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

Thompson AACR 2004
Part II: endemic Burkitt lymphoma
A tale of two infections
First described by Denis Burkitt in 1958

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)
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

- 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.

Rochford, Cannon, Moormann
NatRevMicro 2005

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
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?

Early-age primary EBV infection and malaria-associated EBV reactivation

Peak age-incidence of eBL

Erosion of EBV-specific T cell immunosurveillance

Highest prevalence of symptomatic *Pf*-malaria

Development of premunition to malaria: semi-protective immunity permissive of asymptomatic parasitemia

*Rochford 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

<table>
<thead>
<tr>
<th></th>
<th>Age Group (years)</th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>1-4 (n, %)</td>
<td>5-9 (n, %)</td>
<td>10-14 (n, %)</td>
<td>All ages (n, %)</td>
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<tr>
<td><strong>Kisumu</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of subjects</td>
<td>34 (32)</td>
<td>37 (35)</td>
<td>35 (33)</td>
<td>106 (100)</td>
</tr>
<tr>
<td>EBV seropositive</td>
<td>32 (94)</td>
<td>37 (100)</td>
<td>35 (100)</td>
<td>104 (98)</td>
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<tr>
<td>Mean hemoglobin</td>
<td>9.72</td>
<td>12.25</td>
<td>12.51</td>
<td>11.53</td>
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<tr>
<td>Subjects with P.f. positive smear</td>
<td>26 (77)</td>
<td>27 (73)</td>
<td>29 (83)</td>
<td>82 (77)</td>
</tr>
<tr>
<td>Mean body temperature (°C)</td>
<td>36.80</td>
<td>36.76</td>
<td>36.89</td>
<td>36.82</td>
</tr>
<tr>
<td><strong>Nandi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of subjects</td>
<td>39 (30)</td>
<td>50 (38)</td>
<td>41 (32)</td>
<td>130 (100)</td>
</tr>
<tr>
<td>EBV seropositive</td>
<td>36 (92)</td>
<td>50 (100)</td>
<td>41 (100)</td>
<td>127 (98)</td>
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<tr>
<td>Mean hemoglobin</td>
<td>12.18</td>
<td>12.82</td>
<td>13.29</td>
<td>12.77</td>
</tr>
<tr>
<td>Subjects with P.f. positive smear</td>
<td>3 (8)</td>
<td>7 (14)</td>
<td>11 (26)</td>
<td>21 (16)</td>
</tr>
<tr>
<td>Mean body temperature (°C)</td>
<td>37.21</td>
<td>37.18</td>
<td>37.09</td>
<td>37.16</td>
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</table>
## HLA Class I-restricted EBV peptide selection for IFN-γ ELISPOT assays

<table>
<thead>
<tr>
<th>EBV protein</th>
<th>Cycle</th>
<th>Amino Acids</th>
<th>Peptide Sequence</th>
<th>HLA-restriction</th>
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<tbody>
<tr>
<td>BZLF1</td>
<td>Lytic</td>
<td>190-197</td>
<td>RAK FKQ LL</td>
<td>HLA B8</td>
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<tr>
<td>BMLF1</td>
<td>Lytic</td>
<td>280-288</td>
<td>GLC TLV AML</td>
<td>HLA A2</td>
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<tr>
<td>BRLF1</td>
<td>Lytic</td>
<td>148-156</td>
<td>RVR AYT YSK</td>
<td>HLA A3</td>
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<tr>
<td>BRLF1</td>
<td>Lytic</td>
<td>28-37</td>
<td>DYC NVL NKE F</td>
<td>HLA A24</td>
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<tr>
<td>EBNA 3A</td>
<td>Latent</td>
<td>379-387</td>
<td>RPP IFI RRL</td>
<td>HLA B7</td>
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<tr>
<td>EBNA 3C</td>
<td>Latent</td>
<td>258-266</td>
<td>RRI YDL IEL</td>
<td>HLA B27</td>
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<tr>
<td>EBNA 3B</td>
<td>Latent</td>
<td>217-225</td>
<td>TYS AGI VQI</td>
<td>HLA A24</td>
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<tr>
<td>EBNA 3A</td>
<td>Latent</td>
<td>325-333</td>
<td>FLR GRA YGL</td>
<td>HLA B8</td>
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<tr>
<td>EBNA 3A</td>
<td>Latent</td>
<td>596-604</td>
<td>SVR DRL ARL</td>
<td>HLA A2</td>
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<tr>
<td>EBNA 3A</td>
<td>Latent</td>
<td>603-611</td>
<td>RLR AEA QVK</td>
<td>HLA A3</td>
</tr>
<tr>
<td>EBNA 3B</td>
<td>Latent</td>
<td>657-666</td>
<td>VEI TPY KPT W</td>
<td>HLA B44</td>
</tr>
</tbody>
</table>
EBV-specific IFN-γ ELISPOT responses deficient in children 5-9 yrs old from malaria holoendemic area

* p-value < 0.03

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 exist 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<sup>dim</sup> T cells.
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 change over time 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-\(\gamma\) 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

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?

a Healthy cells

b Missing self

c Induced self-ligands

d ADCC

NK cells and Cancer, Morvan and Lanier 2016
Study Design: BL cases matched to 2 controls

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

Kisumu:
=> 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 family receptors:**
  - NKG2A (Inhibition)
  - NKG2C (Activation)
- **NK cell Activation Receptors (NARs):**
  - NKG2D
  - 2B4(CD244) et NKTβ/NKTα
  - DNAM-1(CD226)
  - CRACC
  - CD160
  - CD161
- **Activating NK cells:**
  - CD57 (memory-like NK subset when associated with NKG2C)
  - CD69
  - CD25
  - NKP44

#### Functional markers

- IFNγ
- MIP1b
- TNFα
- CD107a
- Perforin
- Granzyme a/b

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**Collaboration with Galit Alter:**

Four panel flow experiments she used for CMV
(Cosgrove et. Al, 2014)
NATURAL KILLER CELLS

- Commonly described as \textbf{CD3}^- lymphocytes, NK cells are usually gated on \textbf{CD56}^+ cells.
- Can be supplemented by CD16 or NKp46 staining in flow cytometry.
INNATE BUT WITH ADAPTIVE FEATURES

Multiple “subsets” within CD56$^{\text{dim}}$CD16$^{\text{pos}}$ NK cells

- **Canonical** NK cells: Cytotoxic Immunoregulatory subset
- **Adaptive** NK cells: Effector subset trained for Immuno-surveillance
  - “**Memory-like**” NK cells: Faster cytokinetic and cytotoxic responses
NK CELLS IN KENYAN CHILDREN

EBV+/Malaria-
Low malaria transmission

EBV+/Malaria+
High malaria transmission

Cancer eBL children

Nandi

Kisumu

BL low EBV

BL high EBV

NK cells CD56 positives

NK cells CD16 positives

After phenotype analysis: neither canonical, neither “memory-like” profile

Forconi et. al, Blood Advances, 2018
Picture for BL survivors:
NK $\text{CD56}^{\text{bright}}\text{CD16}^{\text{neg}}$ increased and
NK $\text{CD56}^{\text{neg}}\text{CD16}^{\text{pos}}$ decreased (restoration to 'normal')?
CD56<sup>NEG</sup>CD16<sup>POS</sup> HIGHEST PROPORTION IN BL PATIENTS WITH HIGHER EBV LOADS
KIR3DL1 (NK inhibition signal) increases with malaria/EBV and BL diagnosis

*NKG2A important for AIM

Inhibition signal

Saunders 2015 Imm. Reviews

Farag 2002 Blood
Strong similarities in genes expression between CD56\textsuperscript{neg}CD16\textsuperscript{pos} and CD56\textsuperscript{dim}CD16\textsuperscript{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?
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 Treg

It's T cells

It's NK cells

It's antibodies

Yes, you are all right.
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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Thrasher Research Fund (Moormann)
Denis Burkitt Fellowship (Moormann)
University of Massachusetts Center for Clinical and Translational Science (UL1TR001453).(AMM and JAB)