The rapid antigen-reactive T cell enrichment procedure enables direct *ex vivo* BKV-specific CD4+ T cell characterization

Michaela Niemöller, Mario Assenmacher, and Anne Richter
Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Introduction

BK virus (BKV) is a ubiquitous polyomavirus, which persists in a latent and asymptomatic state in healthy individuals after primary infection. In immunocompromised patients BKV reactivation may occur, which can result in, e.g., hemorrhagic cystitis after allogeneic stem cell transplantation or loss of the allograft after renal transplantation. The detailed characterization of BKV-specific T cells is the basis for a better understanding of the BKV immune response. This in turn provides the foundation for the development of strategies towards the prevention or treatment of infections in immunocompromised patients, for example, the adoptive transfer of virus-specific T cells.

Results

1 BKV-specific T cells are rare in healthy donors

PBMC from six randomly selected healthy donors were stimulated with various peptide pools for immunodominant antigens of BKV, JCV, CMV, influenza, EBV, and AdV, and for positive control with a mixture of CMV/EBV/influenza MHC class I–restricted peptides. After 6 hours, IFN-γ production within the CD4+ and CD8+ T cell compartments was analyzed by intracellular staining using the Rapid Cytokine Inspector. CMV-, EBV-, and AdV-specific IFN-γ+ T cells were clearly detectable in several donor samples. In contrast, IFN-γ+ BKV- and JCV-specific T cells were detectable only at very low frequencies, between 0.01 and 0.03%, in five out of six donor samples.

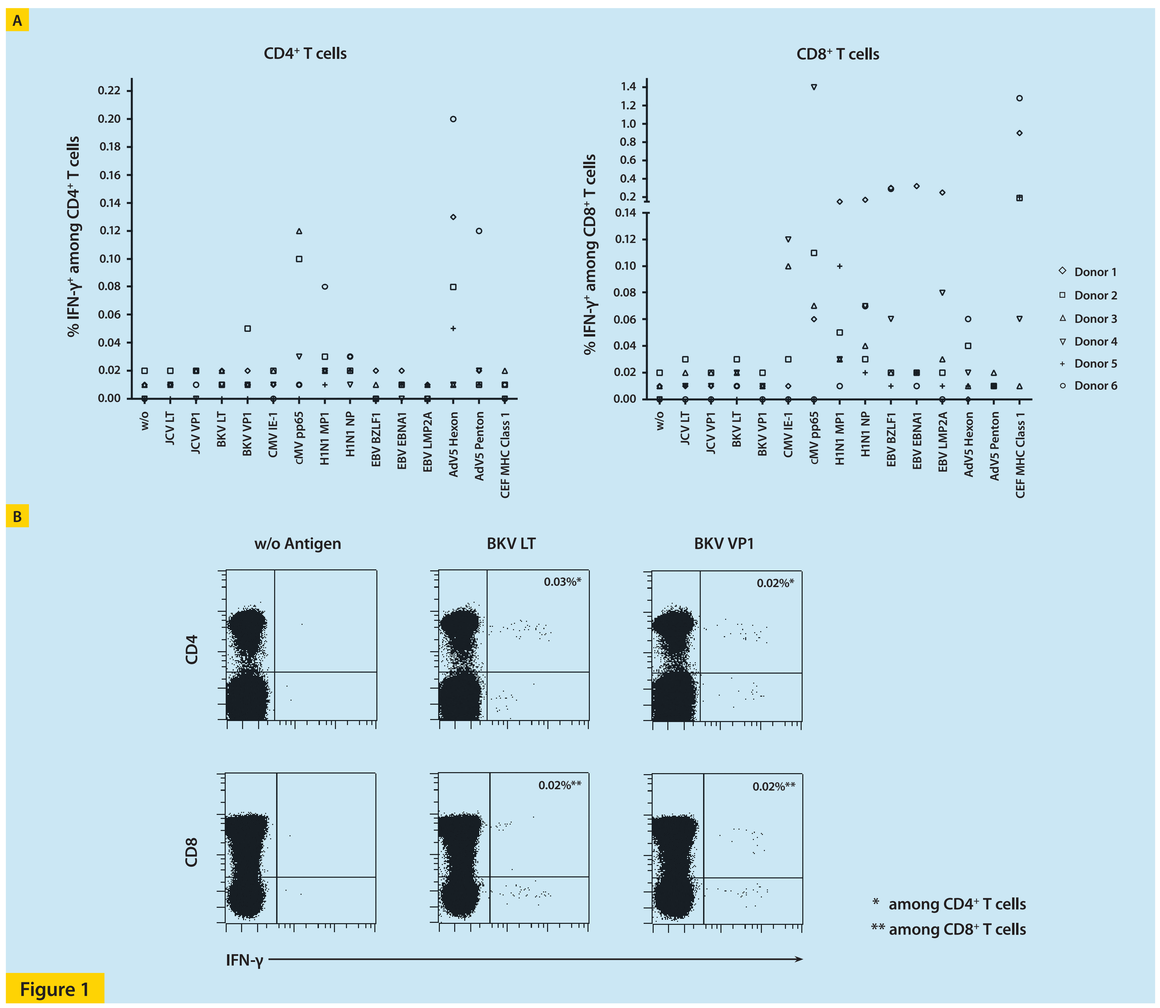


Figure 1

2 Procedure for the rapid antigen-reactive T cell enrichment (Rapid ARTE)

Recently, a protocol for the sensitive functional analysis of different rare antigen-specific CD4+ T cell repertoires directly *ex vivo* was developed. This so-called antigen-reactive T cell enrichment (ARTE) approach involves the magnetic enrichment of antigen-activated CD154+CD4+ T cells to subsequently uncover their phenotypic and functional profile by flow cytometry[1,2]. We further streamlined the ARTE protocol with regard to incubation times and hands-on time (Rapid ARTE). This new protocol allows the stimulation of up to 2.5×10^7 PBMC. After six hours cells are fluorescently labeled for detection of various cell surface markers, such as CD4. At the same time cells are magnetically labeled for CD154. All steps are performed directly in the cell culture plate without cell harvesting and washing, which simplifies the procedure and reduces hands-on time. After surface labeling, the cells are fixed, harvested, and directly applied onto a column for magnetic enrichment of antigen-activated CD154+ T cells. The cells retained in the column are permeabilized, stained intracellularly for cytokines and CD154, and washed directly on the column to avoid centrifugation steps. Finally, CD154+ T cells are eluted from the column for subsequent flow cytometry analysis.

Cell stimulation
Stimulate 1–2.5×10^7 PBMC/mL/well with peptide pools in a 24-well plate. — **6 h**

Cell surface labeling and fixation
Remove 800 µL cell culture supernatant.
Add antibodies for cell surface labeling (incl. CD154-Biotin). — **2 min shaking + 3 min incubation**
Add Anti-Biotin MicroBeads. — **2 min shaking + 8 min incubation**
Add 300 µL 3.7% Inside Fix. — **2 min shaking + 13 min incubation**

Enrichment of labeled CD154+ cells, permeabilization, and intracellular staining
Apply the fixed cell suspension onto the column placed in a separator.
Wash with 500 µL of PEB buffer.
Wash with 500 µL Inside Perm.
Apply CD154 and Anti-Cytokine antibodies for intracellular staining.
Wash with 500 µL Inside Perm.
Wash with 500 µL PEB buffer. — **10 min incubation**

Elution of CD154+CD4+ T cells
Remove column from the separator and place it on a suitable collection tube.
Pipette 500 µL PEB buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

Flow cytometric analysis
Automated sample acquisition.
Enumeration and characterization of antigen-activated T cells.

Hands-on time: 30 min
Total procedure: ≈ 7 h

Figure 2

3 Enumeration of BKV-specific CD154+CD4+ T cells

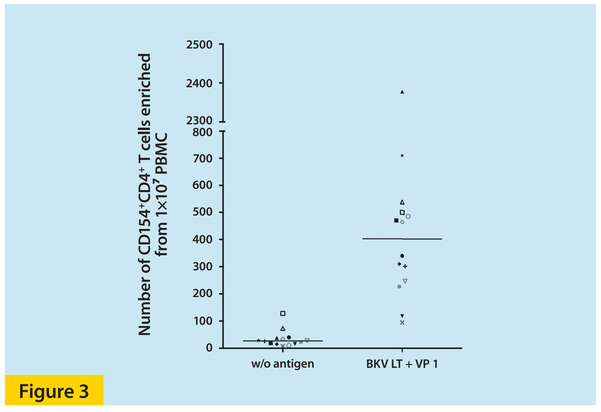


Figure 3

In blood samples BKV-specific T cells are present only at very low frequencies, in the range of the detection limit of flow cytometry analysis. Therefore, we used the Rapid ARTE protocol for enumeration of BKV-specific T cells. Up to 2.5×10^7 PBMC from 14 healthy donors were left untreated or stimulated with BKV peptide pools covering the complete sequence of the large T antigen (LT) and virion protein VP1. Both proteins are immunodominant targets for T cell immunity. After six hours, cells were treated as described in figure 2, and the absolute numbers of enriched CD154+CD4+ T cells were determined. In blood samples of each donor, BKV-reactive CD4+ T cells were found. Absolute numbers ranged between 87 and 2340 per 1×10^7 PBMC.

4 Characterization of BKV-specific T cells using Rapid ARTE

The Rapid ARTE approach allowed us to process larger cell numbers compared to detection by flow cytometry alone. Therefore, the new approach enabled the reliable characterization of functional subsets of the entire BKV-reactive CD154+CD4+ T cell pool, i.e., TNF-α–, IFN-γ–, IL-10–, and IL-2–producing T cells. PBMC (2.5×10^7) from six healthy donors were processed as described in figure 2. Subsequently, the cytokine profile of the BKV-reactive CD4+ T cells was analyzed (A). TNF-α and IL-2 were expressed by the majority of BKV-specific T cells in all samples analyzed for these cytokines. In contrast, the frequency of IFN-γ+ cells among BKV-reactive CD4+ T cells varied between 27 and 74% in the different donor samples (B). Furthermore, IL-10–producing T cells could be identified at very low frequencies, which could not have been detected by flow cytometry without pre-enrichment.

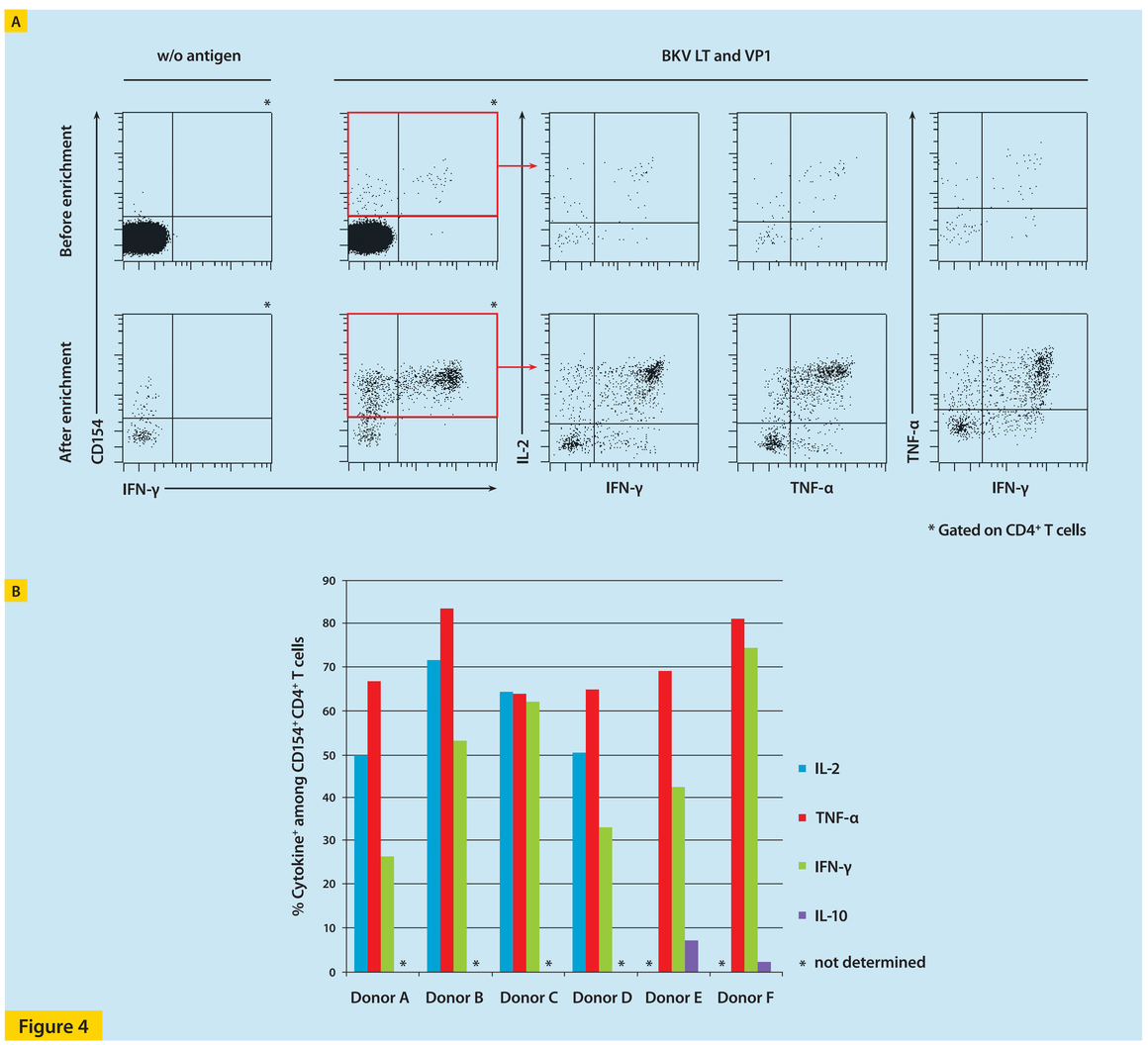


Figure 4

Conclusion

- We developed a rapid (7 h) and convenient protocol to detect and characterize very rare antigen-specific CD4+ T cell subpopulations in blood samples.
- The process does not involve any centrifugation step and requires only minimal hands-on time.
- High-resolution characterization of rare T cell repertoires is achieved combining magnetic cell enrichment and multiparameter flow cytometry analysis of CD154+ T cells.
- In contrast to conventional flow cytometry analysis, this approach enabled us to identify BKV-specific T cells and specify their function.

References

1. Bacher, P. *et al.* (2013) J. Immunol. 190: 3967–3976.
2. Bacher, P. *et al.* (2013) Cytometry A 83: 692–701.

Ziel2.NRW
Regionale Wettbewerbsfähigkeit und Beschäftigung

EUROPÄISCHE UNION
Investition in unsere Zukunft
Europäischer Fonds
für regionale Entwicklung

Ministerium für Innovation,
Wissenschaft und Forschung
des Landes Nordrhein-Westfalen