Epstein-Barr virus infections and diagnosis of PTLD in transplant recipients

Prof.dr. Jaap M. Middeldorp
PTLD after solid organ (SOT) and bone marrow / stem cell (SCT) transplantation

Post-Transplant Lympho Proliferative Disease

- Reversible B-cell hyperplasia to malignant lymphoma
- B-lymphocyte derived
- <1 month to >10 yrs after transplantation

EBV+, Pleomorphic B cell proliferations, Poly-, oligo-, monoclonal
EBV Infection

- Immunodeficiency or suppression will lead to (oligo)clonal proliferation

- Tonsil Naïve B Cell Blast

- Germinal Center

- Dividing Memory B Cell

- Resting Memory B Cell

Infected B Cell

- B-blast Growth Program (Latency III)

Clonal Expansion

“In EBV carriers in vivo”

EBV+ B-cell proliferation & spread is well controlled by immune defences;

In blood ~1/10^5 B-cells infected (1 cell/ml)
In vivo EBV mimics B-cell germinal center reaction

Saliva

Virus

gp350, gp42

Antigen + Ab

CTL

Naive B Cell

Activated B Lymphoblast

CD21, MHC-II

EBNA2-6

EBNA1

LMP1,2

Growth

Default

CTL

Epithelium

Lymphoid Tissue

Circulation

Antigen + T Cell Help

Germinal Center

Hypermutation

Isotype Switching

Epigenetic gene silencing

Memory B Cell

Cell Division

EBNA1 Only

Latency

EBNA1 Only

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Lymphoid environment affects EBV+ B cell behaviour

Differences in SCT vs SOT conditions affect homeostasis

• Fully naïve immune system vs suppressed immune system
• Destroyed lymphoid architecture vs intact architecture
• Systemic vs local allogenic interactions
• Deranged vs normal lymphoid trafficking
• Different amounts of “self” exposed

• Differences in EBV biology & PTLD manifestation in SOT/SCT

Undefined issues:
• What triggers PTLD development at very early stages?
• Reflection of PTLD tissue dynamics in circulation?
• Are circulating B-cells direct PTLD derived?
PTLD pathogenesis is more complex

- Immune suppression
  - EBV-specific CD8+ T-cell (cytotoxic)
  - IL4, IL21
  - CD4+ Th cells
  - CD40L
  - EBV-infected B-cell
  - EBV
  - Autocrine Cytokines
    - IL6, IL10
  - Survival
  - Anti-apoptosis
  - Growth

- Neoinfection by lytic EBV replication
- Allogenic triggering + cytokines
- B-cell hyperplasia (reversible)
- Malignant PTLD ('late stage')

Risk factors:
- D/R EBV status
- Level of HLA mismatch
- Level of immunodeficiency
- Level & type of IST
- T-cell depletion

Heterogeneity is related to PTLD cell morphology.
- Small PTLD cells express EBNA2 without LMP1,
- Intermediate-sized cells EBNA2 and LMP1
- Large PTLD cells express LMP1 without EBNA2
EBV latent protein expression in PTLD is heterogeneous at the single cell level.

LMP1 is generally expressed in larger blastoid (proliferating) cells.
A role for lytic EBV replication in PTLD?

EBNA-1 RT-PCR

"Lytic" EBNA1 F-promotor transcripts

Y3K spliced transcripts (latency type III)
Conclusion 1.

- PTLD is not merely a proliferation of EBV+ B-cells expressing the growth program (Lat-III), but more heterogeneous.

- In (lymphoid) tissue more complex factors, including (allo-) reactive T-cells & cytokines, influence EBV gene expression and determine the fate of EBV+ B cells. SOT is clearly different from SCT situation.

- In PTLD B-cells “in situ” EBV gene expression is (still) regulated in a specific way, possibly mimicking the germinal center selection process, with occasional lytic viral replication and re-infection.

- From PTLD lesion EBV+ B cells enter into the circulation, which we can utilize for diagnostics.
PTLD = localised disease in lymphoid tissues, with spill-over into perifery...

Question: Utility of EBV-DNA/-RNA in circulation as marker for PTLD? Are EBV + cells in circulation direct images of PTLD cells in tissue?
Tools for PTLD diagnosis and monitoring

Quantification of EBV load is essential: Real-time PCR
Standardisation & internal controls

**EBNA-1-based LightCycler PCR**

Conserved segment of EBNA-1 (BKRF-1)

**DNA target:** EBNA1, highly conserved region, single copy

**Sample prep:** Silica-based isolation from GuSCN extracts (automation, standardisation)

Real-time PCR

Standardisation 

Internal controls
Tools for PTLD diagnosis and monitoring:

**EBV-RNA expression profiling:**

Multiprimed real-time RT-PCR or QT-NASBA/NucliSens

(latency type 3 genes):

- EBNA-1 Cp/Wp (QK, Fp)
- EBNA-2,
- LMP-1, LMP-2a
- BARTs (miRNA)
- BHRF-1 (Cp/Yp)
- (ZEBRA)
- (lytic antigens EA-d, VCA, MA)
Clinical specimens for EBV DNA load monitoring

Unfractionated whole blood:
* EB-virions
* EBV+ B cells
* EBV+ tumor cells
* lysed EBV+ cells

Serum/plasma:
* EB-virions (?)
* EBV DNA from lysed cells (DNA = largely fragmented)

Cell fractions:
* Leukocytes
* EBV+ B-cells
* EBV+ tumour cells

0.1 ml blood required

Stevens et al., Meth.Mol.Biol. (2005) 292; 1-16
Viral load in parallel plasma versus whole blood in 50 SOT patients at peak EBV-DNA levels

EBV is mostly cell-associated in SOT patients
### 1b) PREDOMINANT CELL-BOUND DISTRIBUTION OF CIRCULATING EBV DNA IN SOT RECIPIENTS with PTLD

<table>
<thead>
<tr>
<th>SOT pat. (no. samples)</th>
<th>EBV DNA load range in plasma (copies/ml)</th>
<th>EBV DNA load range in whole blood (copies/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (23)</td>
<td>&lt;2,000</td>
<td>8,600-98,200</td>
</tr>
<tr>
<td>B (6)</td>
<td>&lt;2,000</td>
<td>&lt;2,000-308,400</td>
</tr>
<tr>
<td>C (3)</td>
<td>&lt;2,000</td>
<td>7,000-7,200</td>
</tr>
<tr>
<td>D (3)</td>
<td>&lt;2,000</td>
<td>2,600-3,800</td>
</tr>
<tr>
<td>E (1, post Rtx)</td>
<td>58,400,000</td>
<td>54,300,000</td>
</tr>
</tbody>
</table>

**EBV DNA load in simultaneous plasma and whole blood samples of 5 SOT patients, with multiple follow-up**

### 1a) MORE HETEROGENEOUS DISTRIBUTION OF CIRCULATING EBV DNA IN SCT RECIPIENTS with PTLD

<table>
<thead>
<tr>
<th>SCT pat.</th>
<th>EBV DNA load in plasma (copies/ml)</th>
<th>EBV DNA load in PBMCs (copies/10⁶ cells)</th>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1,426,344</td>
</tr>
<tr>
<td>2</td>
<td>3,740</td>
<td>6,216</td>
</tr>
<tr>
<td>3</td>
<td>376,000</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>26,900</td>
<td>17,471</td>
</tr>
<tr>
<td>5</td>
<td>20,100</td>
<td>109</td>
</tr>
</tbody>
</table>

**EBV DNA load in simultaneous plasma and whole blood samples of 5 SCT patients**

**Preferred diagnostic sample is whole blood**
EBV DNA loads in whole blood of Lung Tx (SOT) recipients with or without PTLD

Retrospective study 1700 blood samples, 100 patients, weekly sampling.

<table>
<thead>
<tr>
<th>Patient</th>
<th>PTLD(^1)</th>
<th>follow-up period (months)(^2)</th>
<th>Total no. samples</th>
<th>EBV DNA load range (DNA copies/ml blood)</th>
<th>Samples before PTLD diagnosis</th>
<th>EBV DNA load at PTLD diagnosis (DNA copies/ml blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tested</td>
<td>Above Q-PCR cut-off value</td>
<td>Tested</td>
<td>Above Q-PCR cut-off value</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>13</td>
<td>29</td>
<td>23 (79%)</td>
<td>3,400-308,000</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>19</td>
<td>38</td>
<td>34 (89%)</td>
<td>2,400-98,000</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
<td>15</td>
<td>15</td>
<td>1 (8%)</td>
<td>5,500</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>55</td>
<td>17</td>
<td>23</td>
<td>19 (83%)</td>
<td>2,700-108,800</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>67</td>
<td>13</td>
<td>19</td>
<td>5 (28%)</td>
<td>2,400-11,900</td>
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<td>6</td>
<td>78</td>
<td>13</td>
<td>17</td>
<td>1 (82%)</td>
<td>2,000-13,600</td>
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<tr>
<td>7</td>
<td>-</td>
<td>15</td>
<td>16</td>
<td>3 (19%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>14</td>
<td>13</td>
<td>1 (8%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>26</td>
<td>36</td>
<td>0 (0%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>6</td>
<td>17</td>
<td>0 (0%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>8</td>
<td>9</td>
<td>0 (0%)</td>
<td>-</td>
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</tr>
<tr>
<td>12</td>
<td>-</td>
<td>8</td>
<td>8</td>
<td>0 (0%)</td>
<td>-</td>
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<tr>
<td>13</td>
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<td>8</td>
<td>8</td>
<td>0 (0%)</td>
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<tr>
<td>14</td>
<td>-</td>
<td>6</td>
<td>10</td>
<td>0 (0%)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Clinical cut-off 2000 copies/ml

Stevens et al. Leuk Lymph (2002) 43; 831-840
Balancing between infection and rejection
Immunomodulation after solid organ transplantation

Opportunistic Infection, PTLD
Increasing EBV load

“Safe zone”

Rejection
(transplant dysfunction, transplant loss)

Monitoring dynamics in EBV load effective for prediction for PTLD development.
Clinical cut-off level for “alert signal”
EBV DNA load has very short doubling time \textit{in vivo}: frequent monitoring for detecting EBV dynamics is essential

<table>
<thead>
<tr>
<th></th>
<th>Mean increase (EBV DNA copies/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1 (PTLD 1):</td>
<td>8,500</td>
</tr>
<tr>
<td>Patient 1 (PTLD 3):</td>
<td>8,500</td>
</tr>
<tr>
<td>Patient 2 (FK506 switch):</td>
<td>3,000</td>
</tr>
<tr>
<td>Patient 2 (PTLD):</td>
<td>5,000</td>
</tr>
<tr>
<td>Patient 3 (PTLD after BMT):</td>
<td>20,000</td>
</tr>
</tbody>
</table>

EBV DNA load fluctuation:

- **Patient 1 (PTLD 1):**
  - Initial: 8,600 copies/ml
  - Final: 66,600 copies/ml
  - Time: 7 days
  - Mean increase: 8,500 copies/day

- **Patient 1 (PTLD 3):**
  - Initial: 15,800 copies/ml
  - Final: 308,400 copies/ml
  - Time: 35 days
  - Mean increase: 8,500 copies/day

- **Patient 2 (FK506 switch):**
  - Initial: 40,400 copies/ml
  - Final: 98,200 copies/ml
  - Time: 20 days
  - Mean increase: 3,000 copies/day

- **Patient 2 (PTLD):**
  - Initial: 5,600 copies/ml
  - Final: 39,800 copies/ml
  - Time: 7 days
  - Mean increase: 5,000 copies/day

- **Patient 3 (PTLD after BMT):**
  - Initial: 0 copies/ml
  - Final: 200,000 copies/ml
  - Time: 10 days
  - Mean increase: 20,000 copies/day

**Graph:**

- **X-axis:** Days after LTx
- **Y-axis:** Mean increase (EBV copies/ml/day)
- **Legend:**
  - Increase
  - Decrease

**Graph Note:**

- PTLD events marked on the graph.
PRIMARY EBV INFECTION IS THE MAJOR RISK FACTOR FOR PTLD

132 Lung transplantations

119 EBV-positive recips
10 PTLDs (8.4%)

13 EBV-negative recipients
4 PTLDs (30.8%)
EBV QT-PCR and Serology

PTLD developing in LTx after switch in immunosuppressive treatment

Days after LTx

EBV DNA copies/mL blood

- 0
- 20000
- 40000
- 60000
- 80000
- 100000
- 120000

Cyclosporin A

FK 506

Switch to FK506

PTLD

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Elevated EBV DNA in blood of SOT recipients is mostly (B-)cell-associated.

**A) Primary EBV-infected LTx recipient**

- **EBV DNA copies/ml blood**
- **Days after LTx**
- **PTLD 1**
- **PTLD 2**
- **PTLD 3**

Value of detection of EBV-DNA in plasma is limited!

EBV-DNA associates with quiescent circulating “memory” B-cells.
Elevated EBV DNA in blood of SCT recip’s is not/less (B-)cell-associated

Detection of EBV-DNA in plasma is common and its association with B-cells is more variable.

Circulating plasma EBV-DNA proved to be fragmented
Guideline for pre-emptive monitoring & treatment:

Risk assessment prior to Tx: (D/R status, HLA match, etc).
In “at risk” patients:

SOT: **early post-Tx (0-3 Mo):** weekly EBV-DNA load, (<2000 c/ml)
  → when elevated, retest for EBV-DNA load in few days
  → when increased, reduce IST*

SOT: **later post-Tx (>3 Mo):** test regularly (visits) & upon clinical signs
  → when above clin. cut-off, retest for DNA load next week
  → when >2x increased, adjust IST*

SCT: **early post-Tx (0-6 Mo):** **weekly** EBV-DNA load, (<1000 c/ml)
  → when elevated, retest for EBV-DNA load in few days
  → when >2x increased, reduce IST or apply Rituximab*

* When accompanied by symptoms, elevated B-cell counts, e.a.
  (otherwise, retest within few days or 1 week)
Preemptive intervention

= intervention guided by elevated EBV loads to prevent PTLD

In SOT recipients:
- **immunomodulation** (temporary lowering of IS)
- (rituximab)
- infusion of *ex vivo* generated autologous EBV-specific T-cells

In SCT/BMT recipients:
- **rituximab** (anti-CD20) + reduced IST
- infusion of donor EBV-reactive T-cells
- infusion of *ex vivo* activated donor EBV-CTLs

By frequent EBV-DNA monitoring and timely intervention PTLD can be eliminated effectively at early stage
Relation between EBV DNA and lung function post LTx: VCmax and FEV1
Relation between EBV DNA, VCmax and FEV1
Preemptive decrease of IS in lung Tx (SOT) (guided by EBV DNA load)

1990-6/2001 (no EBV load monitoring):
- 165 Lung transplantations
- 23 PTLD (13.9%)

- 151 Lung transplantations
- 2 PTLD (0.7%)

Significant reduction PTLD: High clinical importance!!
Significant reduction in BOS: Organ function improved!!
In SCT/BMT-PTLD with Rituximab (RTx) treatment
Decreasing EBV DNA loads predict good response

If EBV load
2x >1000c/ml plasma
>2000c/ml whole blood
Infuse Rituximab (anti-CD20)

EBV load (copies/ml plasma)

Responders
Non-responders

0 hrs <72 hrs 0 hrs >72 hrs
Preemptive decrease of IS + RTx in stem cell Tx (SCT) (guided by EBV DNA load)

Meijer & Cornelissen (2008)
Curr.Opin.Haematol. 15: 576
• EBV DNA load monitoring post-Tx is very useful in a preemptive approach.
• “Sec” DNA level is not very useful, only suggestive >c/o
• Frequent EBV DNA load testing is required!

• Rising EBV-DNA load predicts aberrant EBV behaviour, most likely leading to PTLD if left untreated.
• Preemptive treatment guided by EBV-DNA dynamics can prevent serious PTLD complications, discriminate PTLD from rejection/GVHD and improve overall Tx outcome in both SOT and SCT.
Tools for PTLD diagnosis and monitoring:

EBV-RNA expression profiling in whole blood:

Multiprimed real-time RT-PCR or QT-NASBA/NucliSens

(latency type 3 genes):

- EBNA-1 Cp/Wp (QK, Fp)
- EBNA-2,
- LMP-1, LMP-2a
- BARTs (miRNA)
- BHRF-1 (Cp/Yp)
- (ZEBRA)
- (lytic antigens EA-d, VCA, MA)
EBV RNA profiling in circulating B-cells in whole blood of PTLD patients

PTLD patients:
- BARTs
- U1A snRNP

PTLD overview:
(19 samples, 14 patients)
- EBNA-1: 2/19
- LMP-1: 3/19
- LMP-2: 3/19
- EBNA-2: 0/19
- BARTs (miRNA): 18/19
- U1A snRNP: 19/19

Latency type III (growth program): Never detected

Latency type II (default program): Occasionally at abundant PTLD (SCT>>SOT)

Latency-1 (latent program): Frequently detected (BARTs), mostly without EBNA1

In PTLD circulating cells are “resting “memory type, in contrast to PTLD in tissue. Increase in number reflects PTLD process, but switched-off gene expression.
Viral load dynamics & latent gene expression in SOT patient with PTLD

SOT recipient 1

2a

BART RNA +
EBNA1 RNA-
LMP1 RNA-
LMP2 RNA+

BART RNA +
EBNA1 RNA-
LMP1 RNA-
LMP2 RNA+

BART RNA +
EBNA1 RNA+/-
LMP1 RNA+
LMP2 RNA+

PTLD 1

PTLD 2

PTLD 3

EBV DNA copies/ml blood

Days after LTx

0 50 100 150 200 250 300 350 400 450 500

0 50000 100000 150000 200000 250000 300000

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Viral load dynamics & latent gene expression in SOT patient with PTLD

SOT recipient 2

<table>
<thead>
<tr>
<th>Sample nr</th>
<th>EBV DNA load (copies/ml blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>~64000</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>12</td>
<td>~170000</td>
</tr>
</tbody>
</table>

EBV DNA load: LMP1 mRNA, LMP2 mRNA, EBNA1 mRNA, EBNA2 mRNA, BARTs RNA

- LMP1 mRNA
- LMP2 mRNA
- EBNA1 mRNA
- EBNA2 mRNA
- BARTs RNA

All samples negative
Viral load dynamics & latent gene expression in **SCT** patients with PTLD & Rituximab treatment

**SCT patient 1**

- **Plasma EBV load:** 1000 copies/ml
- **Cellular EBV load:** 1,426,344 copies/10^6 PBMC
- **EBV RNA profile:** BARTs +, EBNA1 +, LMP1-, LMP2 +

**SCT patient 2**

- **Plasma EBV load:** 3,740 copies/ml
- **Cellular EBV load:** 6,216 copies/10^6 PBMC
- **EBV RNA profile:** BARTs +, EBNA1 +, LMP1-, LMP2 +

**SCT patient 3**

- **Plasma EBV load:** 300,000 copies/ml
- **Cellular EBV load:** 376,000 copies/10^6 PBMC
- **EBV RNA profile:** BARTs +, EBNA1 -

**SCT patient 4**

- **Plasma EBV load:** 26,900 copies/ml
- **Cellular EBV load:** 17,471 copies/10^6 PBMC
- **EBV RNA profile:** BARTs +, EBNA1 +, LMP1-, LMP2 +
Viral load dynamics & latent gene expression in SCT patient with multiple PTLD episodes

**SCT patient 5**

- **Plasma EBV load:** 20,000 copies/ml
- **Cellular EBV load:** 4109 copies/10^6 PBMC
- **EBV RNA profile:** BARTs +, EBNA1+, LMP1+, LMP2+

**Conclusion 3:**
- EBV-RNA profiling has limited value over EBV-DNA load.
- BART RNA (miRNA) most frequently detected.
- In SCT more EBNA1 mRNA, more proliferating cells.
- EBNA2 mRNA never detected (no latency type III).
Overall Conclusions ...

- Increasing EBV DNA loads precede PTLD and can predict relapse
- Whole blood is preferred universal sample type
- Low EBV DNA load has high negative predictive value
- Dynamic changes in EBV DNA load reflect PTLD behaviour
- EBV-RNA in blood has little additive value...role of LMP2 & BARTs
- Decreasing EBV load corresponds with beneficial therapy response

Prevent PTLD by monitoring EBV-DNA load dynamics !!

Therapeutic intervention guided by EBV DNA load dynamics to prevent PTLD also has beneficial effects on long-term Tx-outcome
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Department of Hematology

Jan Cornelissen
Rik Brooimans
Question time

Epstein-Barr virus (EBV)

Benign

Persistent

Malignant

Prof. dr. Jaap M. Middeldorp

VU medisch centrum