Detection of Epstein-Barr Virus Specific Functional T Cells by Flow Cytometry

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INTRODUCTION

The ability to evaluate EBV specific T cell responses post-transplant is currently an unmet need for managing patients at risk of developing post-transplant lymphoproliferative disorder (PTLD). Functional impairment or lack of a T cell response to EBV has been predicted to drive the development of PTLD. Research indicates that T cells capable of producing multiple cytokines, polyfunctional T cells (PFCs), are generally absent in patients that have developed PTLD, but are present in individuals that have prevented virus reactivation and can control the transformation that causes PTLD (1, 2). Additionally, the decline of viral load in primary infections over time correlates with the appearance of polyfunctional T cells (3).

We developed a whole blood assay that measures CD4 and CD8 T cell responses to stimulation with various EBV proteins/peptides. Cells expressing CD69 along with IFNγ, TNFα, or IL-2, and polyfunctional T cells co-expressing 2 or 3 cytokines along with CD69 were measured. A positive response was defined as a 3-fold increase in the number of activated T cells over unstimulated controls. The peptides used to stimulate the CD8 T cells were from the lytic protein BZLF1 and the latent proteins EBNA 3A/3C. EBV specific CD4 T cells were detected by stimulating with an EBV whole cell lysate. This assay was validated using whole blood from apparently normal adult donors who self-reported as previously infected with EBV or were seropositive.

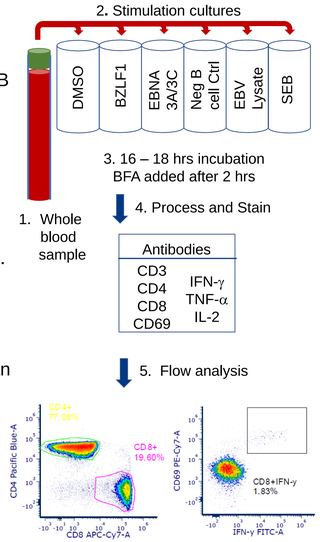
CD8 T cell responses to EBV were the predominant responding cells in normal, healthy adult volunteers, but responses to the different peptides/proteins were variable from donor to donor. Polyfunctional CD8 T cells were detected in most responding donors. EBV specific CD4 T cell responses were much lower than CD8 T cell responses, but were also polyfunctional.

The ability to monitor the presence or absence of EBV specific, highly functional T cells will be an important tool, along with monitoring viral load, in managing patients post-transplant as their immune system recovers.

MATERIALS AND METHODS

Figure 1. Assay Method

1. Sodium heparin whole blood samples are added to culture wells.
2. Samples are stimulated with BZLF1, EBNA 3A/3C, B cell negative control, EBV Lysate, DMSO vehicle or SEB positive control.
3. Brefeldin A (BFA) is added after 2 hours of incubation and culture continues overnight.
4. Cultures are processed the next day and cells stained with fluorochrome labeled antibodies.
5. Flow cytometry analysis defines the T cell populations using antibodies to CD3, CD4 and CD8. Functional CD4 or CD8 T cells are identified by intracellular cytokine staining using CD69, IFN-γ, TNFα, and IL-2 labeled antibodies.
6. Positive responses to stimulation with EBV proteins/peptides are described as 3-fold higher than background with at least 15 cells in the responding population.



Serology testing was performed using EBNA-1 IgG and VCA IgG ELISA test systems according to the manufacturer's instructions. EBNA-1 is a latent protein and the VCA is the viral capsid antigen comprised of several lytic phase proteins.

RESULTS

Table 1. Correlation of EBV T cell responses with EBV sero-reactivity

	EBV T cell non-responder	EBV T cell responder
EBV seronegative	6	0
EBV seropositive	0	20

Table 1. Sero-reactivity to EBV VCA antigen (lytic phase) and EBNA-1 antigen (latent phase) was measured by ELISA. IgG specific for at least 1 of the EBV antigens was detected in 20 of 26 samples tested. Samples from all sero-positive donors tested in the T cell immunity panel had CD4 and/or CD8 T cell responses to stimulation with at least one of the EBV proteins/peptides. Six donors that were EBV seronegative did not have any EBV specific T cell responses 3-fold above background, but did respond to SEB.

Figure 2a. CD4 T cell responses to stimulation with EBV cell lysate

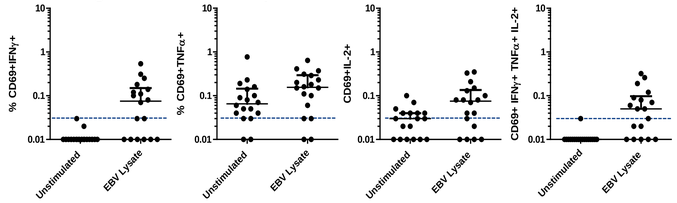


Figure 2b. CD8 T cell responses to stimulation with EBV BZLF1 and EBNA1

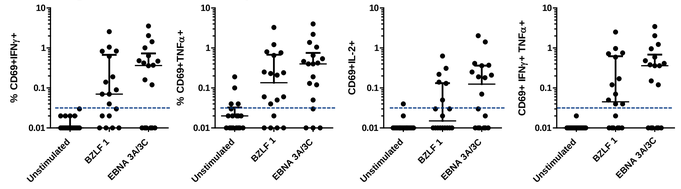
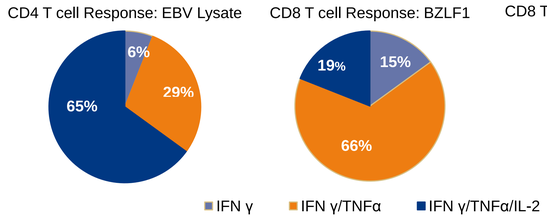


Figure 2. EBV Specific CD4 and CD8 T cell responses from normal healthy donors. Results from 18 normal, healthy donors tested during validation of the T cell assay are shown above. Background responses across all donors varied, especially the TNFα and IL-2 responses. EBV specific polyfunctional responses from both CD4T cells (Fig. 2a) and CD8 T cells (Fig. 2b.) were detected in EBV seropositive donors. All seropositive samples had CD8 T cell responses to stimulation with either BZLF1 or EBNA 3A/3C peptide pools and 9 of the 12 seropositive samples had CD4 T cell responses to stimulation with EBV lysate.

Figure 3. Average Polyfunctional proportions of responding T cells from normal, healthy donors.



RESULTS

Figure 4a. EBV Specific T cell Response in Immunosuppressed Patient with Active EBV Infection

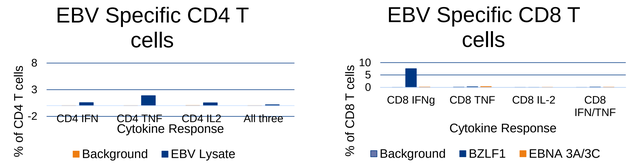


Figure 4b. EBV Specific T cell Response in Normal, Healthy donor; no active infection.

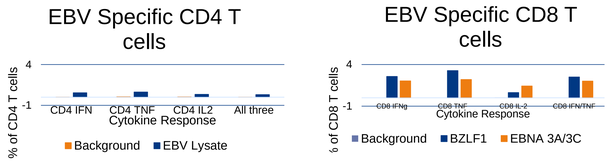


Figure 4. EBV Specific T cell responses from an immunosuppressed patient with an active EBV infection (Fig. 4a) had very few polyfunctional T cells when compared with the T cell response from a normal healthy donor with no evidence of active infection (Fig. 4b). The large CD8 response of the patient was stimulated by the peptides from the lytic protein BZLF1, where the normal donor responded to both the lytic and latent antigens. While the number of responding CD4 and CD8 T cells were lower in the normal donor, the proportion of responding cells that were polyfunctional was higher.

CONCLUSIONS

- Correlation between antibody responses to EBV specific proteins and T cell responses to EBV specific proteins was 100%. All seronegative donors failed to generate a T cell functional response when stimulated with EBV proteins or peptides and all seropositive donors did generate a T cell functional response to an EBV proteins or peptides. Note that the serology testing utilized lytic and latent protein targets that are different from the protein/peptides used to measure T cell mediated immunity.
- Samples from seropositive, normal, healthy donors respond to stimulation with the EBV peptides BZLF1 and EBNA 3A/3C. Most CD8 T cells expressing INFγ and CD69 also express TNFα indicating these are highly functional CD8 T cells.
- Responses of CD4 T cells in samples from seropositive, normal, healthy donors were lower than the CD8 T cell responses from the same donors, but were also highly functional, producing IFNγ, TNFα and IL-2.
- The response of an immunosuppressed patient with an activated EBV infection was distinct from the normal healthy donor responses in that the vast majority of responding CD8 T cells only produced IFNγ. CD4 response was predominantly TNFα and had a lower proportion of polyfunctional CD4 T cells.

References:

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2. Ning, RJ, XQ Xu, KH Chan and AKS Chiang. 2011. Long-term carriers generate Epstein-Barr virus (EBV)-specific CD4⁺ and CD8⁺ polyfunctional T-cell responses which show immunodominance hierarchies of EBV proteins. Immunnol. 134: 161-171.
3. Lam, JKP, KF Hui, RJ Ning, XQ Xu, KH Chan and AKS Chiang. 2018. Emergence of CD4+ and CD8+ polyfunctional T cell responses against immunodominant lytic and latent EBV antigens in children with primary EBV infection. Front. Microbiol. 9:416 doi: 10.3389/fmicb.2018.00416.