Monitoring of virus-specific T cells as a prognostic marker after pediatric kidney transplantation

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Background

Immunosuppressive therapy after solid organ transplantation

=> disturbed balance between virus-replication & cellular immune defense

=> increased risk of viral complications, especially by cytomegalovirus (CMV), Epstein-Barr virus (EBV) and polyoma BK virus (BKV)

Trough level monitoring of immunosuppressants

=> insufficient to estimate the intensity of immunosuppression

Virus serology and virus DNA

=> insufficient to predict the individual risk and course of viral infections and to decide on the necessity and duration of antiviral prophylaxis/therapy

Virus-specific T cells control virus replication.
Hypothesis

Virus-specific T cells may serve as an indicator for “overimmunosuppression” and a prognostic marker for viral infections.

Individual effect-related drug-monitoring:
Optimization of post-transplant management by individual steering of immunosuppressive and antiviral therapy based on virus-specific T cells.
**Method: Cytokine Flowcytometry**

**Basic principle:**
After stimulation with virus-antigens, the activation of virus-specific CD4+ and CD8+ T cells leads to upregulation of CD69 and intracellular production of cytokines (IFNγ & TNFα).

=> Identification of CD4+ and CD8+ T cells, in which intracellular cytokine production is stimulated by virus-antigen and visualized by fluorescent antibodies
Method: Cytokine Flowcytometry
(established 2006 in cooperation with M.+U. Sester, Homburg (Saar))

1.) **Stimulation** of leukocytes by virus-antigen

2.) **Fixation** of leukocytes and lysation of erythrocytes

3.) **Immunostaining** by fluorescent antibodies against CD4, CD8, CD69, cytokines [IFNγ & TNFα]

4.) **Flowcytometry**: Measurement of fluorescence-marked lymphocytes
   => Identification of CD69-pos. & IFN γ-/TNFα-pos. T cells
   (=virus-specific T cells)
Method: Cytokine Flowcytometry

1. Stimulation

- Heparinized whole blood sample (450 µl)

- In vitro stimulation with virus-antigen in the presence of costimulatory antibodies (CD28 and CD49d)

  => Upregulation of CD69 and induction of intracellular cytokine production (IFNγ & TNFα)

positive control: stimulation with staphylococcus aureus enterotoxin B (SEB)

negative control: stimulation with control antigen
  (without virus-specific antigens)

- Incubation in polypropylene tubes at 37°C at 6% CO₂ for a total of 6 hours:
  after 2 hours addition of brefeldin A (BFA)

  => Block of cytokine secretion => Intracellular accumulation of cytokines
Method: Cytokine Flowcytometry

2. Fixation and lysation

• Addition of EDTA for 15 min

• Addition of Becton Dickinson lysing solution for 10 min
  => Lysation of erythrocytes and fixation of leukocytes

• Centrifugation and suction of supernatant

• Wash with FACS buffer (PBS, 5% FCS; 0.5% BSA; 0.07 NaN₃)

• Storage overnight at 4°C or immediate continuation of processing
**Method: Cytokine Flowcytometry**

3. Immunostaining

- Permeabilization of fixed leukocytes with FACS buffer containing 0.1% saponin for 10 min at room temperature
  
  => Offering a possibility for anti-cytokine-antibodies to pass cell membrane and bind to intracellular cytokines

- Centrifugation and suction of supernatant

- Staining for 30-45 min (in the dark, at room temperature) using saturating conditions of fluorescent antibodies against CD4, CD8, CD69 and IFNγ or TNFα (BD Biosciences)

- Wash with FACS buffer

- Centrifugation and suction of supernatant

- Fixation with 1% paraformaldehyde (PFA)
Method: Cytokine Flowcytometry

4. Flowcytometry

- Analysing of fluorescence-marked lymphocytes on a FACS Calibur / FACS Verse (BD Biosciences) using Cellquest/FACSuite software

- Identification of virus-spec. T cells as CD69-pos. and IFNγ/TNFα-pos. T cells

  => Frequency of virus-specific CD4+/CD8+ T cells is determined by percentage of CD4+/CD8+ T cells that were activated by virus-antigen (upregulation of CD69) to produce cytokines (IFNγ & TNFα).

- Calculation of percentage of virus-specific T cells by subtraction of the frequency obtained by respective negative control

- Quantification of absolute numbers of virus-specific T cells based on absolute number of blood leukocytes/lymphocytes analyzed in parallel
Method: Cytokine Flowcytometry

Differentiation of white blood cells (R1 = lymphocytes)

Differentiation of lymphocytes (R2 = CD4-positive T cells)
Differentiation of CD4-positive T cells

(red circle = CD69-pos. & IFNγ-pos. CD4+T cells = CMV-specific CD4+T cells)

SEB (positive control)  CMV-antigen  control antigen (negative control)

CMV-seropos. patient  CMV-seroneg. patient
• Significant correlation between IFNYγ- and TNFα-pos. CMV-spec. CD4+T cells (Spearman r= 0.993, p<0.0001)

• Significant correlation between IFNYγ- and TNFα-pos. ADV-spec. CD4+T cells (Spearman r= 0.802, p<0.0001)
Study populations

**Main population:**
Pediatric patients (0-18 years) during the first two years after KTx with Basiliximab, Prednisolone, low-dose Ciclosporine A and Everolimus

**Smaller populations:**
- Pediatric patients after KTx with Basiliximab, Prednisolone, Mycophenolate Mofetil and Ciclosporine A or Tacrolimus
- Pediatric patients after heart or liver transplantation
- Adult patients after KTX with polyoma BK virus (BKV)-infection
Monitoring of virus-specific T cells in our lab

1. Cytomegalovirus (CMV)-specific T cells since March 2006

2. Adenovirus (ADV)-specific T cells since March 2006

3. Herpes simplex virus (HSV)-specific T cells since January 2009

4. Polyoma BK virus (BKV)-specific T cells since February 2009

5. Epstein-Barr virus (EBV)-specific T cells since January 2010
Prevalence and follow-up of virus-specific T cells

- Prevalence of CMV-/HSV-sp. CD4+ T cells corresponded with IgG-seropositivity.
- CMV-, ADV- & HSV-sp. CD4+ T cells were permanently detectable.
- In contrast to CD4+ T cells, virus-sp. CD8+ T cells showed lower levels and were only temporarily detectable.
- Virus-sp. CD4+ T cells fluctuated depending on the grade of immunosuppression.
Monitoring of virus-specific T cells

1. Polyoma BK virus (BKV)-specific T cells
   => Polyomavirus-associated nephropathy

2. Cytomegalovirus (CMV)-specific T cells
   => Primary CMV-infection/-reactivation

3. Cytomegalovirus (CMV)-, Herpes-simplex virus (HSV)- and Adenovirus (ADV)-specific T cells
   => Grade of immunosuppression ("Overimmunosuppression")

4. IVIST study
   => "Effect-related drug-monitoring"

4. Epstein-Barr virus (EBV)-specific T cells → results still pending
Polyomavirus-associated nephropathy (PVAN) leads to decreased graft function and graft loss. Prognostic markers for the outcome of polyoma BK virus (BKV)-infections are missing.

**Hypothesis:** BKV-specific T cells may serve as an indicator for the individual susceptibility to BKV-associated complications and may help to steer treatment.

**Method**

- 26 children after KTx (aged 1-17 years, median 10 years, 69% ♂) with current or previous detection of BKV-DNA in blood
- Measurement of BKV-specific CD4 and CD8 T cells (BKV-CD4 and CD8 Tvis) at different times
- Analysis of BKV-DNA by PCR
**Results:** BKV-specific CD4 and CD8 T cells (BKV-CD4/CD8 Tvis)

- The majority of our study group (22 out of 26 children) showed BKV-CD4 Tvis (up to 8.3 cells/µl).
- Only 15 patients temporarily showed BKV-CD8 Tvis (up to 3.3 cells/µl).
Patient characteristics

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<th>Patient no.</th>
<th>Age [yr]</th>
<th>Sex</th>
<th>Blood-BKV-DNA (maximum) [copies/ml]</th>
<th>Persistence of BKV-DNA (&gt;3 months)</th>
<th>BKV-CD4 Tvis (maximum) &lt;0.75/µl</th>
<th>BKV-CD4 Tvis (maximum) &gt;0.75/µl</th>
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Florid PVAN (violet):
Low BKV-CD4 Tvis with persistency of blood-BKV-DNA (>3 months)

Asymp. BKV infection (blue):
High BKV-CD4 Tvis without persistency of blood-BKV-DNA

# = Tvis-measurement after disappearance of BKV-DNA in blood
Follow-up of BKV-CD4 and CD8 Tvis

Symptomatic BKV infection ⇔ Asymptomatic BKV infection

Biopsy proven florid PVAN

- Persistency of blood-BKV-DNA
- Lack or very low levels of BKV-CD4 Tvis (<0.75 cells/µl)
- No BKV-CD8 Tvis

Asymptomatic BKV infection

- No persistency of blood-BKV-DNA
- High levels of BKV-CD4 Tvis (>0.75 cells/µl)
- Temporary detection of BKV-CD8 Tvis
Follow-up of BKV-Tvis after therapeutic intervention

- Minimization of immunosuppression because of BKV infection: Tacrolimus-withdrawal => Everolimus and low-dose steroid
- After minimization of immunosuppression: Increase of BKV-CD4 Tvis and decrease of BKV-DNA
In case of BKV-DNA-detection in blood after KTx, levels of BKV-CD4 Tvis correlate with the individual risk of BKV-associated complications:

Low levels of BKV-CD4 Tvis (<0.75 cells/µl) => Increased risk of florid PVAN
Sufficient levels of BKV-CD4 Tvis (>0.75 cells/µl) => Asymptomatic BKV-infection

Serving as a marker of the individual BKV-specific immune defense, levels of BKV-CD4 Tvis may represent the risk of florid PVAN and optimize individual timing of therapeutic interventions.

Conclusion
Cytomegalievirus (CMV)-specific T cells

CMV-infection leads to decreased graft survival and significant morbidity.

Hypothesis:
CMV-specific T cells may serve as a prognostic marker of CMV-infections to estimate the necessity and duration of antiviral therapy.

Method: Prospective longitudinal study

- 37 pediatric patients (aged 1-17 years, median 13 years, 57%♂)
- Monitoring of CMV-specific CD4+ and CD8+ T cells during the first year after kidney transplantation (KTx)
- Immunosuppression: Basiliximab, Prednisolone, Ciclosporine A & Everolimus (n=34), Ciclosporine A & MMF (n=1), Tacrolimus & MMF (n=2)
- In case of significant CMV-DNA-detection, start of antiviral therapy
Results: Donor (D)/Recipient (R)-CMV-constellation and post-Tx CMV-infections/-reactivations

- Prevalence of CMV-sp. CD4+T cells (24%) corresponded to CMV-IgG-seropositivity.
- After KTx: 5 primary CMV-infections and 4 CMV-reactivations
Primary CMV-infections: Two symptomatic & three asymptomatic courses

Asymptomatic prim. CMV-infections
- initial boost of CMV-sp. CD4+ T cells combined with CMV-seroconversion
- no significant detection of CMV-DNA
- no antiviral therapy

Symptomatic prim. CMV-infections
- initial boost of CMV-DNA
- delayed increase of CMV-sp. CD4+T cells simultaneous with decrease of CMV-DNA after start of (Val-) Ganciclovir-therapy
CMV-reactivations:
Two symptomatic & two asymptomatic courses

**Symptomatic CMV-reactivations**
- initial boost of CMV-DNA with transient disappearance of CMV-sp. CD4+T cells
- slow re-increase of CMV-sp. CD4+T cells simultaneous with decrease of CMV-DNA after start of (Val-)Ganciclovir-therapy

**Asymptomatic CMV-reactivations**
- high levels of CMV-sp. CD4+T cells
- spontaneous disappearance of CMV-DNA
- no antiviral therapy
Comparison of CMV-specific CD4+ and CD8+T cells in case of CMV-reactivations

Symptomatic CMV-reactivation

- transient disappearance of CMV-sp. CD4+T cells with re-increase of CMV-sp. CD4+T cells after start of antiviral therapy
- low levels of CMV-sp. CD8+T cells

Asymptomatic CMV-reactivation

- persistent high levels of CMV-sp. CD4+T cells
- low levels of CMV-sp. CD8+T cells
Prevalence of CMV-sp. CD4+T cells corresponded to CMV-IgG-positivity. In contrast to CD4+T cells, CMV-sp. CD8+ T cells showed lower levels and were only temporarily detectable.

=> CMV-sp. CD4+T cells: more applicable for immunomonitoring.

CMV-specific CD4+T cells correlate with individual susceptibility to symptomatic CMV-infections/-reactivations after KTx:

Sufficient levels of CMV-specific CD4+T cells (>2 cells/µl)

=> sufficient CMV-specific immune defense
=> no CMV-associated complications

Absence/decrease of CMV-specific CD4+T cells (<2 cells/µl)

=> increased risk of symptomatic CMV-infections/-reactivations

**Perspective:**

Serving as prognostic marker for individual risk of viral diseases, monitoring of CMV-specific CD4+T cells may improve post-Tx management and optimize individual timing and duration of antiviral therapy.
Immunomonitoring by CMV- and ADV-specific T cells

Hypothesis:
A low number of virus-specific T cells after Tx may serve as an indicator of overimmunosuppression.
A high number of virus-specific T cells after Tx may serve as an indicator of underimmunosuppression.

Method: Prospective longitudinal study

- 37 pediatric patients (aged 1-17 years, median 13 years, 57% ♂)
- Monitoring of cytomegalovirus (CMV)- and adenovirus (ADV)-specific CD4+T cells during the first year after kidney transplantation (KTx)
- Monitoring of viral infections and virus-DNA (especially CMV & EBV)
- Immunosuppression: Basiliximab, Prednisolone, Ciclosporine A & Everolimus (n=34), Ciclosporine A & MMF (n=1), Tacrolimus & MMF (n=2)
Results: Prevalence of CMV- & ADV-specific T cells and post-Tx viral infections

- Prevalence of CMV-sp. CD4 T cells corresponded with CMV-IgG-seropositivity.
- CMV- & ADV-sp. CD4 T cells were permanently detectable and fluctuated depending on the grade of immunosuppression.

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<th>Prim. Infections / reactivations</th>
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<td>Polyoma BK virus (BKV)</td>
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CMV-reactivations: Follow-up of CMV- & ADV-sp. T cells

Two symptomatic courses ⇔ Two asymptomatic courses

Symptomatic CMV-reactivations

- transient disappearance of CMV-sp. CD4+T cells with DNA-boost
- re-increase of CMV-sp. CD4+T cells simultaneous with DNA-decrease after Valganciclovir-start
- low levels of ADV-sp. CD4+T cells (<2 cells/µl)

Asymptomatic CMV-reactivations

- high levels of CMV- and ADV-sp. CD4+T cells (>2 cells/µl)
- spontaneous disappearance of CMV-DNA without antiviral therapy
Correlation of virus-sp. CD4+T cells & immunosuppression

**Sufficient immune defense**
- temporary decrease of virus-sp. CD4+T cells during initial post-Tx period with rapid re-increase
- in the presence of high levels of virus-sp. CD4+T cells (>2/µl): no significant viral complications (e.g. no symptomatic EBV-/CMV-infections/ reactivations)

**Insufficient immune defense**
- in case of persistent low levels of CMV- & ADV-sp. CD4+T cells (<2/µl): increased risk of viral diseases (especially primary EBV-infections with persistent EBV-DNA-load)
Correlation of CMV- & ADV-sp. CD4+T cells with EBV-DNA

In case of high EBV-DNA load, CMV- and ADV-sp. CD4+T cells were significantly lower than without relevant DNA-detection.

Spearman \( r = -0.69 \) & \( p < 0.001 \) (CMV-sp. CD4-T cells)
Spearman \( r = -0.49 \) & \( p = 0.001 \) (ADV-sp. CD4-T cells)
Conclusion

After KTx, CMV- and ADV-specific CD4+T cells represent not only virus-specific but also general cellular immune defense. Accordingly, they correlate with individual susceptibility to viral infections:

- Sufficient levels of CMV- & ADV-specific CD4+T cells (>2 cells/µl) => no symptomatic viral infections/reactivations
- Decrease of CMV- & ADV-specific CD4+T cells (<2 cells/µl) => increased risk of viral complications (e.g. by EBV)

No conclusion according to underimmunosuppression is possible, because no events as acute rejections or donor specific antibodies were detected.

Perspective:
Serving as an indicator of “overimmunosuppression”, monitoring of virus-specific CD4+T cells may improve post-Tx management and optimize individual timing of antiviral therapy and dosing of immunosuppression (effect-related drug-monitoring).

19% of our study group showed neither CMV- nor ADV-specific CD4+T cells => additional measurement of HSV-specific CD4+T cells to establish further parameters for T cell-monitoring
Monocentre, randomized, open-labeled study to steer immunosuppressive and antiviral therapy by measurement of virus-specific CD4+ T cells in addition to trough level monitoring after pediatric kidney transplantation

=> Effect-related drug monitoring

Study start: 2010
Control group (n=32)

Pediatric kidney transplantation (KTx)

Study start & Randomisation
4 weeks after KTx

Intervention group (n=32)

Steering of immunosuppression by trough level monitoring

Additional steering of immunosuppressive drugs by levels of CMV-, ADV- & HSV-CD4 Tvis

Study design:

Month 2-6 post KTx:
Low-dose Prednisolone
Cyclosporine A (C0 50-100 µg/l ); Everolimus (C0 3-6 µg/l)

Month 7-24 post KTx:
Prednisolone withdrawal (in case of normal protocol biopsy)
Cyclosporine A (C0 30-75 µg/l); Everolimus (C0 2-5 µg/l)
Example: Patient of intervention group (Tvis monitoring)

- 12-year-old girl; stable graft function (s-creatinine 58-65 µmol/l)
- Donor CMV-pos./ Recipient CMV-neg. (=>Valganciclovir-prophylaxis 3 months)
- Asymptomatic CMV-infection (CMV-seroconversion without DNA-detection)
Kidney transplantation: Basiliximab, Prednisolone, Ciclosporine A

4 weeks after transplantation: Study start
Low-dose Prednisolone
Reduction of Ciclosporine A-dose to 50%
Start of Everolimus
  => Target trough levels (LC-MS/MS): Ciclosporine A 50-100 µg/l
    Everolimus 3-6 µg/l

6 months after transplantation: Control biopsy
Prednisolone withdrawal (within 3 months)
Reduction of target trough levels for Ciclosporine A and Everolimus
  => Target trough levels (LC-MS/MS): Ciclosporine A 30-75 µg/l
    Everolimus 2-5 µg/l
Antiviral therapy (IVIST-study)

Antiviral prophylaxis (Valganciclovir) in case of high risk constellation (Donor CMV-positive/Recipient CMV-negative):

=> control and intervention group: prophylaxis for 3 months

Preemptive antiviral therapy ((Val-)Ganciclovir) in case of significant detection of CMV-DNA in blood:

=> control group: antiviral therapy for 3 months
=> intervention group: antiviral therapy, until a sufficient and stable number of CMV-specific CD4+T cells are detectable
Frequency of T cell-monitoring (IVIST-Study)

Study start: 4 weeks after kidney transplantation (KTx)

Month 2-3 after KTx: biweekly

Month 4-12 after KTx: monthly

Month 13-24 after KTx: bimonthly

Study end: 2 years after KTx
Primary and secondary endpoints of IVIST-study

Primary endpoint:
GFR 2 years after transplantation

Secondary endpoints:
Number of viral and bacterial infections
Number of CMV-infections/-reactivations
Duration of antiviral therapy in case of CMV-infection/-reactivation
Number of AEs in association with antiviral therapy
Number of AEs in association with immunosuppression
Number of hospitalizations/SAEs
Trough levels of Ciclosporine A and Everolimus
Follow-up

First patient first visit: April 15, 2010

Extension from mono- to multicenter trial: December 2011

Planned recruitment: 64 pediatric patients at the age of 1-16 years
(32 patients in each group)

Cumulative recruitment: 41 patients
64% of planned recruitment
(Tvis group 22; control group 19)
### Status (update March 2015)

<table>
<thead>
<tr>
<th></th>
<th>Tvis group (n=22)</th>
<th>Control group (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Mean±SD) [years]</td>
<td>11.0 ± 3.9</td>
<td>11.5 ± 4.1</td>
</tr>
<tr>
<td>Gender</td>
<td>12 ♂ (55%)</td>
<td>11 ♂ (58%)</td>
</tr>
<tr>
<td>CMV-prophylaxis</td>
<td>10 (46%)</td>
<td>7 (37%)</td>
</tr>
<tr>
<td>Completed study period</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Drop-outs</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Death</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

6 Drop-outs because of change of immunosuppressive regimen
1 Death: Drowning in combination with hypertrophic cardiac disease
DEVELOPMENT SAFETY UPDATE REPORT (DSUR)

Clinical trial title: A multicenter, randomized, open-labeled study to steer immunosuppressive and antiviral therapy by measurement of virus- (CMV, ADV, HSV) specific T cells in addition to determination of trough levels of immunosuppressants in pediatric kidney allograft recipients. An explorative study.

Trial identification number: IVIST01

Date of regulatory approval: 14 August 2009

Date of any changes to the regulatory approval:
- 09 February 2011: First Amendment
- 16 December 2011: Second Amendment (extension from mono- to multicenter trial)

Data lock point: 14 August 2014

Reporting period: 15 August 2013 – 14 August 2014

Number of report: 5

Version of report: 1.0 final

In conclusion, no relevant new safety information arose during the reporting period. Thus, the IVIST01 trial will be continued as planned.
Aim of IVIST01-trial

Improvement of graft function and reduction of infections by avoidance of too intensive immunosuppressive therapy and drug toxicity after kidney transplantation

 Novel concept of personalization of immunosuppressive management by Tvis monitoring

=> Effect-related drug monitoring by Tvis
Thank you!

IVIST-Participating centers:
Cologne (C. Taylan, M. Geßner, L.T. Weber)
Hamburg (A. Lehnhardt, M. J. Kemper)
Rostock (H. Staude, M. Wigger)

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