# Track® CMV ELISpot

Immune monitoring of CMV-specific cell-mediated immunity

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Lophius Biosciences GmbH - White Paper - 2016 - T-Track® CMV ELISpot

## Why monitoring CMV-specific CMI?

Dear reader,

Within this white paper you will find general information about cellmediated immunity (CMI) and novel immune monitoring strategies based on the measurement of antigen-specific T cell responses.

Since cytomegalovirus (CMV) continues to be one of the most common complications after solid-organ transplantation [1], improvement of CMV management by innovative diagnostic solutions remains of clinical relevance and will be addressed within the following pages.

With the aim to support your work as a clinician, diagnostician and/or physician towards personalized medicine, we introduce the *in vitro* T-Track<sup>®</sup> CMV diagnostic ELISpot kit as a solution to monitor CMVspecific CMI and thereby evaluate the potential risk for CMV disease in immunocompromised patients, such as transplant recipients.

Read more about our patented T-activation<sup>®</sup> technology for enhanced immune stimulatory capacity and immunogenicity of proteins, enabling the development of innovative and highly sensitive diagnostic systems. Lophius Biosciences is your partner for immune diagnostic solutions.

#### T cell-mediated immunity

As part of the adaptive immune system, cell-mediated immunity (CMI) involves the activation of antigen-specific T cells that can help to eliminate pathogens that reside inside host cells.

T cells recognize fragments of proteins that have been partially digested inside an antigen-presenting cell (e.g. dendritic cell, macrophage). The resulting peptides are carried to the surface of the presenting cell bound to major histocompatibility complexes (MHC), which present the fragments to T cells [2].

Processing of antigens from intracellular pathogens (e.g. viruses) involves the endogenous pathway and results in the presentation of cytosolic peptide fragments on the cell surface in association with MHC class I molecules, ultimately resulting in the activation of epitope-specific cytotoxic T cells (CTL or CD8<sup>+</sup> T cells). Specialized antigen-presenting cells (APC) can also present peptides derived from endocytosed extracellular proteins in the context of MHC class II, via the exogenous pathway, resulting in the activation of T helper cells (or CD4<sup>+</sup> T cells). In addition, APCs can cross-present peptides derived from endocytosed proteins with MHC class I molecules to cytotoxic CD8<sup>+</sup> T cells (cross-presentation pathway) [2–4].

Activation of T cells is induced by interaction of their T cell receptor with a specific epitope/MHC complex, leading to proliferation and differentiation into effector cells capable to attack infected target cells. Once a helper T cell has been activated to become an effector cell, it can help activate other cells by secreting a variety of cytokines and by displaying costimulatory proteins on its surface [5].

### Effector T cells

After a naive T cell is activated, effector molecules produced by the armed effector T cells fall into two broad classes: cytotoxins (e.g. perforin that creates holes in the target cell membrane), which are released by cytotoxic CD8<sup>+</sup> T cells, and cytokines (e.g. IFN-γ, interleukins), which are synthesized *de novo* by all effector T cells.

Cytokines are a diverse group of small soluble proteins secreted by one cell that can alter the behaviour or properties of the cell itself or of another cell. The following exposure to different cytokines can lead to the differentiation of naive CD4<sup>+</sup> T (T helper or Th) cells into distinct types of effector T helper cells, called Th1, Th2 or Th17 [5]. Each type of effector T cell is specialized in distinct host defense reactions against e.g. microbes, parasites or fungi, changing either microbicidal properties of macrophages or facilitating initiation of the humoral immune response. Th2 cells express B cell-activating effector molecules, while Th1 cells express effector molecules that change the microbicidal properties of macrophages [5].

Cytokine secretion, subsequent proliferation and differentiation of naive T cells to effector T cells represent major components orchestrating cell-mediated immunity. Eventually, protection against various pathogens correlates with the development of an antigen-specific T cell immune response [5,6], which can be analyzed by various methods.

The Enzyme-Linked Immunosorbent Spot (ELISpot) assay provides both qualitative and quantitative information about cytokines secreted by – and thus the activation state of – antigen-specific T cells [7,8,29]. Its application as a sensitive *in vitro* immune monitoring tool will be described within the following chapters.

#### **CMV-specific CMI**

Immunological protection against CMV involves CD4<sup>+</sup>, CD8<sup>+</sup>, natural killer (NK) and natural killer T (NKT) cells [6]. A virus-specific T cell response develops within 6 weeks after primary antigen exposure and is dominated by a CD8<sup>+</sup> T cell response targeting mainly the CMV immediate early-1 (IE-1) antigen. CD4<sup>+</sup> T helper cells release cytokines to activate further cytotoxic T cells and maximize bactericidal activity of e.g. macrophages. They are also associated with long-term recovery, predominantly targeting the CMV lower matrix phosphoprotein 65 (pp65) [9–11].

In addition to the interaction of APC with antigen-specific T cells, costimulatory receptors on the surface of both T cell and APC are required to assure efficient immune protection [12]. Furthermore, NKT cells become rapidly activated and attack infected cells. To enhance their cytotoxic capacity, NKT cells release IFN-γ and IL-4 [6,13]. In addition to cytokines, NK cells secrete cytotoxic proteins and enzymes to induce either apoptosis or osmotic cell lysis [6,14]. Upregulation of inhibitory receptors by T cells, such as programmed cell death protein 1 (PD-1), can on the other hand lead to persistent virus replication. This causes a loss of function and is commonly known as T-cell exhaustion [15].

The highly variable frequency of CMV-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells correlates with varying levels of protection [9–11,17,18] and can be quantified by various methods. In immunocompetent individuals, primary CMV infection and reactivation are typically asymptomatic [6]. As a result, many people are unaware that they have been infected [16]. On the other hand, immunocompromised individuals such as transplant patients are at higher risk of symptomatic CMV infection and reactivation. To improve immune monitoring for effective CMV management, correlates of protection from CMV disease need to be identified.

### Need for improved CMV diagnostic solutions

Through its direct and indirect effects, CMV is associated with significant clinical illness, allograft loss, and mortality after transplantation as well as the leading cause of congenital infections worldwide [1,6,19].

Reported allograft nephropathy or even allograft loss illustrate the challenge of delayed-onset primary CMV disease and its impact on transplantation outcome despite antiviral prophylaxis [1]. It is also the most common non-genetic cause of childhood hearing loss and an important cause of neurodevelopmental delay, driven by non-primary maternal infection [19]. Beyond the direct clinical manifestations of CMV syndrome or tissue-invasive disease, the virus indirectly increases predisposition to allograft rejection and opportunistic infections [1].

Antiviral CMV treatment is costly, has serious side effects and decision to treat is still solely based on viral load detection. Since the strongest risk factor for CMV disease is a lack of CMV-specific immunity, CMV immunodiagnostic assays should assess a potential way to improve individualized CMV management strategies [1].

## CMV diagnosis and immunological monitoring

Viral load testing is the basis for post-transplantational diagnosis and monitoring of CMV infection and disease, as well as for patient management (e.g. decision to initiate antiviral preemptive therapy and monitoring response to therapy). The standard method of viral load testing is "quantitative nucleic acid amplification testing" (QNAT – real-time-PCR-based measurement of CMV DNAemia), which requires expensive equipment and reagents and is only in the process of being standardized [1]. Nevertheless, QNAT has better precision, broader linear range and less risk of contamination than regular PCR-based tests and is more amenable than the semi-quantitative CMV antigenemia assay [1,20].

Since T cells are crucial for the control of CMV, adjunctive immune monitoring of CMV-specific cell-mediated immunity might predict individuals at increased risk of CMV disease after transplantation and may be useful in guiding preemptive or prophylactic antiviral therapies. Interestingly, accumulating data suggests that immune monitoring in combination with viral load monitoring may be valuable to overcome major challenges in CMV management and to guide therapy decisions [1].

To meet the needs for improved CMV diagnostic solutions for clinical application, an assay should be easy-to-use, standardized, reproducible, highly sensitive, and amenable to either widely available platforms or shipping to measurement centers.

Current immune monitoring assays for CMV are based on MHC multimer staining (by FACS), intracellular staining (ICS) of cytokines (by FACS), and interferon-gamma (IFN- $\gamma$ ) release assays (IGRAs) [9,10,17,18,21–24]. Each of these assays present limitations in terms of sensitivity (ELISA), ability to measure cell functionality (Multimer staining) and standardization capability (ICS-FACS), as well as in their ability to predict CMV disease [1,25,26]. T cell-based IFN-γ ELISpot assays rely on the detection of IFN-γ-secreting CMV-reactive effector cells after stimulation of whole blood or peripheral blood mononuclear cells (PBMC) with CMV-specific antigens or peptides [1,8,28-30]. It is as yet the most sensitive assay, measures CMV-specific cell functionality at a single-cell level and presents the potentiality for standardization.

As opposed to all existing competitor products, one outstanding advantage of the CE-marked *in vitro* diagnostic IFN-γ ELISpot test T-Track<sup>®</sup> CMV is to measure the functionality of a broad range of CMV-specific effector cells, including CD8<sup>+</sup> T cells (cytotoxic T cells or CTL), CD4<sup>+</sup> T cells (T helper or Th), natural killer (NK) cells and NKT cells. The patented T-activation<sup>®</sup> technology enhances the immune stimulatory capacity of CMV immediate early-1 protein (IE-1) and of phosphoprotein 65 (pp65) proteins, reflecting more closely the uptake, processing and presentation of natural antigens, and thus resulting in a high assay sensitivity [4,28-30].

Because of the recall of a wide CMV-specific T cell repertoire (CD8<sup>+</sup> and CD4<sup>+</sup>), and of the bystander activation of CMV-reactive cells of the innate immunity (NK, NKT or NKT-like), a positive test result is expected in most CMV-seropositive individuals, independently of their HLA antigens<sup>1</sup>. Following an easy, standardized and reproducible procedure, results are available within 24 hours and can be analyzed by specialized laboratories and diagnostic departments.

<sup>1</sup> T-**Track**<sup>®</sup> **CMV** positive test results were observed in 97% CMV-seropositive healthy donors [29] and in 90% hemodialysis patients [30]. Therefore **T-Track**<sup>®</sup> **CMV** shows high sensitivity in both immunocompetent and immunocompromised individuals.

#### T-activation<sup>®</sup> Technology

The T-Track<sup>®</sup> CMV ELISpot kit is based on the *in vitro* stimulation of mononuclear cells of the peripheral blood with two immunogenic CMV-specific proteins:

- T-activated<sup>®</sup> IE-1
- T-activated<sup>®</sup> pp65

In contrast to peptides and unmodified proteins, T-activated<sup>®</sup> proteins (formulated with Lophius' patented T-activation<sup>®</sup> buffer) are processed and presented via the exogenous (MHC-II) pathway and endogenous (MHC-I) pathway (cross-presentation) by functional antigen-presenting cells (APC), thus mimicking a natural infection (Figure 1).



Figure 1 - Presentation of T-activated® antigens by APC via the exogenous (MHC-II) pathway and the endogenous (MHC-I) pathway (crosspresentation). The specific interaction of a peptide / MHC complex with a T cell receptor (TCR) on the surface of a T helper (Th) or cytotoxic T cell (CTL) results in T cell activation and secretion of IFN-γ. Therefore, T-activated<sup>®</sup> proteins result in a more efficient and HLA antigenindependent stimulation of a broad spectrum of clinically-relevant subpopulations of antigen-reactive effector cells (CD4<sup>+</sup> and CD8<sup>+</sup> T cells and bystander activation of NK and NKT-like cells), as outlined in Figure 2 [4,29].



# 10

#### Advantages of the T-activation® Technology

- Enhanced immune stimulatory capacity and immunogenicity of proteins
- Presentation by APC along MHC-II and MHC-I (cross-presentation) pathways
- Closely mimicking the natural uptake, processing and presentation of antigens
- Stimulation of a broad spectrum of clinically relevant effector cells (Th, CTL, NK, NKT-like cells)
- HLA type-independent stimulation
- Applicable for multiple immunological assays and read-out systems, including ELISpot, FACS, ELISA

#### Fields of application:

- T cell-based diagnostic solutions for viral infections like CMV, EBV, BKV
- Transplantation medicine, autoimmune diseases, cancer, ...
- Improved immunogenicity / enhanced performance of vaccines
- Enhanced assay sensitivity, e.g. for monitoring immune responses against tumor-associated antigens

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- Banas et al. "Clinical validation of T-Track<sup>®</sup> CMV to monitor CMV-specific cell-mediated immunity in kidney transplant recipients". Submitted

# T-Track<sup>®</sup> CMV ELISpot kit

The CE-marked *in vitro* diagnostic test is a highly-standardized and readyto-use ELISpot assay. The test enables highly sensitive detection of CMVspecific effector cells and measurement of CMV-specific CMI in healthy and immunosuppressed individuals. Possible immune monitoring applications of T-Track<sup>®</sup> CMV ELISpot assay are:

- Measurement of the reactivity of effector cells against CMV antigens and / or assessment of CMV-specific immune reconstitution
- Assessment and follow-up of CMV-specific immunocompetence after iatrogenic immunosuppression (immunosuppressants or T celldepleting antibodies)
- Assessment and follow-up of CMV-specific immunity under or after antiviral treatment / CMV prophylaxis
- Assistance in antiviral therapy decision-making, together with CMV virus load determination



#### Test principle and interpretation

Peripheral blood mononuclear cells (PBMC) are isolated from Li-heparinized whole blood by density gradient centrifugation, adjusted to the required cell density and seeded on an ELISpot membrane coated with IFN-γ-specific antibodies. After stimulation for 17-21 hours with two CMV-specific antigens (T-activated® immediate-early IE-1 protein and phosphoprotein pp65) and Phytohemagglutinin (PHA) as a positive control, cells are removed and secreted IFN-γ that was captured by IFN-γ-specific antibodies is detected by another enzyme-conjugated INF-γ-specific detection antibody. Following addition of a soluble substrate, an enzymatic reaction produces an insoluble colored precipitate and spots are revealed. Thereby one spot represents the footprint of a single antigen-reactive IFN-γ-producing effector cell (Figure 3).



Figure 3 – T-Track® CMV results showing exemplary findings from allogenic hematopoietic stem cell transplantation (HSCT) patients: in each case only 1 exemplary well per measurement is shown; numbers on the upper right side of each well indicate the number of detected reactive CMV-specific effector cells. The operator control verifies proper assay performance.

A negative test result or a low or decreasing CMV-CMI might indicate an increased risk potential for CMV reactivation requiring treatment.

Note: the described method allows a semi-quantitative assessment of CMV-specific immunocompetence in CMV-seropositive patients, but is not suitable for the detection of a CMV infection.

#### Model for evaluating the risk for CMV disease

T-Track<sup>®</sup> CMV test results should only be interpreted in the context of the overall clinical picture. It is advisable to carry out the T-Track<sup>®</sup> CMV ELISpot in parallel to other CMV-specific diagnostic tests (such as CMV DNAemia PCR or pp65 antigenemia) and to evaluate the results in consideration of existing symptoms.

To eventually improve assessment of the risk for CMV disease, the following model (Figure 4) illustrates a possible risk stratification of clinicallyrelevant CMV reactivation post-transplantation based on CMV-specific CMI. T cell-based control of CMV replication could be affected after immunosuppressive treatment and/or T cell-depleting therapy post-transplantation. In this model, a high and stable CMV-specific CMI indicates a reduced risk for clinical complications following CMV reactivation, whereas a low and/or decreasing CMV-specific CMI could imply an increased risk for post-transplantational CMV disease.



A patient risk stratification matrix and recommendations for therapy decisions is proposed based on viral load (VL) detection together with CMV-specific cell-mediated immunity measurement using T-Track<sup>®</sup> CMV (Figure 5). T-Track<sup>®</sup> CMV results should only be interpreted by the physician in combination with another CMV-specific diagnostic test (such as CMV DNAemia PCR or pp65 antigenemia) and in the context of the overall clinical picture.



Observation of a decreasing or low viral load with an increasing or high CMV-specific CMI might indicate a low risk for CMV complication (Figure 5, upper left quadrant). Antiviral therapy might not be necessary or might be discontinued. Adjustment of immunosuppressive treatment might not be necessary. In this case, occasional viral load monitoring in parallel to T-Track<sup>®</sup> CMV measurement would be recommended.

Observation of an increasing viral load and simultaneous low or decreasing CMV-specific CMI might indicate a high risk for CMV reactivation and related clinical complications (Figure 5, lower right quadrant). Frequent monitoring of viral load in parallel to T-Track<sup>®</sup> CMV would be recommended. Immuno-suppressive treatment might be adjusted. Decision to start or continue anti-viral therapy is per clinician's assessment based on test results (viral load and T-Track<sup>®</sup> CMV) and the patient's overall clinical picture.

In case of intermediate or borderline viral load but stable CMV-CMI, frequent monitoring in parallel to T-Track<sup>®</sup> CMV might help stratify the risk of future CMV complications and guide the clinician in the decision to initiate, delay or discontinue antiviral therapy.

## Technical and clinical validation of T-Track<sup>®</sup> CMV

T-Track<sup>®</sup> CMV assay development and performance characteristics in healthy individuals

"An optimized IFN-y ELISpot assay for the sensitive and standardized monitoring of CMV"

In this study, performance characteristics of T-Track<sup>®</sup> CMV IFN-γ ELISpot assay for the monitoring of CMV-specific CMI were validated in healthy individuals. Results show that stimulation with T-activated<sup>®</sup> proteins results in improved assay sensitvity and a HLA antigen-independent application. T-Track<sup>®</sup> CMV demonstrates robust performance in terms of assay variability (≤22%), precision and linearity.

 Barabas S., Spindler T., Kiener R., Tonar C., Lugner T., Batzilla J., Bendfeldt H., Rascle A., Asbach B., Wagner R., Deml L. (2017). An optimized IFN-γ ELISpot assay for the sensitive and standardized monitoring of CMV protein-reactive effector cells of cell-mediated immunity. BMC Immunol. 18(1):14

#### Validation of T-Track® CMV in hemodialysis patients

"T-Track<sup>®</sup> CMV is a reliable and sensitive assay for the detection of CMV-CMI in patients eligible for kidney transplantation"

This multicenter observational study aimed to determine Lophius Biosciences' T-Track® CMV sensitivity in hemodialysis patients and compare it to that of competitor products. T-Track® CMV showed a 90% sensitivity in CMV-seropositive patients, compared to 73% for QuantiFERON®-CMV and 77% for six selected iTAg<sup>™</sup> MHC Tetramers.

Banas B., Böger C.A., Lückhoff G., Krüger B., Barabas S., Batzilla J., Schemmerer M., Köstler J., Bendfeldt H., Rascle A., Wagner R., Deml L., Leicht J., Krämer B.K. (2017). Validation of T-Track<sup>®</sup> CMV to assess the functionality of cytomegalovirus-reactive cell-mediated immunity in hemodialysis patients. BMC Immunol. 18(1):15

# CMValue study: Clinical validation of T-Track<sup>®</sup> CMV in kidney transplant recipients

"T-Track CMV is a sensitive immune-monitoring tool in immunosuppressed renal transplant recipients"

This multicenter observational study aimed to validate the suitability of Lophius Biosciences' T-Track® CMV to assess the functionality of CMVspecific cell-mediated immunity in immunocompromised patients following kidney transplantation. T-Track® CMV showed high sensitivity prior to transplantation (before onset of immunosuppressive therapy) and over 6 months post-transplantation (under immunosuppressive therapy).

 Banas et al. Clinical validation of a novel ELISpot-based in vitro diagnostic assay to monitor CMV-specific cell-mediated immunity in kidney transplant recipients. Submitted

#### AlloProtectCMV study: Clinical validation of Lophius Biosciences' T-Track<sup>®</sup> CMV in allo-HSCT recipients

*"Identification of a possible prognostic marker for higher risk of recurrent CMV reactivation"* 

This on-going multicenter observational study in a cohort of allogenic hematopoietic stem cell transplantation (allo-HSCT) recipients aims to validate the suitability of Lophius' T-Track<sup>®</sup> CMV assay to assess the functionality of CMV-reactive effector cells and to predict recurrent CMV reactivation in allo-HSCT patients.

# CMV-CMI study: Cell-mediated immunity for prevention of CMV disease in SOT patients

"T-Track<sup>®</sup> CMV as a decision guidance for the duration of antiviral prophylaxis following SOT"

This on-going interventional randomized controlled trial in high-risk solid-organ transplant recipients aims to adapt the duration of antiviral prophylaxis according to the result of Lophius' T-Track<sup>®</sup> CMV assay. The two main end-points of the study are clinical outcome and health-economic benefit.

#### Validation of T-Track<sup>®</sup> CMV during pregnancy

"T-Track<sup>®</sup> CMV can detect impaired CMV-specific immunity during and after pregnancy"

 Reuschel E., Barabas S., Zeman F., Bendfeldt H., Rascle A., Deml L., Seelbach-Goebel B. (2017). Functional impairment of CMV-reactive cellular immunity during pregnancy. J Med Virol. 89(2):324-331

Updated international consensus guidelines on the management of cytomegalovirus in solid-organ transplantation - Kotton et al. (2013). Transplantation 96: 333-360.

CE-marked T-Track<sup>®</sup> CMV as a commercially-available immune monitoring assay to predict CMV disease.

### A solution to evaluate the risk for CMV disease

As presented within this white paper, cell-mediated immunity is critical for the control of CMV infection and reactivation. Immune monitoring of functional CMV-specific effector cells could predict individuals at increased risk of CMV-related complications.

With the highly-sensitive, reliable and standardized immune monitoring tool T-Track<sup>®</sup> CMV we offer an easy-to-use diagnostic IFN-γ ELISpot kit, measuring the functionality of clinically-relevant CMV-reactive effector cells. In association with viral load detection, the test might allow a better evaluation of the risk for CMV disease in immunocompromised patients and may help guide clinicians in their treatment decision-making, for an improved and individualized patient management.

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#### About Lophius Biosciences GmbH

Lophius Biosciences is a privately-held German biotechnology company focusing on the development and marketing of innovative immune diagnostic systems to improve therapy control and personalized treatment of patients in the area of transplantation, infectious and autoimmune diseases.

The company's developments are based on its expertise in cell-mediated immunity as well as on its proprietary T-activation<sup>®</sup> and Reverse T Cell Technology platforms. Whereas the T-activation<sup>®</sup> technology platform allows an efficient stimulation of a broad spectrum of clinically-relevant immune effector cells to accurately measure the cell-mediated immunity, the Reverse T Cell Technology platform can distinguish between active and memory T cells to develop innovative diagnostics.

With its T-Track<sup>®</sup> CMV leading product, based on T-activation<sup>®</sup> technology, Lophius offers a highly sensitive, reliable and standardized CE-marked in vitro diagnostic solution to measure the functionality of CMV-specific cellmediated immunity. T-Track<sup>®</sup> CMV assists clinicians in the risk stratification of CMV disease in immunocompromised patients, toward an improved and individualized patient management.

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