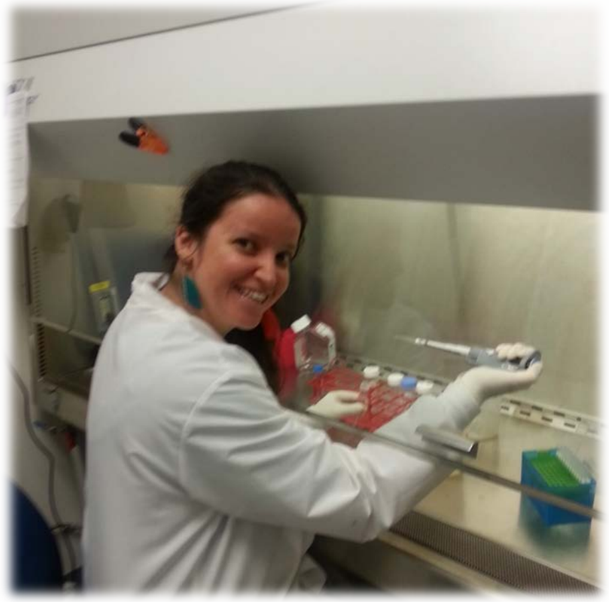
Iterated heat map for every single markers



ACCENSE: Automated Classification of Cellular Expression by Nonlinear Stochastic Embedding. Shekhar PNAS 2014

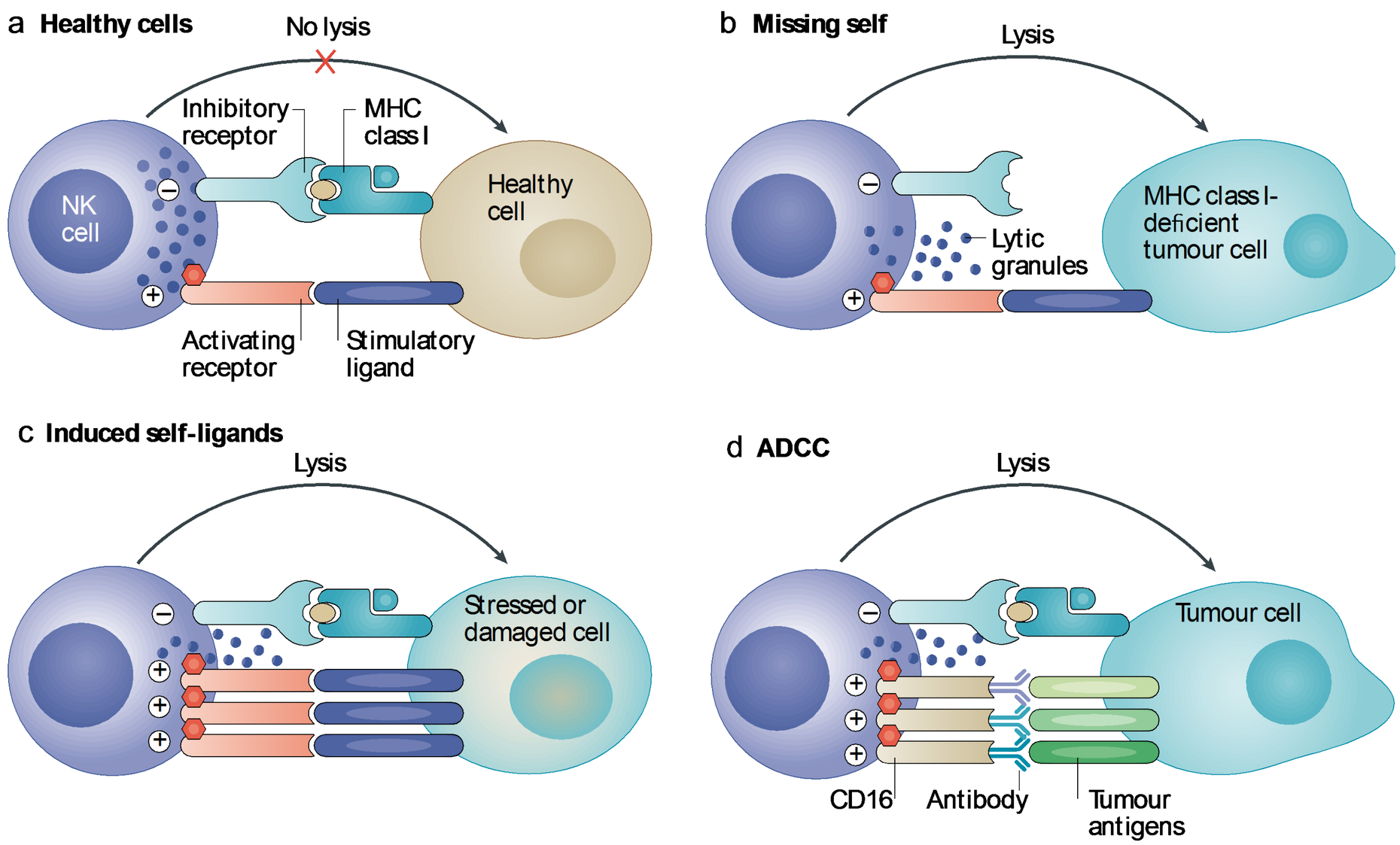
WHAT IS THE ROLE OF NATURAL KILLER (NK) CELLS IN EBV IMMUNOSURVEILLANCE IN YOUNG CHILDREN?

- How do Natural Killer (NK) cells contribute to control of EBV during early-age primary infections and during lytic reactivation in young children?
- How do *Plasmodium falciparum* malaria co-infections influence the balance between NK and T cell control over EBV-infected B cells?
- What is the role of NK cells in eBL pathogenesis versus survival?



Catherine Forconi
post-doc

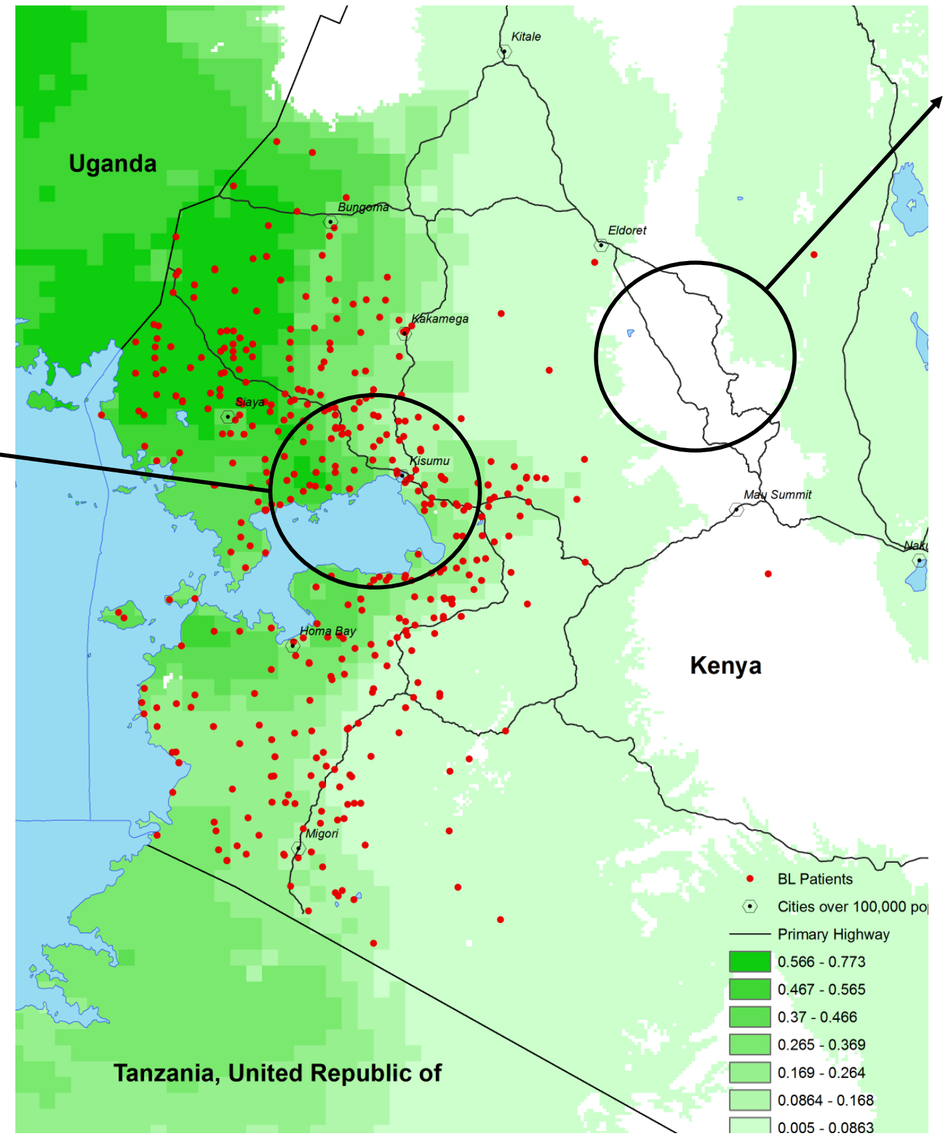
What is the functional capacity of NK cells in children with BL?



NK cells and Cancer, Morvan and Lanier 2016

Study Design: BL cases matched to 2 controls

Kisumu:
=> Children **EBV⁺/Malaria⁺**



Buckle et al *International Journal of Cancer* 2016 Sep 15;139(6):1231-40

Nandi (normal control group):
=> Children **EBV⁺/Malaria⁻**

Burkitt lymphoma (BL) patients (cases)
Red dots indicate home of children diagnosed with Burkitt Lymphoma
=> **BL+/EBV⁺/Malaria⁺**

CHARACTERIZATION OF NK CELLS ($CD56^+CD3^-$) WITHIN OUR STUDY POPULATIONS

Phenotypic markers

- KIRs (Inhibition and Activation)
- Natural Cytotoxicity Receptors (NCRs):
  - CD16
  - NKp46, NKp30
- CD94/NKG2 familly receptors:
  - NKG2A (Inhibition)
  - NKG2C (Activation)
- NK cell Activation Receptors (NARs):
  - NKG2D
  - 2B4(CD244) et NKTB/NKTA
  - DNAM-1(CD226)
  - CRACC
  - CD160
  - CD161
- Activating NK cells:
  - CD57 (memory-like NK subset when associated with NKG2C)
  - CD69
  - CD25
  - NKp44

Functional markers

IFNg
MIP1b
TNFa
CD107a
Perforin
Granzyme a/b

Collaboration with Galit Alter:

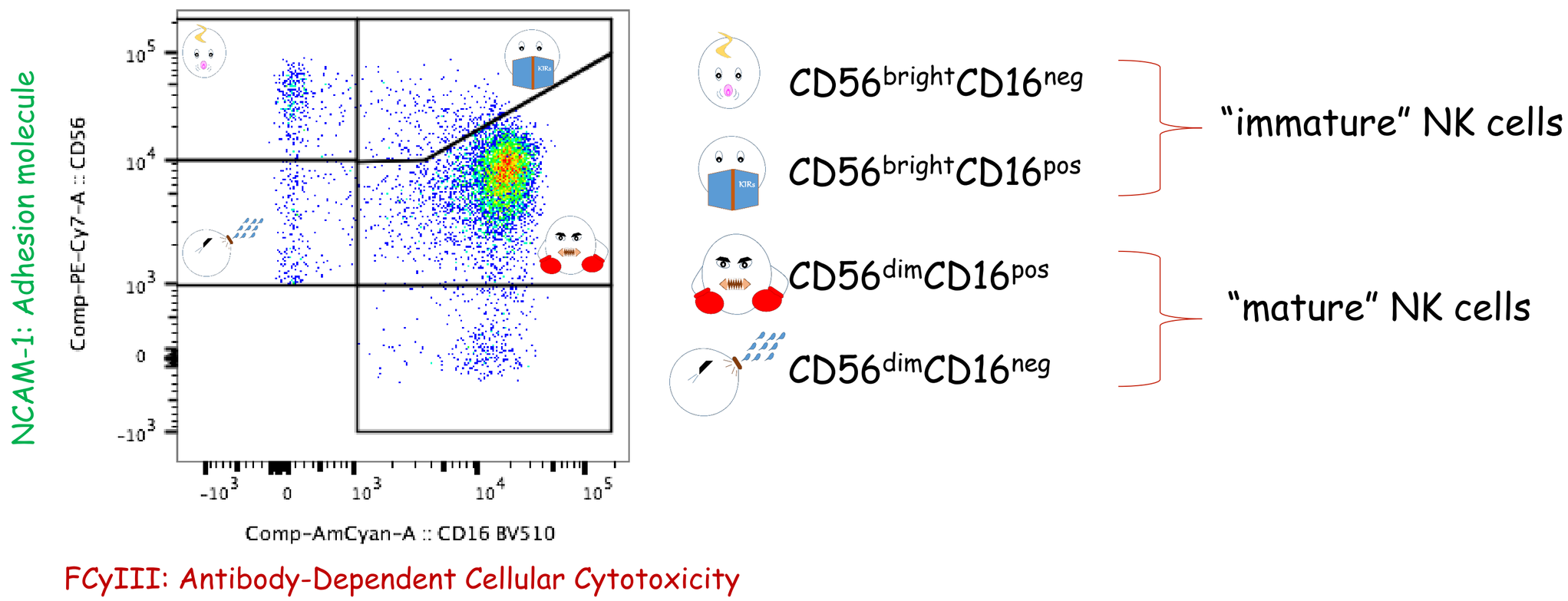
Four panel flow experiments

she used for CMV

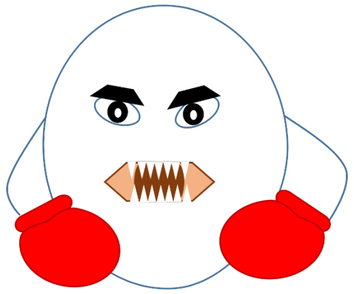
(Cosgrove et. Al, **2014**)

NATURAL KILLER CELLS

- Commonly described as **CD3⁻** lymphocytes, NK cells are usually gated on **CD56⁺** cells.
- Can be supplemented by CD16 or NKp46 staining in flow cytometry



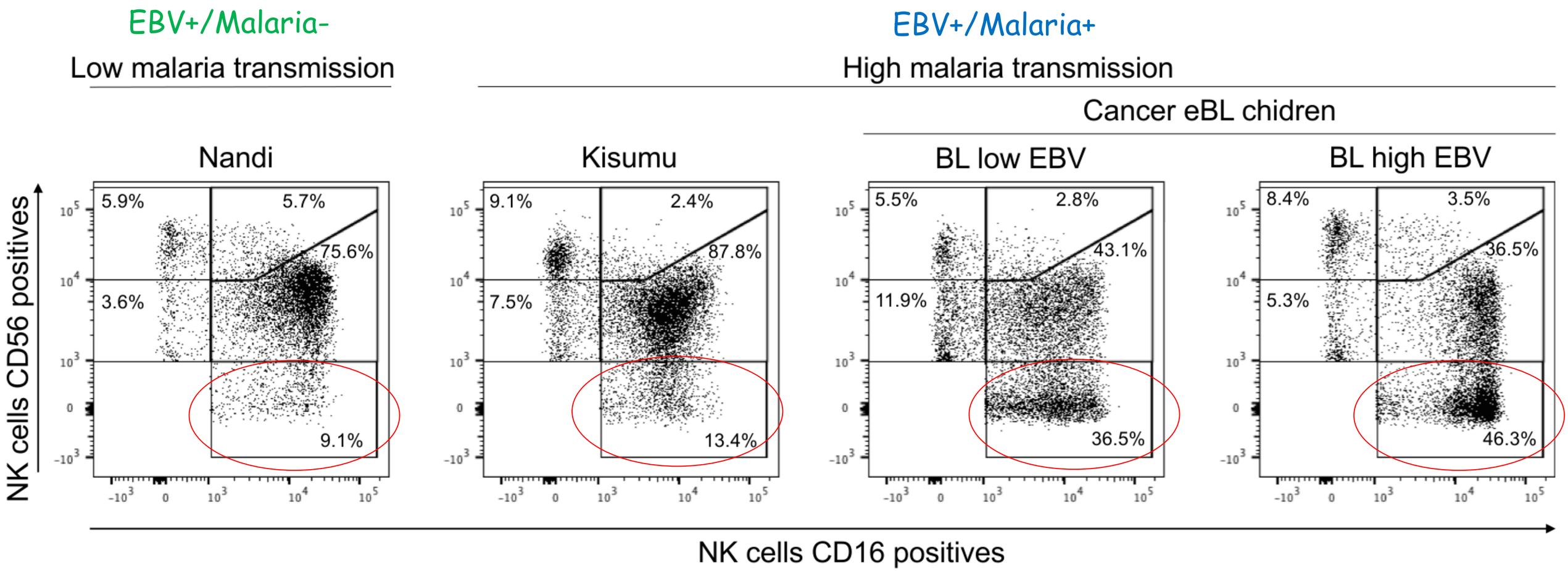
INNATE BUT WITH ADAPTIVE FEATURES



Multiple "subsets" within $CD56^{dim}CD16^{pos}$ NK cells

- Canonical NK cells: Cytotoxic Immunoregulatory subset
- Adaptive NK cells: Effector subset trained for Immuno-surveillance
  - "Memory-like" NK cells: Faster cytokinic and cytotoxic responses

NK CELLS IN KENYAN CHILDREN



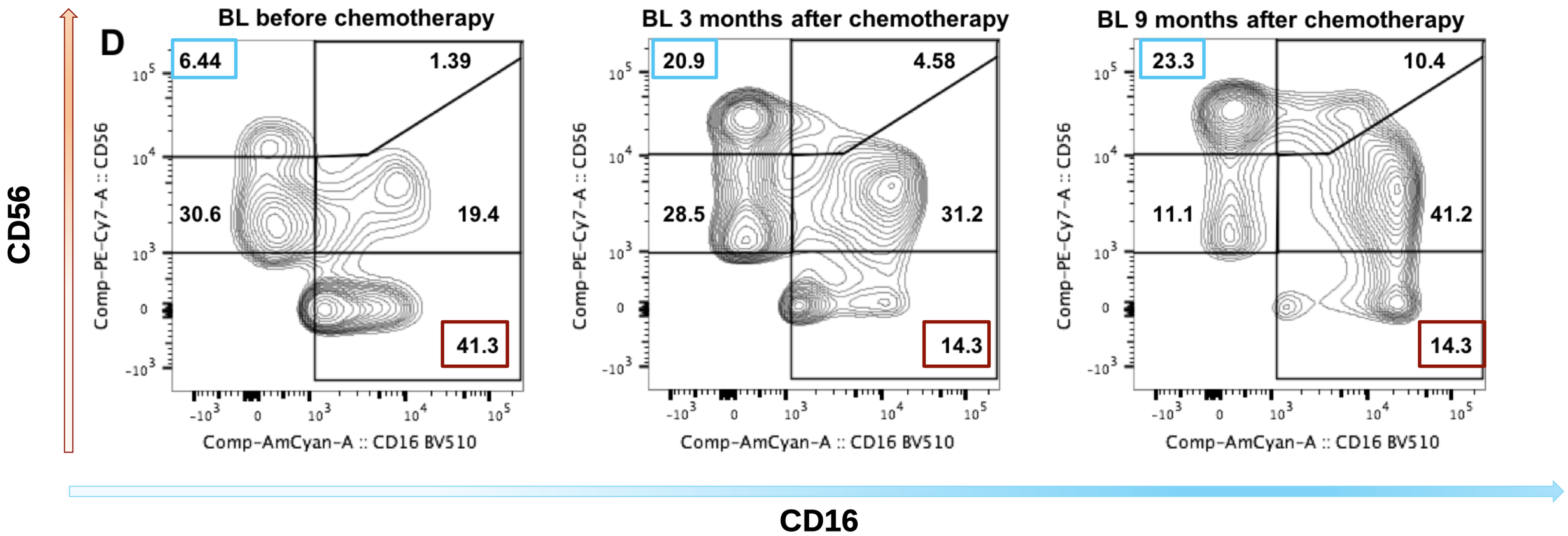
After phenotype analysis: neither canonical, neither "memory-like" profile

Forconi *et. al*, Blood Advances, 2018

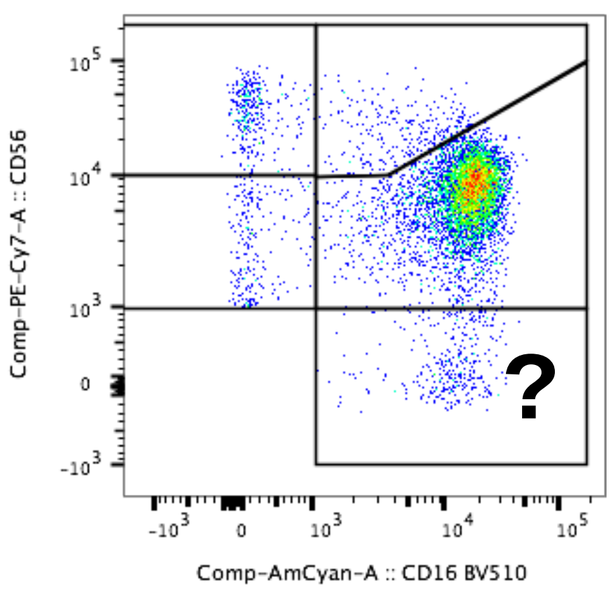
Picture for BL survivors:

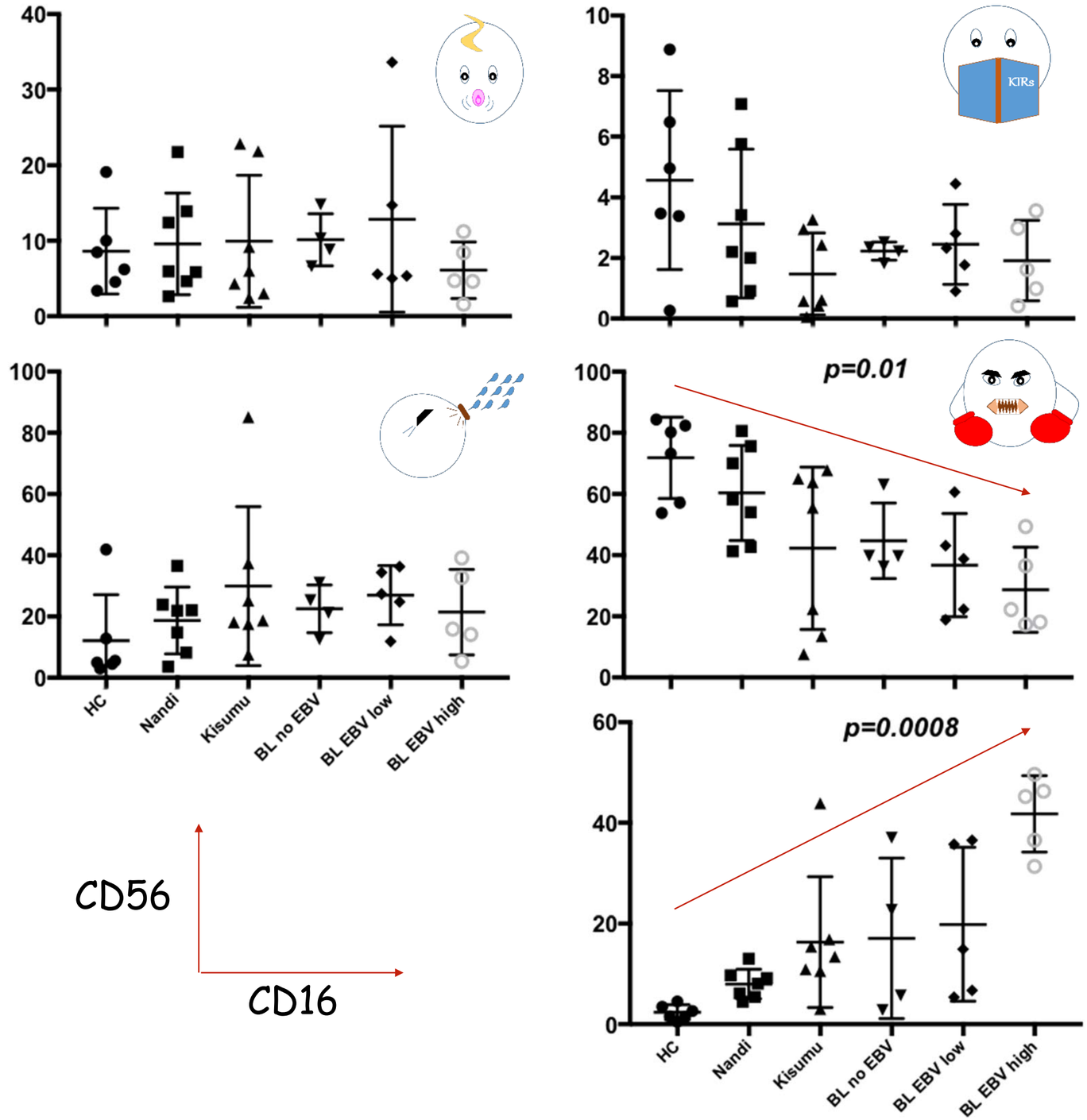
NK CD56^{bright}CD16^{neg} increased and

NK CD56^{neg}CD16^{pos} decreased (restoration to 'normal')?

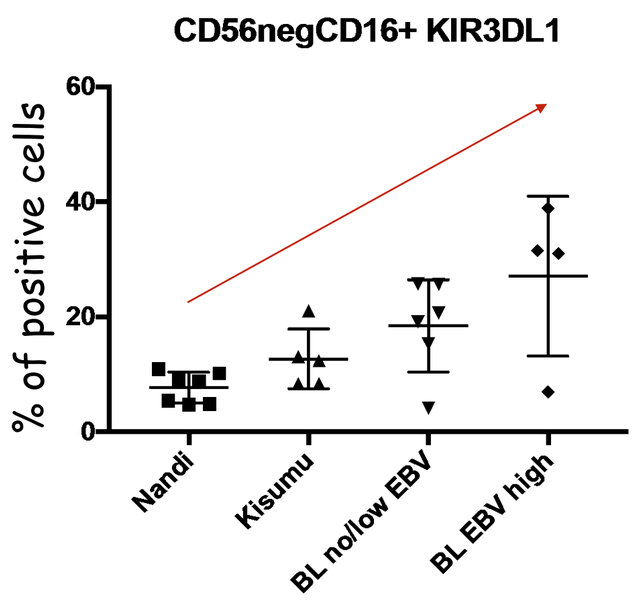


$CD56^{NEG}CD16^{POS}$ HIGHEST PROPORTION IN BL PATIENTS WITH HIGHER EBV LOADS





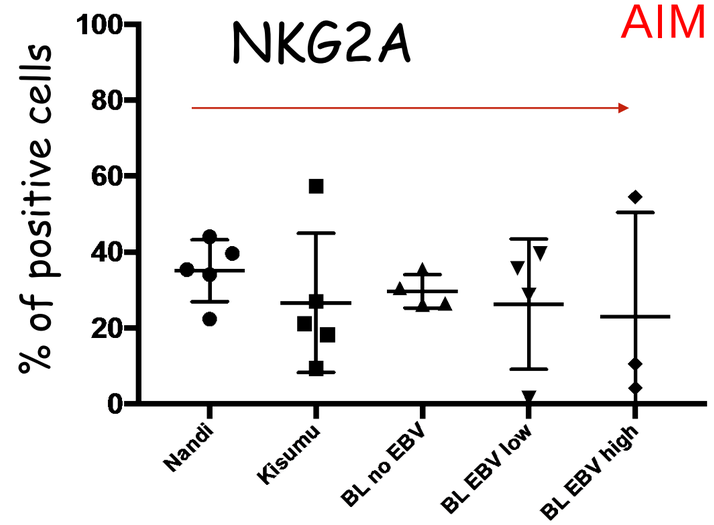
KIR3DL1 (NK inhibition signal) increases with malaria/EBV and BL diagnosis

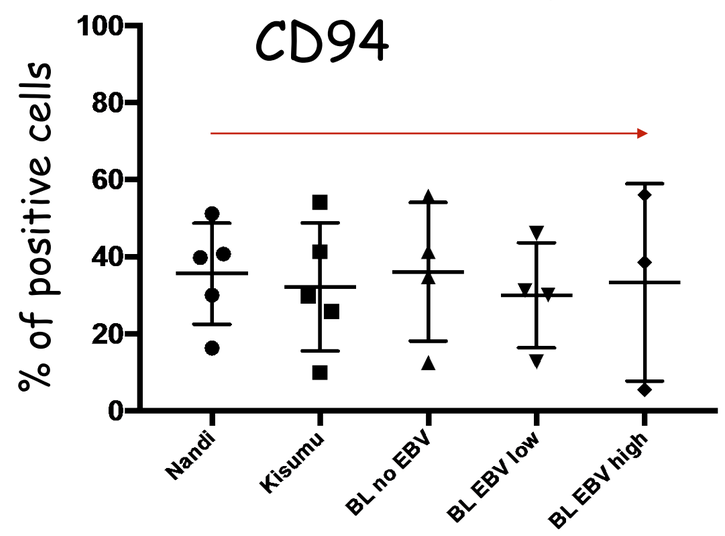


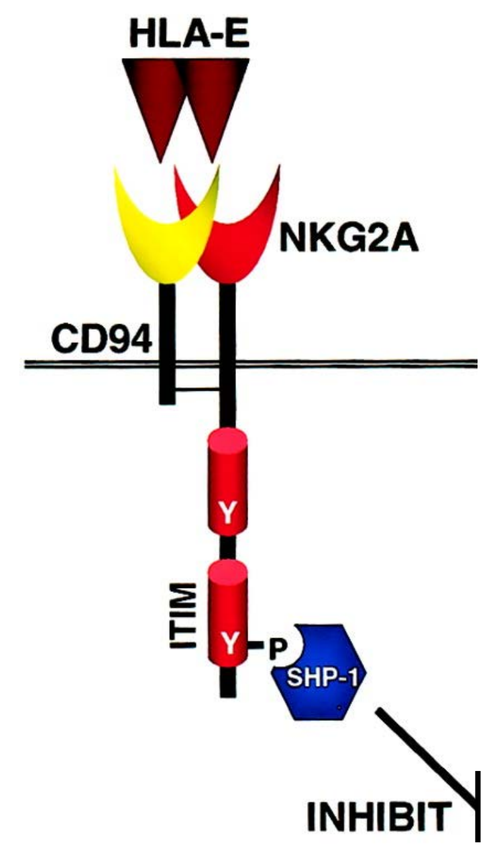
Inhibition signal

Saunders 2015 Imm. Reviews

*NKG2A important for AIM

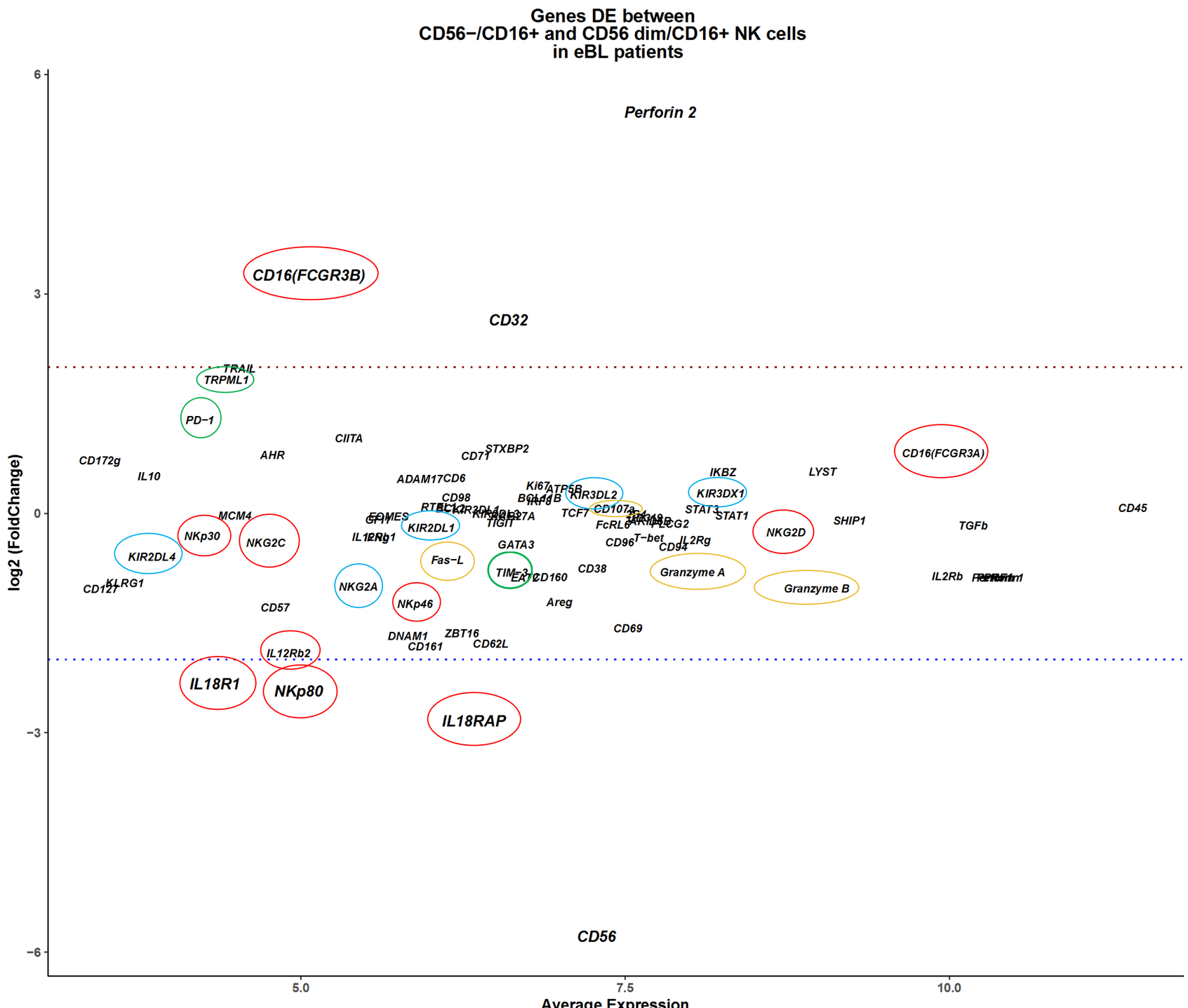






Farag 2002 Blood

Strong similarities in genes expression between $CD56^{neg}CD16^{pos}$ and $CD56^{dim}CD16^{pos}$ NK cells



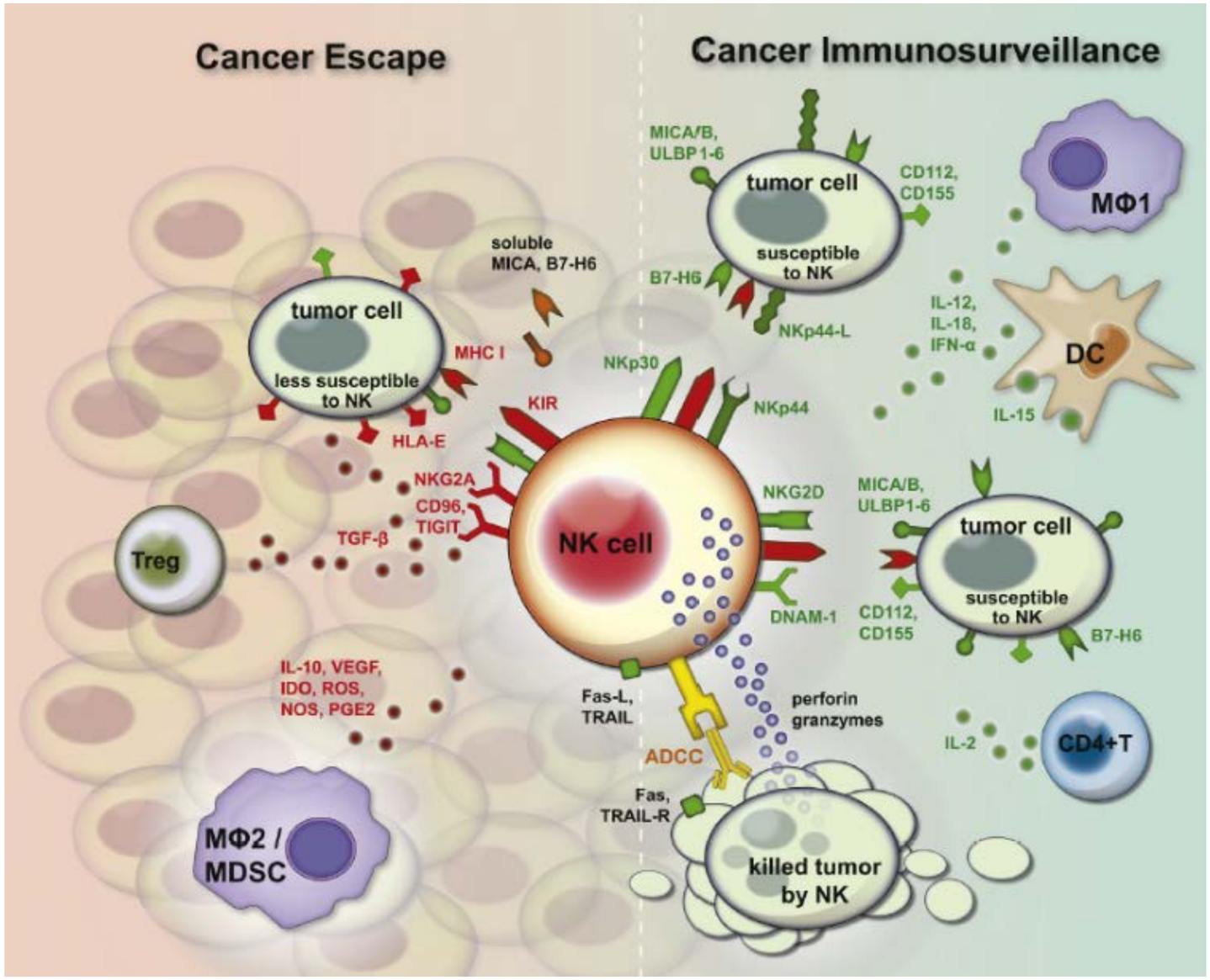
NK activation

NK cytotoxicity

Inhibition of NK cells

Exhaustion of NK cells

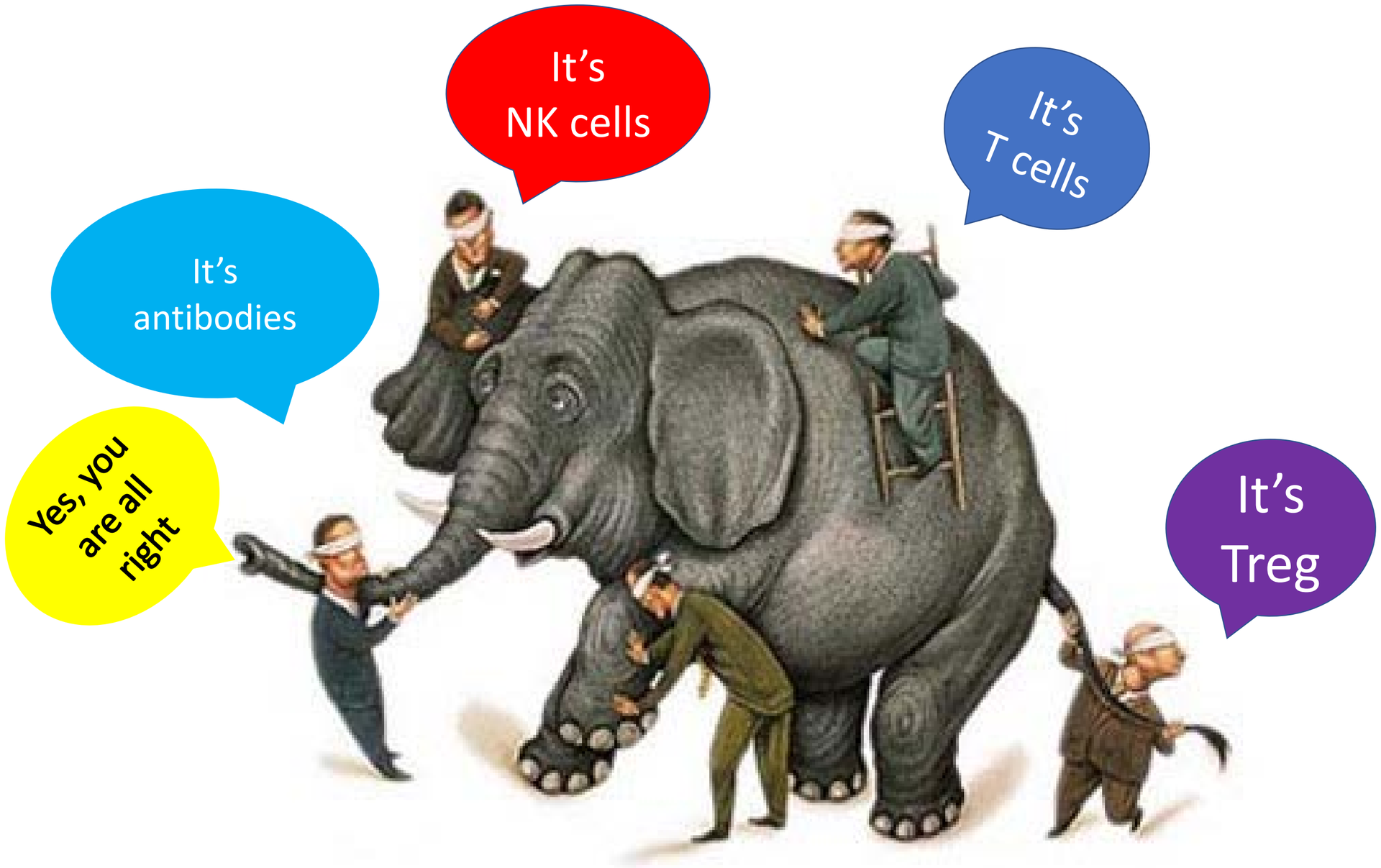
The ultimate clinical question :
How do we design NK immunotherapy for these children?



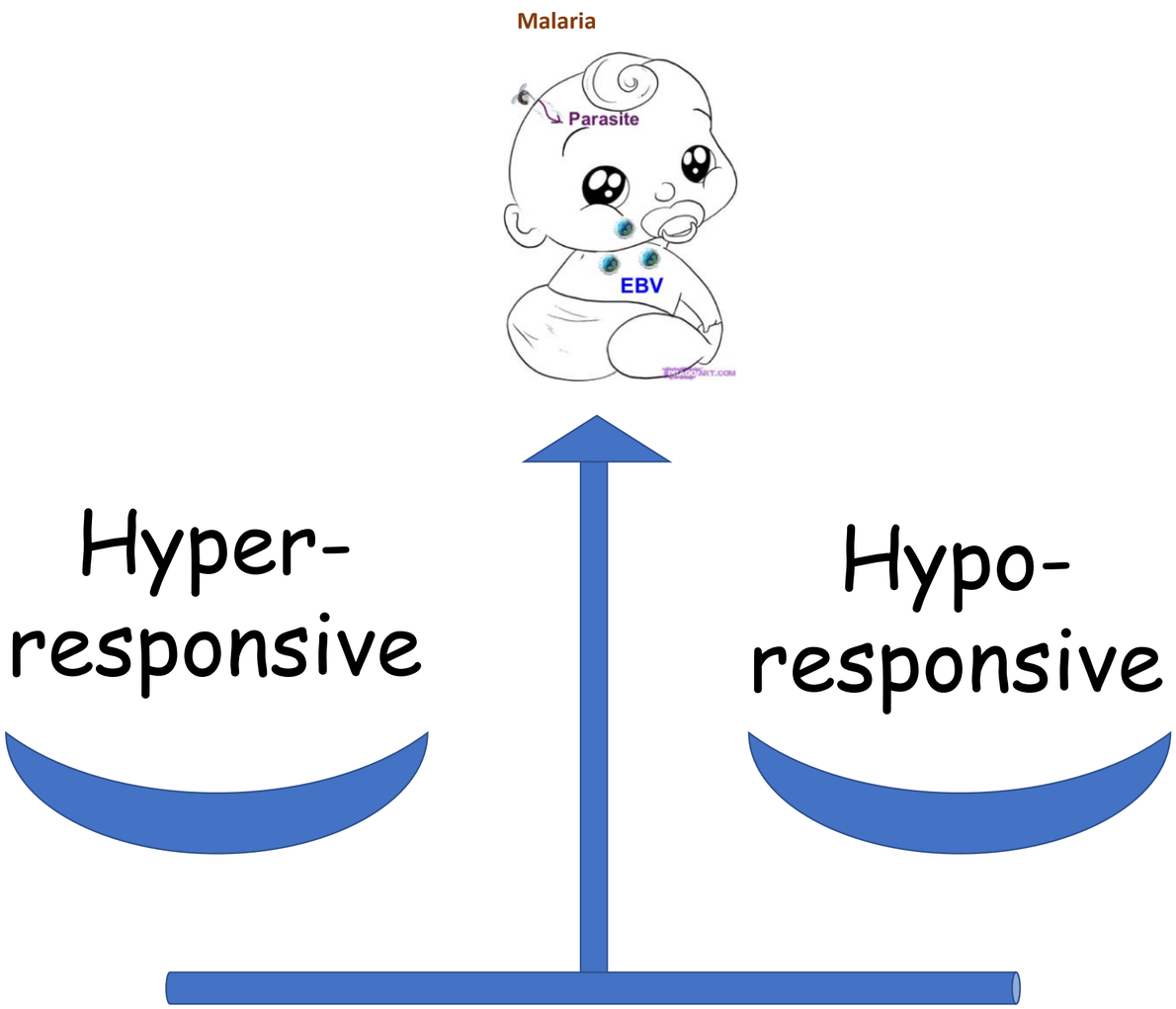
Pahl ImmunoBio 2017

Immune surveillance (anti-viral and anti-tumor) questions to ponder for eBL

1. What cell types control EBV infections in young children?
   - Relative contributions by Innate, unconventional T cells and effector-memory T cells?
   - Does this shift as child ages (and DC mature) to become more adult-like EBV-specific immunity?
2. When do malaria (or other immune-modulating) infections adversely impact EBV immunity?
   - Malaria prevents efficient priming to EBV or does it incrementally eroded anti-virial immunity?
3. What mediates EBV-specific immunity in children diagnosed with eBL?
   - Are immune-defects specific to EBV?
   - Do they persist or can they be ‘rebooted’ after chemotherapy?


It's
NK cells
It's
T cells
It's
antibodies
Yes, you
are all
right
It's
Treg

Is there an immunologic balance achieved to protect children against malaria that puts children at risk for EBV-associated BL?



Pathogen clearance but risk of immuno-pathology
or
chronic infection with 'muted' immunity

Questions to ponder for treating eBL in Africa

- Which immuno-therapeutic approaches should be taken for this EBV-associated cancer?
- Are there special considerations related to immune response and regulation in children that differ from adults?
- Which parasitic infections are immune modulating enough to be able to influence response to an immunotherapy for eBL? (is this a pre-existing condition?)
- What are the implications for immunotherapies for eBL and EBV vaccines to prevent this pediatric cancer in Africa?

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- Pamella Omollo
- Nursing staff and parents of BL patients

University of Zurich

- Christian Münz

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