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Phenotype and functionality of pathogen specific T cells in chronic infections and implications for novel diagnostic and therapeutic approaches

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Abbreviations

APC	Allophycocyanin
APC	Antigen presenting cells
ART	Antiretroviral therapy
A-TB	Active tuberculosis
AUC	Area under the curve
B	Blocking
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette-Guérin
BFA	Brefeldin A
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
CFP-10	Culture filtrate antigen 10
CFSE	Carboxyfluorescein succinimidyl ester
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T-lymphocyte antigen 4 (CD152)
Cy-(5.5/7)	Cyanine-(5.5/7)
DMSO	Dimethy lsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot (assay)
ESAT-6	Early secretory antigenic target 6
FACS	Fluorescence activated cell scanning

FCS	Fetal calf serum
FI	Fold increase
FITC	Fluorescein-Isothiocyanate
FSC	Forward scatter
G	G-force
Gln	Glutamin
H1N1	Influenza-A-Virus H1N1 (A/H1N1)
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HSA	Human serum albumine
IFN	Interferon
Ig	Immunoglobulin
IGRA	Interferon gamma release assay
IL	Interleukin
INH	Isonicotinic acid hydracide
IQR	Interquartile range
LTB	Latent tuberculosis
M.	Mycobacterium
MDR-TB	Multidrug-resistant tuberculosis
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein-1

PD-L	Programmed cell death protein ligand
PdmH1N1	Pandemic H1N1
PE	Phycoerythrin
Pen/Strep	Penicillin/Streptomycin
PerCP	Peridinin-Chlorophyll-Protein
PFA	Paraformaldehyde
PPD	Purified protein derivate
QFT-G	QuantiFERON Tb-Gold
ROC	Receiver operating characteristic
RPM	Revolutions per minute
SEB	Staphylococcal enterotoxin B
SI	Stimulation index
SSC	Side scatter
TIM-3	T cell immunoglobulin mucin-3
TNFα	Tumor necrosis factor alpha
T-TB	Treated tuberculosis
WHO	World Health Organization
α	alpha/anti
β	beta
γ	gamma

Summary

Cellular immunity is pivotal for the control of pathogens. An impairment of cellular immunity, as observed after HIV infection or upon immunosuppressive medication after organ transplantation, increases the risk of infectious complications. This risk seems to depend on both the quantity and functional quality of the pathogen specific immune response.

Current diagnostic approaches to monitor pathogen specific T cell responses like commercially available interferon-γ release based ELISA and Elispot assays are able to quantify immune responses but do not assess T cell phenotype and functionality.

This thesis therefore concentrated on evaluation of functional and phenotypical changes in pathogen specific T cell responses during different stages of chronic infections. Another aim was to use these characteristics as biomarkers for monitoring disease activity.

Our studies on Cytomegalovirus (CMV) infection in kidney transplant recipients revealed that T cell functionality during CMV primary infection and reactivation is impaired. This is reflected by a decreased ability to produce an array of mainly proinflammatory cytokines and a constrained proliferative capacity. We could show that the impaired functionality is linked to the expression of the inhibitory T cell receptors PD-1, TIM-3 and CTLA-4 and at least partly reversible via inhibitory pathway blockade. Furthermore, our data indicate that expression analysis of inhibitory receptors on CMV specific T cells might be able to predict reactivation and relapse. Interestingly, we found that the parallel loss of the co-stimulatory receptors CD27 and CD28 on CD4 T cells occurs rapidly after primary CMV infection and is closely linked to CMV serostatus. In the future analysis of $CD28^{-}CD27^{-}$ CD4 T cells, especially in combination with anergy markers, might be used as a method for the rapid, stimulation independent assessment of CMV specific immunity.

Our studies on tuberculosis infection revealed that both functional and phenotypical analysis of *M. tuberculosis* specific T cell responses allow the diagnosis of patients with active infection with high specificity and reasonable sensitivity. The highest diagnostic power was achieved by combined analysis of the T cell receptors CD27 and CTLA-4.

The study on HIV patients in Tanzania and Germany, which was done in collaboration with Dr. Schütz, provided basic insight on the impact of regular exogenous or endogenous antigen rechallenge on the maintenance of pathogen specific T cell responses in HIV-immunodeficiency.

The results of this thesis clarify that a combination of functional and phenotypical markers for characterisation of pathogen specific T cells allows a more precise estimate of disease activity in vivo. In the future, this might be used for improved immune monitoring and contribute to an individualised guidance of therapy.

Zusammenfassung

Die zelluläre Immunantwort spielt eine essentielle Rolle bei der Kontrolle von Krankheitserregern. Eine Schwächung der zellulären Immunität, wie sie nach HIV-Infektion oder unter immunsuppressiver Medikation im Rahmen der Organtransplantation beobachtet werden kann, geht mit einem erhöhten Risiko für infektiöse Komplikationen einher. Dieses scheint sowohl von der Quantität als auch von der Qualität der Erreger-spezifischen Immunantwort abzuhängen. Gebräuchliche diagnostische Verfahren zum Nachweis Erreger-spezifischer T-Zellantworten, wie die auf Interferon-γ Freisetzung basierenden ELISA und Elispot Methoden, erlauben eine quantitative Analyse der spezifischen Immunantwort, liefern jedoch keine Informationen über deren Phänotyp und Funktionalität.

Daher lag der Fokus dieser Doktorarbeit auf der detaillierten Charakterisierung grundlegender phänotypischer und funktioneller Veränderungen in der Erreger-spezifischen Immunantwort während unterschiedlicher Stadien chronischer Infektionen. Ein weiteres Ziel bestand darin, diese Charakteristika als neue Biomarker zum Monitoring der Erregeraktivität zu evaluieren.

Unsere Studien zur Charakterisierung der spezifischen Immunantwort gegen das Cytomegalievirus (CMV) bei nierentransplantierten Patienten zeigten, dass die T-Zellfunktionalität während CMV-Primärinfektion und Reaktivierung beeinträchtigt ist. Dies spiegelt sich in einer reduzierten Produktion von überwiegend proinflammatorischen Zytokinen und einer eingeschränkten proliferativen Fähigkeit wider. Wir konnten einen Zusammenhang zwischen der erhöhten Expression der inhibitorischen T-Zellrezeptoren PD-1, TIM-3 und CTLA-4 und einer verminderten Funktionalität nachweisen, welche durch deren Blockade zumindest teilweise wiederhergestellt werden konnte. Des Weiteren legen unsere Daten nahe, dass die Expressionsanalyse von inhibitorischen T-Zellrezeptoren auf CMV spezifischen T-Zellen Reaktivierungsereignisse und Rezidive vorhersagen kann. Interessanterweise zeigte sich, dass ein paralleler Verlust der beiden kostimulatorischen Rezeptoren CD27 und CD28 auf CD4 T-Zellen zeitnah nach CMV-Primärinfektion auftritt und eng mit dem CMV-Serostatus korreliert. Die Analyse der $CD27^{-}CD28^{-}$ CD4 T-Zellen, insbesondere in Kombination mit Anergiemarkern könnte zukünftig als Methode zur

schnellen stimulationsunabhängigen Bewertung der CMV-assoziierten Immunität eingesetzt werden.

Unsere Studien zur Tuberkuloseinfektion zeigten, dass sowohl die Analyse von funktionellen als auch phänotypischen Veränderungen auf *M. tuberculosis* spezifischen CD4 T-Zellen dazu geeignet ist, eine aktive Tuberkulose mit hoher Spezifität und guter Sensitivität zu diagnostizieren. Bei kombinierter Analyse der beiden T-Zellrezeptoren CD27 und CTLA-4 war die diagnostische Aussagekraft am höchsten.

Die in Kooperation mit Dr. Schütz in Tansania und Deutschland durchgeführte Studie bei HIV-infizierten Patienten lieferte grundlegende Erkenntnisse zur Bedeutung von wiederkehrendem endogenem und exogenem Antigenkontakt für das Aufrechterhalten Erreger-spezifischer Immunantworten im Kontext der Immundefizienz nach HIV-Infektion.

Die Ergebnisse der hier vorliegenden Arbeit verdeutlichen, dass eine Kombination phänotypischer und funktioneller Marker zur Analyse pathogenspezifischer T-Zellen eine präzisere Abschätzung der Erregeraktivität in vivo erlaubt. Dies könnte zukünftig zur verbesserten Immundiagnostik von chronischen Infektionen genutzt werden und einen wichtigen Beitrag bei der individuellen Therapiesteuerung leisten.

1 Introduction

1.1 The human immune system

The human immune system functions to protect the organism from invading pathogens and to eliminate degenerated and carcinogenous cells. It consist of the innate and the adaptive immune system, which work together to keep up the integrity of the body.

1.1.1 The innate immune system

The first line of defense consists of physical (skin and mucosa), chemical (pH value of skin and gastrointestinal passage, antimicrobial enzymes and peptides) and microbacterial (symbiotic bacteria which compete with pathogenous bacteria) barriers, which prevent the majority of pathogens from entering tissues and the blood system. The few pathogens which overcome these barriers are attacked by the effector cells of the innate immune system, predominantly comprising macrophages, granulocytes and NK cells. They are assisted by the humoral parts of the innate immune system such as the complement system, acute phase proteins, lectins and cytokines. Pathogens are detected via structures foreign to the body, such as peptidoglycan and mannose, or infected cells are unspecifically recognised by the lack of major histocompatibility complex (MHC) molecules, which are expressed on all healthy cells of the body. Long-term control of pathogen cannot be achieved by the innate immune system alone, and therefore requires induction of adaptive immunity.

1.1.2 The adaptive immune system

The adaptive immune system comprises T and B lymphocytes, as well as antibody producing plasma cells, which are specifically induced towards individual pathogens. Induction and expansion of these cells occurs in secondary lymphoid tissues, where B- and T cells encounter foreign antigen presented by antigen-presenting cells (APC). The cells of the adaptive immune system carry antigen specific receptors called T cell receptor (TCR) and B cell receptor (BCR). During the development of the immune system a repertoire of approximately 10^{11} T and B lymphocytes evolves, each carrying their individual antigen specific BCR and TCR. Many of the immature lymphocytes recognise self-antigens but are eliminated during the maturation process in the thymus (T cells) and the bone marrow (B cells). A few autoreactive cells may escape this negative selection. These cells are kept in

check by the mechanisms of peripheral tolerance to prevent the onset of autoimmune diseases. After maturation in the bone marrow, naive B cells migrate into secondary lymphoid tissues. Upon antigen presentation and activation by T helper cells, they differentiate into antibody producing plasma cells and memory B cells. During maturation in the thymus, T cells differentiate into T helper (CD4) T cells, cytotoxic (CD8) T cells and regulatory (CD4 $CD25^{high}$ $CD127^{low}$) T cells. As with B cells, these naïve T cells home to secondary lymphoid tissues for potential recognition of foreign antigen. Whereas the main function of CD8 T cells is killing of infected or abnormal cells, CD4 T cells have a broad array of functions, such as activating CD8 T cells and B cells and recruiting leukocytes to the site of infection. Regulatory T cells are a subpopulation of CD4 T cells, which down-regulate immune responses and play an important role in preventing immune pathology and autoimmunity. CD8 T cells predominantly recognize 8-10 amino acid long peptides of cytosolic origin, which originate from intracellular antigens and are presented via MHC class I molecules on APC or infected somatic cells. CD4 T cells predominantly recognize 13-17 amino acid long antigen-peptides of extracellular origin, which are presented by APC in an MHC class II restricted manner. Yet, the mechanisms of cross-presentation and autophagy enable the presentation of extracellular antigens in a MHC class I restricted manner and cytosolic antigens in a MHC class II restricted manner, respectively (JANEWAY et al., 2007).

1.2 Antigen recognition, maturation and differentiation of T cells

Naïve T cells remain functionally inactive until contact with their specific antigen epitope, which needs to be presented via MHC molecules on professional APC in the lymphoid organs in the presence of co-stimulatory receptors (Figure 1). Antigen mediated activation of naïve T cells induces proliferation and differentiation into many effector and a few central memory T cells. After antigen clearance further T cell proliferation is dampened by inhibitory T cell receptor signalling, and inhibitory cytokines secreted by regulatory T cells. Together, these signals mediate apoptosis of excessive effector cells and quiescence of memory T cells (Figure 1).

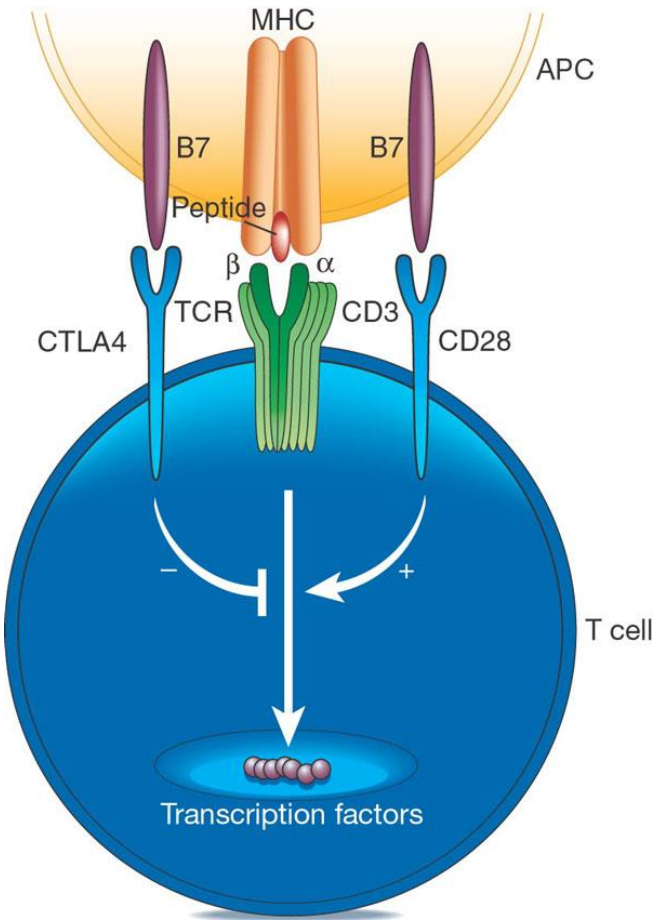
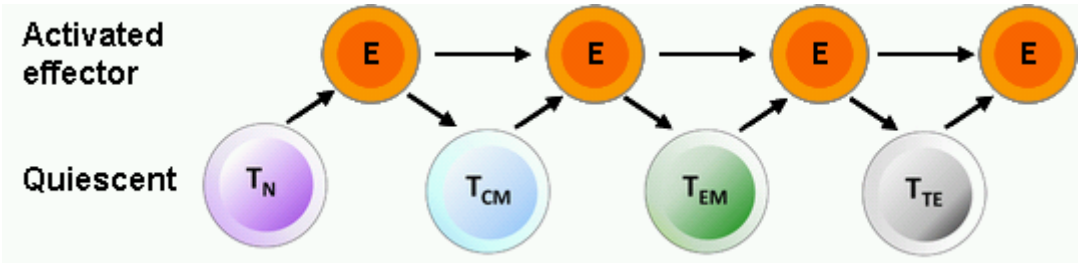


Figure 1: Antigen presentation. Antigens are presented by antigen presenting cells (APC) as small peptides in the context of MHC class I and class II molecules to antigen specific CD8 or CD4 T cells, respectively. Co-stimulatory (via CD28) and co-inhibitory signals (via CTLA-4) lead to T cell activation and inhibiton, respectively. Reprinted by permission from Macmillan Publishers Ltd: Nature, (APPELBAUM, 2001)

Continuous antigen specific stimulation of T cells leads to further differentiation into effector memory T cells and finally into terminally differentiated effector memory T cells (T_{TE}). In contrast to naïve T cells, activation of antigen experienced memory T cells can occur independent of co-stimulation and is mediated not only by professional APC in secondary lymphoid tissues but also by antigen presenting somatic cells (JANEWAY et al., 2007). Each of this differentiation stages is marked by the expression of specific maturation markers (Figure 2). Typical maturation markers comprise functionally relevant co-receptors needed for T cell activation (e.g. CD28, CD27) and T cell homeostasis (e.g. CD127) as well as chemokine receptors needed for lymph-node homing (e.g. CCR7 and CD62L) and two isoforms of CD45, CD45RA and CD45R0, which facilitate T cell activation. Whereas naïve T cells express all T cell activation markers and chemokine receptors, these molecules are lost in a stepwise fashion with increasing differentiation (Figure 2).



CD45RA	+	-	-	+
CCR7	+	+	-	-
CD62L	+	+	-	-
CD28	+	+	-/+	-
CD127	+	+	+/-	-
CD27	+	+	+/-	-

Figure 2: Memory T cell differentiation. Expression of typical markers of differentiation in naïve T cells (T_N), central memory T cells (T_{CM}), effector memory T cells (T_{EM}) and terminally differentiated effector memory T cells (T_{TE}). Figure adapted from (MAHNKE et al., 2013).

1.3 Persistent pathogens and their interplay with the immune system

Whereas most infections are efficiently cleared after primary infection by a combined effort of innate and adaptive immunity, a few pathogens are able to evade clearance and establish livelong persistence in the host. Common pathogenic strategies to establish persistence are concealment in immune privileged compartments (e.g. neurons), interference with the host immune response, and the establishment of phases without metabolic activity and replication, a state termed latency.

Cytomegalovirus (CMV) and *Mycobacterium tuberculosis* (*M. tuberculosis*) are two clinically relevant persistent pathogens that constantly challenge the adaptive T cellular immune response of the host. For both pathogens, the maintenance of sufficient amounts of antigen-specific T cells is crucial to control infection. Whereas a fair amount of data on quantitative analysis of pathogen specific T cell responses exists, there is little knowledge on the phenotype and functionality of these cells in diverse clinical situations. Likewise, the dynamics of antigen-specific T cell characteristics upon chronic antigen stimulation in relation to rapid antigen clearance is poorly characterised and may be studied by comparing

patients with persistent infection with individuals after vaccination, where time, dosing and duration of antigen challenge are clearly defined. This might help to define phenotypical and functional differences between chronic and self-limiting infections. If different states of pathogen replication and disease activity could be linked to function and phenotype of the pathogen specific immune response, this might be of importance to improve current diagnostic strategies.

To date it is still not fully understood what drives the differences in frequency and maintenance of antigen specific immune responses against different chronic pathogens. Patients with primary HIV infection suffer from a massive initial loss in CD4 T cells in general, and it is unclear how restoration of antigen specific CD4 T cell responses that re-emerge thereafter is driven. In stem cell transplant recipients it has been observed that the re-emerging immune responses after severe lymphopenia do not necessarily resemble that of the stem cell source (GANDHI et al., 2003). It is therefore of great interest, to elucidate if restoration of antigen specific T cell responses is predominantly driven by equal expansion of precursor T cells or if it is driven by antigen prevalence. As the prevalence of *M. tuberculosis* is high in sub-saharian Africa but low throughout Europe, analysis of *M. tuberculosis* specific immunity in HIV infected patients might be a means to elucidate the influence of antigen prevalence on restoration of antigen specific responses.

1.3.1 Cytomegalovirus infection

Cytomegalovirus (CMV), also known as human herpesvirus 5 (HHV-5) contains a double stranded DNA of 230.000 basepairs and belongs to the herpesviridae family (subfamily betaherpesviridae). Like all herpesviridae, CMV establishes live long latency upon primary infection. In the western world the seroprevalence ranges from 30 to 70 percent and increases with age (CANNON et al., 2010; HEALTH, 2010). CMV infection occurs most commonly in the first three years of life via breast feeding and smear infections, and in young adults via sexual contacts. Primary infection in immunocompetent individuals is usually asymptomatic with flu-like symptoms accompanied in some cases by lymph node swelling and lymphopenia. CMV is transmitted via all cell-containing bodily fluids, as the virus is strictly cell associated (BOWDEN et al., 1995). CMV infects nearly all cell types but live long persistence is only established in leukocytes, with monocytes as the predominant reservoir. After primary infection, virus is shedded in high loads in urine and saliva with

prolonged shedding being observed in congenitally and perinatally infected children. Sporadic reactivation is self-limited and only minimal amounts of virus are shedded in this situation (ROSS, BOPPANA, 2005). Whereas CMV is effectively controlled by the immune system in immunocompetent individuals it causes serious infectious complications in congenitally infected infants and in immunocompromised individuals. CMV infection is the most common congenital infection (0.5-1% of infants infected) and primary CMV infection of the mother during pregnancy results in 30 to 40 percent congenital infection of the fetus. Eight to ten percent of infected infants are born with severe mental retardation. Another 10 to 15 percent of children appear asymptomatic at birth but develop long-term damages, in particular hearing loss years after. CMV transmission from seropositive mothers has a significantly lower incidence and a reduced risk for fetal damage, compared to primary infection during pregnancy. Perinatal and postnatal infections are usually asymptomatic except in premature or immunocompromised infants (FOWLER et al., 1992; ROSS, BOPPANA, 2005; STAGNO et al., 1986).

1.3.1.1 Cytomegalovirus infection in transplant recipients

Infection with human CMV is one of the most common infectious complications after organ transplantation. Clinical CMV disease is marked by fever, pneumonia, hepatitis, gastrointestinal complications, encephalitis or retinitis. Furthermore, CMV infection negatively affects graft function in the long-term (BRITT, ALFORD, 1996; FISHMAN, RUBIN, 1998). Unlike immunocompetent individuals, where viral replication is largely contained by immunological control mechanisms, immunosuppressive drug treatment after transplantation may disrupt the balance between immunological control and viral replication (SESTER et al., 2001; SESTER et al., 2005). The overall risk of CMV disease post-transplant is mainly determined by the donor and recipient serostatus, the type of organ transplanted and the dosing of immunosuppressive drugs (LOWANCE et al., 1999; SMILEY et al., 1985; SPEICH, VAN DER BIJ, 2001). CMV seronegative transplant recipients of a seropositive donor are at particularly increased risk, due to the following reasons. Firstly, the lack of a pre-existing CMV specific immunity enables a rapid, unlimited replication of the virus until a protective antiviral response is generated. Secondly, the induction of a protective antiviral immune response is impaired in the presence of immunosuppressive drugs following transplantation. Finally, higher initial viral loads may be transferred via the transplanted

organ, than in natural infection. CMV seropositive recipients of a seropositive or seronegative organ are at moderate risk for CMV disease post-transplant. In this situation, a pre-existing protective CMV specific immunity may be quantitatively and functionally affected by the type and dosage of immunosuppressive drugs after transplantation (KOTTON et al., 2013b). The use of T cell depleting antibodies for induction therapy further increases the risk for CMV disease in this patient cohort post-transplant (ISSA, FISHMAN, 2009).

Minimisation of CMV infectious complications after transplantation implies the need for preventive and therapeutic strategies adapted to the individual risk profile of a patient. These measures include either antiviral prophylaxis for the first 3 to 6 month post-transplant or pre-emptive therapy, where antiviral therapy is guided by regular monitoring of viral load or antigenemia. Both approaches have led to a substantial decrease in CMV disease in the last decade (KOTTON et al., 2013b). Antiviral prophylaxis is strongly recommended for CMV seronegative recipients of a seropositive organ, as it reduces the risk of transplant transmitted CMV primary infection or delays primary infection to a later time point post-tranplant, where immunosuppression is already reduced. In seropositive transplant recipients with pre-existing CMV specific immunity, pre-emptive therapy is a viable alternative to prophylaxis. The advantages of pre-emptive therapy are reductions in treatment costs and side effects of medication, yet one has to take into account the need of close monitoring scheme and the associated diagnostic costs (KOTTON et al., 2013b). An additional disadvantage of universal prophylaxis is the occurrence of late onset disease after completion of antiviral prophylaxis (KOTTON et al., 2013b). A possible but more costly solution to prevent occurrence of late onset disease would be a hybrid approach with prolonged close viral load or antigenemia monitoring after completion of prophylaxis. Yet, one general methodological limitation of viral load and antigenemia monitoring, is that it measures viral reactivation and hence cannot predict it. Whereas this is sufficient to prevent CMV disease it cannot prevent asymptomatic infections, which have been shown to have a detrimental impact on graft survival (SAGEDAL et al., 2004).

An approach to more specifically identify patients at risk for infectious complications may include monitoring of CMV specific immunity. Previous work by our group suggests, that complementary monitoring of the individual CMV specific immunity in a pre-emptive setting might help to identify patients with CMV infection and/or disease and hence reduce a

negative impact of CMV on graft survival (SESTER et al., 2001; SESTER et al., 2005; SESTER et al., 2008b). Furthermore, the assessment of a patient´s CMV specific immunity after completion of prophylaxis might help to adjust further monitoring and treatment according to the individual immunocompetence (MANUEL et al., 2013).

1.3.2 *Mycobacterium tuberculosis* infection

Infection with *M. tuberculosis* is a leading cause of morbidity and mortality worldwide, accounting for nearly 1.3 million deaths annually and ranks second among deaths attributed to infectious diseases (WHO, 2013). Tuberculosis (TB) mortality is highest in developing countries, as malnutrition and HIV coinfection are major risk factors for active *M. tuberculosis* disease and sufficient medication is often not available (WHO, 2013). The primary route of infection is through bacteria containing droplets, which enter the lungs via the upper airways. *M. tuberculosis* resides and replicates primarily in macrophages. These cells are effective in targeting and killing microbes, but are reprogrammed by *M. tuberculosis* (HOUBEN et al., 2006). Upon uptake in phagosomes, *M. tuberculosis* halts phagosome maturation and uses them as their primary reservoir. After primary infection, *M. tuberculosis* is typically contained by an array of immune cells, consisting of macrophages, fibroblasts, B- and T lymphocytes. This formation of cells is called granuloma and prevents dissemination of bacteria. Inside the granuloma, *M. tuberculosis* can enter a state of dormancy, which allows persistence with little or no replication over years and decades. Reactivation of *M. tuberculosis* occurs most likely in the setting of immunosuppression, caused by a destabilization of granulomas. As CD4 T lymphocytes play a crucial role in sustaining granuloma structure and HIV leads to massive CD4 T cell depletion, HIV co-infection is a major promoter of *M. tuberculosis* reactivation, making HIV-TB co-infection one of the biggest challenges to global health (SESTER et al., 2010; WHO, 2013). To make matters worse, multidrug resistant tuberculosis is on the rise in many regions worldwide, mostly due to non-adherence to the elaborate and costly treatment regimens (WHO, 2013). Because of the slow doubling time and a robust metabolism, recommended treatment comprises 6-9 month of therapy with a combination of four different antibiotics. Slow doubling time also impedes diagnostics, as culture of *M. tuberculosis*, which is still regarded the gold standard, can take up to six weeks. Sputum microscopy or PCR are more rapid but less sensitive and rely on patients' ability to produce potentially infected sputum. Therefore, these approaches are

not suited to diagnose novel still asymptomatic infections or screen for latent infections with *M. tuberculosis* (PAI, O'BRIEN, 2008). Immunological assays are indirect methods commonly used for screening of a latent infection with *M. tuberculosis*. The classical immunological assay to diagnose latent tuberculosis infection is the tuberculin skin test, which is nowadays complemented or replaced by blood-based interferon gamma release assays (IGRAs). Both skin test and IGRAs detect an immune response towards mycobacterial antigens and hence provide indirect evidence of prior contact with *M. tuberculosis* by measuring a local inflammatory reaction (skin test) or T cellular IFNγ production (IGRAs) after stimulation with *M. tuberculosis* specific antigens (PAI, O'BRIEN, 2008).

1.4 Principles of assays to determine antigen specific T cells

Antigen specific immune responses can be either assessed serologically by measuring antibodies via ELISA technique, or by assessment of antigen specific T cells. Because antibody detection via ELISA is simple and inexpensive, it is commonly used to assess the infection status with a particular pathogen. Assays for assessment of antigen specific T cells are more laborious but allow more precise determination of antigen specific immunity even in situations where serology might be confounded by passive transfer of antibodies such as in neonates or in patients after plasma transfusion. Furthermore, in contrast to determination of specific antibodies, cellular immunity has been shown to correlate more closely with disease activity in numerous infections, especially when combined with detailed functional and phenotypical analyses of specific T cells (D'SOUZA et al., 2007; DAY et al., 2006; KASPROWICZ et al., 2008; KAUFMANN et al., 2007; PENG et al., 2008; PETROVAS et al., 2006; RADZIEWICZ et al., 2007; SCHMIDT et al., 2014; SCHMIDT et al., 2012a; SESTER et al., 2002b; SESTER et al., 2011b; SESTER et al., 2008b; TRAUTMANN et al., 2006; URBANI et al., 2008).

Various assay principles exist to quantify and characterize antigen specific T cells. Most rely on 6-20h stimulation of whole blood or isolated PBMC with either whole antigen preparations or isolated peptides. Stimulation leads to induction of cytokines and cell activation markers on antigen specific T cells. Cytokines are secreted and can be analysed in the supernatant using ELISA formats (CHANG et al., 1984; TROYE-BLOMBERG et al., 1985). Whereas classical ELISAs cannot assign cytokine production to individual cells, this can be achieved by the ELISPOT technique (CZERKINSKY et al., 1988). In this assay, secreted

cytokines are bound locally via cytokine specific antibodies, which are pre-bound on culture plates. After washing and staining with a secondary antibody, antigen specific cells can be detected as distinct spots in the culture plate. An alternative method to quantify individual antigen specific T cells is intracellular cytokine analyses in combination with flow cytometry (SANDER et al., 1991). In this assay, cytokines induced in response to antigen specific stimulation are accumulated intracellularly by adding an inhibitor of cellular transport (e.g. Brefeldin A) during the stimulation period. After fixation and permeabilisation, cytokines are stained intracellularly, which enables subsequent flow cytometric analyses of specifically activated T cells. An additional advantage of this method is the possibility to co-stain several cytokines and/or additional phenotypical markers to further characterize antigen specific T cells (ELSON et al., 1995; JUNG et al., 1993; SANDER et al., 1991). Another approach to analyse antigen specific T cells is the use of peptide-HLA-multimers (ALTMAN et al., 1996; BROOIMANS et al., 2008; GRATAMA et al., 2001). Multimers are high affinity peptide-HLA complexes, which are covalently bound to a biotin backbone. Due to increased avidity, between 4 and 10 peptide-HLA complexes are bound to a biotin backbone. These multimers can be used as specific reagents to directly bind T cells with the appropriately matching T cell receptor, thus allowing stimulation independent analysis of antigen specific T cells.

1.5 Antigen specific T cell assays as diagnostic tools to detect and monitor CMV infection periods

As outlined above, monitoring of cellular immunity towards CMV has the potential to analyse individual immunocompetence in transplant recipients which may help to minimize infectious complications after transplantation (KOTTON et al., 2013a). Currently available assays to analyse CMV specific T cells rely on a 6-20h stimulation period of blood cells with specific antigens, where cytokines such as IFNγ, TNFα or IL-2 are induced in an antigen-specific manner. As describe above, these cytokines may be detected using ELISA-based assays, ELISPOT-based assays or flow cytometric intracellular staining (FISHMAN, RUBIN, 1998; KOTTON et al., 2013a). A large body of evidence exists that a quantitative decrease of specific immunity is associated with symptomatic CMV infection (KOTTON et al., 2013a; SESTER et al., 2001; WALKER et al., 2007; WESTALL et al., 2008). However, these merely quantitative approaches are not suited to predict asymptomatic episodes of viral reactivation. Whereas ELISA and ELISPOT are methodically limited to quantify stimulation

induced cytokine responses and cannot further characterise T cells in more detail, flow cytometric approaches are able to measure antigen specific T cells via multiple activation marker and cytokine co-expression and to characterise them in detail with phenotypical T cellular markers (JUNG et al., 1993; SANDER et al., 1991).

1.5.1 Phenotypical and functional characterisation of pathogen specific immune responses

As CMV is a livelong persistent pathogen, the interplay between CMV and the immune system is diverse. Despite potent immune escape mechanisms such as down-regulation of MHC molecules on infected cells (MOCARSKI, 2004), T cell immunity against CMV is among the strongest responses directed against a single pathogen and can account for up to 40 percent of the total T cellular response (SESTER et al., 2002b). In addition, CMV seems to modulate T cell responses, as the CMV specific T cell pool consists predominantly of terminally differentiated effector T cells with distinct features of immune senescence. One prominent feature of terminal differentiation is a reduction in telomere length (FLETCHER et al., 2005; VAN DE BERG et al., 2010). Another typical feature of terminal differentiated T cells is the loss in the expression of CCR7, CD27, CD28, CD127, and the re-expression of CD45RA (DERHOVANESSIAN et al., 2009; DERHOVANESSIAN et al., 2011; VAN DE BERG et al., 2008). This phenotype is observed on a majority of CMV specific T cells irrespective of reactivation episodes or CMV associated clinical symptoms. Interestingly, terminally differentiated T cells and CMV seropositivity have been attributed to a so called immune risk phenotype in the elderly, with increased overall susceptibility to infections and a decreased life expectancy (DERHOVANESSIAN et al., 2009). However, a detailed functional and phenotypical analysis of CMV specific immunity as correlate of protection from CMV replication and disease in the setting of organ transplantation had not been performed before.

Studies on immunity towards a variety of other persistent pathogens have shown that chronic infections are associated with alterations in T cell functionality (D'SOUZA et al., 2007; DAY et al., 2006; KASPROWICZ et al., 2008; KAUFMANN et al., 2007; PENG et al., 2008; PETROVAS et al., 2006; RADZIEWICZ et al., 2007; TRAUTMANN et al., 2006; URBANI et al., 2008), which predominantly manifest as a loss in multifunctionality (PANTALEO, HARARI, 2006). Whereas T cell responses during primary infection are dominated by IFNγ single

producing cells, T cell responses in self-limiting infections after antigen clearance are dominated by IL-2 single producing cells. T cells in controlled persistent infections are predominated by multifunctional cells, whereas virus/disease reactivation is associated with a loss of IL-2 production and dominance of IFNγ single producing cells. This model is in line with our previous work on CMV and BKV infection, where we could correlate virus/disease reactivation with a decrease in IFNγ/IL-2 co-expressing cells (SCHMIDT et al., 2014; SESTER et al., 2008b). Apart from analysis of multifunctionality, the inhibitory T cell receptor PD-1 was shown to be overexpressed during chronic lymphocytic choriomeningitis virus (LCMV) infection in mice and seems to be responsible for the observed anergic phenotype with a loss in functionality and proliferative potential (BARBER et al., 2006). Of note, the anergic phenotype was shown to be reversible by inhibitory receptor blockade in vitro. In line with these studies in mice, we have previously shown that CMV specific CD4 T cells in renal transplant recipients with CMV reactivation episodes show reduced functionality. This was marked by a reduced proliferative capacity, a loss in IL-2 production and high PD-1 expression, which was at least partly reversible by in vitro blockade of PD-1 signalling (DIRKS, 2008; SESTER et al., 2008a; SESTER et al., 2008b). More recently, other inhibitory receptors such as TIM-3 and CTLA-4 have also been shown to be associated with viremic episodes in other types of chronic viral infections like HIV, HBV and HCV (BARBER et al., 2006; JONES et al., 2008; KASSU et al., 2010; KHAITAN, UNUTMAZ, 2011; ODORIZZI, WHERRY, 2012; VALI et al., 2010). Thus, apart from assessment of T cell functionality, additional phenotypical analysis of CMV specific T cells may be a promising approach to predict CMV reactivation episodes.

1.5.2 Use of peptide-HLA-multimers as a stimulation independent approach to monitor antigen specific T cell immunity

CMV antigen specific stimulation is well suited for quantitation, phenotypical and functional characterisation of CMV specific immunity (KOTTON et al., 2013b; MANUEL et al., 2013; SESTER et al., 2001; SESTER et al., 2008b; WIDMANN et al., 2008). Yet, these assays require relatively long assay times and the prerequisite of live and fully functional T cells. Therefore, test results may be impaired depending on time until processing. One approach to reduce assay time is the use of peptide-HLA-multimers, where synthetic complexes of peptides and MHC molecules are used as reagent to directly detect antigen specific T cells without the

need for specific stimulation (ALTMAN et al., 1996; BROOIMANS et al., 2008; GRATAMA et al., 2001). As this technique allows for a quantitation and phenotypical characterisation of T cells independent of their capability to produce cytokines, peptide specific responses might be enumerated more accurately in the setting of suspected T cell anergy (DAVIS et al., 2011; LEE et al., 1999). Furthermore, multimer technology can be combined with phenotypical characterisation. Indeed, analysis of PD-1 expression on CD8 T cells bound by CMV specific tetramers correlated with CMV disease in liver transplant recipients (LA ROSA et al., 2008). Of note, most multimer studies to date focus on CD8 T cells due to the poor stability of MHC class II multimers (DAVIS et al., 2011). Therefore analysis of total pathogen specific responses using the multimer technology is limited by the knowledge of the patient's individual MHC status, the availability of peptide-HLA multimer combinations and the relative inability to analyse antigen specific CD4 T cell responses (DAVIS et al., 2011).

1.5.3 $CD28^-CD27^-$ CD4 T cells as a stimulation independent correlate of CMV specific immunity

As outlined above, CMV specific T cells are predominantly of an end-stage differentiated phenotype showing signs of replicative senescence. One feature of this phenotype is the loss of CD28 and CD27 expression on both CD4 and CD8 T cells. Whereas a population of $CD28^-$ $CD27^-$ CD8 T cells is also observed in CMV seronegative individuals, the existence of $CD28^-$ $CD27^-$ CD4 T cells seems to be more specific to seropositive individuals (FLETCHER et al., 2005; LOONEY et al., 1999; POURGHEYSARI et al., 2007; VAN DE BERG et al., 2008; VAN LEEUWEN et al., 2006; VAN LEEUWEN et al., 2004). The presence of $CD28^-CD27^-$ CD4 T cells seems to correlate closely with CMV seropositivity, yet notably not with seropositivity towards other herpesviruses like HSV, EBV or VZV (DERHOVANESSIAN et al., 2011; FLETCHER et al., 2005). CMV induces the generation of $CD28^-CD27^-$ CD4 T cells after infection, as CMV induced activation of plasmacytoid dendritic cells mediates the loss of CD28 and CD27 expression on CD4 T cells via high level IFNα secretion (FLETCHER et al., 2005). The close association of $CD28^-CD27^-$ CD4 T cells with CMV infection might offer a tempting opportunity to exploit this notable T cell subset as a stimulation independent correlate of CMV specific immunity and to explore its value as a monitoring tool in the setting of organ transplantation.

1.6 T cell assays for diagnosis of active *M. tuberculosis* infection

Direct microbiological confirmation of *Mycobacterium tuberculosis* infection is hampered by the low replication rate and the concealment of mycobacteria in locally confined granulomas in the lung. Consequently, diagnosis of active infection by culture may take several weeks and assay sensitivity is considerably poor (PAI, O'BRIEN, 2008). Diagnosis of latent infection with *M. tuberculosis* is indirectly performed by the use of immunological assays that detect specific T cell immunity towards mycobacterial antigens. Numerous studies on the commercially available ELISA based QuantiFERON-TB Gold in tube assay and ELISPOT based T-SPOT.*TB* assay have shown advantages to the classically used tuberculin skin test by their ability to discriminate between BCG vaccination response and actual infection with *M. tuberculosis* bacteria (LALVANI, 2007). This is due to the use of ESAT-6 and CFP-10 proteins as stimulants. Both proteins are encoded in the region of difference 1 (RD1) of the *M. tuberculosis* genome, which is deleted from the genome of the vaccine strain Bacillus Calmette Guérin (BCG). Interestingly, IGRAs have been shown additionally to have a higher sensitivity in immunocompromised patients (SESTER et al., 2004; SESTER et al., 2003). Furthermore, unlike skin testing, they do not require a follow-up visit.

IGRAs have also been extensively studied as tool to identify patients with active tuberculosis. However, they are not suited to specifically diagnose active TB, as they do not sufficiently discriminate active from latent infection (PAI et al., 2006; PAI, O'BRIEN, 2008; SESTER et al., 2011a). Although *M. tuberculosis* specific CD4 T cell frequencies have been shown to be higher in untreated active infection than in successfully treated tuberculosis (SESTER et al., 2011b), the considerable overlap in frequencies between these patient groups precludes their use to distinguish between active and latent *M. tuberculosis* infection, which was supported in a recent meta-analysis (SESTER et al., 2011a). Thus, better biomarkers are needed to discriminate active tuberculosis from latent infection.

Better biomarkers are also needed for the identification of latently infected individuals at risk for progression to active tuberculosis. In general, tuberculosis develops only in about 5 to 10 percent of individuals with latent infection during their lifetime. Whereas healthy immunocompetent individuals are at lowest risk of progression, individuals with impaired immunity are at considerably higher risk. The highest risk group are patients with HIV/TB co-infection, which is an enormous challenge in regions of high tuberculosis incidence (WHO,

2013). Because of the variable progression risk, preventive chemotherapy of latent TB is not generally recommended for healthy immunocompetent individuals but highly recommended in situations of increased risk, such as close contact to a patient with active disease or relevant degree of immunosuppression (DIEL et al., 2009; DIEL et al., 2011). As prophylaxis ranges from three (isoniazide and rifapentine combination therapy) to nine month (isoniazide monotherapy) and is associated with serious side effects and treatment costs, it would be desirable to specifically target only those patients at need of therapy. Yet, both TST and IGRAs as currently used measures to estimate the individual risk of a patient to progress from latent to active TB only have considerably low positive predictive values.

During the last decade some promising novel biomarkers for the diagnosis of active *M. tuberculosis* infection have been described, but none have reached application in clinical routine so far. Conceptually, these biomarkers may also have potential to predict latently infected individuals at risk for progression. One approach might be the use of novel *M. tuberculosis* antigens or peptide pools which seem to correlate with different *M. tuberculosis* infection states (DEMISSIE et al., 2006; DOSANJH et al., 2011; VINCENTI et al., 2003). Furthermore, similar to chronic viral infections, we and others have shown that a loss in T cell multifunctionality can discriminate active from latent infection with high specificity and reasonable sensitivity (HARARI et al., 2011; SESTER et al., 2011b). In addition, studies in mice have indicated that a loss of the co-stimulatory receptor CD27 occurs on *M. tuberculosis* specific T cells in the lung, soon after infection and that reappearance of these cells in the circulation correlates with the degree of lung destruction and disease severity (KAPINA et al., 2007; LYADOVA et al., 2004). First studies in humans suggest that analysis of CD27 on *M. tuberculosis* specific T cells might be a potent biomarker to diagnose active tuberculosis (HARARI et al., 2011; NEMETH et al., 2012; SCHUETZ et al., 2011; SESTER et al., 2011b; STREITZ et al., 2012; STREITZ et al., 2007).

As inhibitory T cell receptors have been shown to correlate with pathogen replication/disease activity in numerous chronic viral infections, expression of these inhibitory receptors on T cells specific for mycobacterial antigens might also correlate with mycobacterial replication. Hence, their combined analysis may represent promising tools for diagnosis of active TB or to identify latently infected individuals with an increased risk for progression to active tuberculosis. Up to now however, the diagnostic potential of inhibitory

T cell receptors, CD27 expression and cytokine profiling on *M. tuberculosis* specific T cells to diagnose active TB and to evaluate the risk for progression in latently infected individuals has not been analysed in a sufficient manner. In addition, it is not known whether certain combinations of biomarkers might be complementary and therefore suited to further enhance diagnostic accuracy.

1.7 Aims of this thesis

Chronic infections are marked by intermittent periods of reactivation, and by the inability of the immune system to eradicate the pathogen. A common feature of chronically active pathogens is the modulation of the pathogen specific immune response.

The aim of this thesis was to investigate the phenotype and functionality of T cells in chronic infections and to evaluate those as diagnostic correlates of disease state and progression. We aimed to focus on cytomegalovirus and *M. tuberculosis* as two different clinically relevant persistent viral and bacterial pathogens to explore both common immunological features as well as differences to establish chronicity. To elucidate potential differences in phenotypical and functional dynamics of the antigen-specific immune response in chronic infections and those in the absence of antigen persistence, we additionally monitored antigen specific immune responses after influenza vaccination. Furthermore, the effect of pathogen prevalence on the maintenance of antigen specific responses was studied in healthy individuals and HIV-infected individuals who suffer from temporary T cell depletion.

As reactivation of CMV infection is a particular complication in immunocompromised patients, a particular focus was placed on the characterisation of specific immunity in transplant recipients with reactivations and primary infections. Biomarkers that were typically found in transplant recipients with active CMV infection were then studied as potential markers to diagnose active infection with other clinically relevant pathogens.

The following aims were specifically addressed in this thesis:

- Does the occurrence of CMV reactivation episodes in transplant recipients correlate with phenotypical and functional changes in CMV specific T cell responses and are these alterations suitable as prognostic biomarkers to assess the risk of CMV reactivation and disease?

- Is the anergic phenotype linked to inhibitory T cell receptor expression and is it reversible via blockade of these receptors?
- Can CMV specific HLA-peptide-multimers or the $CD28^{-}CD27^{-}$ CD4 T cell phenotype be used in combination with anergy markers as rapid stimulation independent tools for monitoring infectious complications in transplant recipients?
- Can biomarkers identified in active CMV infection be applied to immunity after vaccination or infection with *M. tuberculosis*?
- Does pathogen prevalence influence the maintenance of antigen specific immunity in HIV infected individuals after temporary T cell depletion?

2 Materials and methods

2.1 Materials

2.1.1 Plastic materials

Device	Producer
1.5ml tubes	Brand, Wertheim
15ml tubes	Greiner, Frickenhausen
50ml tubes	Greiner, Frickenhausen
FACS-tubes (5ml, 75x12mm)	Sarstedt, Nürnbrecht
Pipette tips	Greiner, Frickenhausen
Precision dispenser-tips (5ml, 1,25ml)	Brand, Wertheim
Single use pipettes (1ml, 2ml, 5ml, 10ml, 25ml)	Sarstedt, Nürnbrecht, Corning Costar, Bodenheim
Culture plates (96-wells)	Greiner, Frickenhausen

2.1.2 Technical equipment

Device	Producer
Graduated pipettes (10µl, 20µl, 100µl, 200µl, 1000µl)	Abimed, Langenfeld
Pipette tips	Greiner, Frickenhausen
Pipetboy	Integra Biosciences, Fernwald
Precision dispenser-tips (5ml, 1,25ml)	Brand, Wertheim
Neubauer counting chamber	Neubauer, Marienberg
MACSmix tube rotator	Miltenyi Biotec
Vortex-Mixer	neoLab Migge Laborbedarf, Heidelberg
Microscope	Nikon, Düsseldorf

Device	Producer
Microscope CRX31	Olympus, Hamburg
Sterile workbench, Clean Air Techniek	Woerden, NL
Sterile workbench, Tecnoflow	Integra Biosciences, Fernwald
Sterile workbench, Hera safe	Heraeus, Hanau
Incubator, Hera Cell	Heraeus, Hanau
Eppendorf-centrifuge, Minifuge	Eppendorf, Hamburg
Centrifuge, Megafuge 1.OR	Heraeus, Hanau
ELISA reader, Victor X4	PerkinElmer, Rodgau
PCR cycler, Light Cycler 480	Roche, Mannheim
Flow cytometers FACS Canto II, FACS Canto A and FACS Calibur	BD Biosciences, Heidelberg

2.1.3 Flow cytometers

The flow cytometers listed in Table 1 were used for analysis of T cell immunity in this thesis.

Table 1: Flow cytometers with the used configurations.

	Laser configuration			Optical filters		Fluorochromes
	Laser	Excitation (nm)	Power (MW)	Long pass	Band pass	
BD FACS Canto II	L1 Solid state (blue)	488	20	502	530/30	FITC
				556	585/42	PE
				655	670 LP	PerCP, Per-CP-Cy5.5
				735	780/60	PE-Cy7
	L2 HeNe (red)	633	16	-	660/20	APC
				735	780/60	APC-H7
	L3 Solid state (violet)	405	60	-	440/50	BD Horizon V450
				502	525/40	BD Horizon V500
BD FACS	L1 Solid state (blue)	488	20	502	530/30	FITC
				556	585/42	PE
				655	670 LP	PerCP, Per-CP-

	Laser configuration			Optical filters		Fluorochromes
	Laser	Excitation (nm)	Power (MW)	Long pass	Band pass	
						Cy5.5
				735	780/60	PE-Cy7
	L2 HeNe (red)	633	16	-	660/20	APC
				735	780/60	APC-H7
BD FACS Calibur	L1 Argon (blue)	488	15	502	530/30	FITC
				556	585/42	PE
				655	670 LP	PerCP
	L2 Semiconductor diode (red)	635	10	-	661/16	APC

2.1.4 Chemicals, buffers, and media

Chemicals	Producer
NaN_3	Serva, Heidelberg
Brefeldin A (BFA)	Sigma, Deisenhofen
BSA (bovine serum-albumin)	Serva, Heidelberg
CFDA-SE	Molecular Probes, MoBiTec, Göttingen
Acetic acid	Sigma, Deisenhofen
EDTA	Sigma, Deisenhofen
Facs Clean	BD Biosciences, Heidelberg
Facs Flow	BD Biosciences, Heidelberg
Facs Rinse	BD Biosciences, Heidelberg
Ficoll	Linaris, Bettingen
FCS (fetal calf serum)	Cambrex Biosciences, Verviers, Belgien
HSA (human serum-albumin)	Baxter BioSience; Heidelberg
L-Glutamin	PAA, Cölbe

Chemicals	Producer
Lysis solution	BD Biosciences, Heidelberg
NaN_3	Fluka, Buchs, Schweiz
Paraformaldehyde	Aldrich-Chemie, Steinheim
PBS	Linaris, Bettingen
Penicillin/Streptomycin (P/S)	PAA, Cölbe
RPMI 1640 (cell medium)	Biochrom AG, Berlin
Saponin	Sigma, Deisenhofen

2.1.5 Buffers and cell culture media

FACS buffer (PBS, 5% FCS, 0.5% BSA, 0.07% NaN_3)

Saponin buffer (PBS, 5% Saponin, 5% FCS, 0.5% BSA, 0.07% NaN_3)

CFDA-SE labelling solution (PBS, 0.1% HSA, 10µM CFDA-SE)

CFDA-SE washing solution (RPMI 1640, 1% L-Glutamin, 1% P/S, 0.5% HSA)

Culture medium (RPMI 1640, 1% L-Glutamin, 1% P/S, 5% FCS)

2.1.6 Reagents for T cell stimulation

All reagents used for T cell stimulation assays are listed in Table 2. The final concentration of reagents in the stimulatory reaction is indicated.

Table 2: Reagents used for T cell stimulation.

Stimulant	Clone	Concentration	Producer
αCD28	L293	1µg/ml	BD Biosciences, Heidelberg
αCD49d	9F10	1µg/ml	BD Biosciences, Heidelberg
CMV antigen	n.a.	32µl/ml	Bio Whittaker, Verviers
CMV control antigen	n.a.	32µl/ml	Bio Whittaker, Verviers
CMV antigen	n.a.	32µl/ml	Virion, Würzburg
CMV control antigen	n.a.	32µl/ml	Virion, Würzburg

Stimulant	Clone	Concentration	Producer
Staphylococcus aureus enterotoxin B (SEB)	n.a.	2.5µg/ml	Sigma, Deisenhofen
PPD	n.a.	7,33µg/ml	Statens Serum Institute, Copenhagen
ESAT-6	n.a.	10µg/ml	Lionex, Braunschweig
CFP-10	n.a.	10µg/ml	Lionex, Braunschweig
pdmH1N1	n.a.	2.5µg/ml	GlaxoSmithKline, London, United Kingdom
Begrivac (Flu09)	n.a.	30µg/ml	Novartis, Marburg
Peptides:	**HLA:**		
NLVPMVATV (pp65)	A2	5µg /ml	Glycotope Biotechnology, Heidelberg
TPRVTGGGAM (pp65)	B7	5µg /ml	Glycotope Biotechnology, Heidelberg
VTEHHDTLLY (pp50)	A1	5µg/ml	Glycotope Biotechnology, Heidelberg
YSEHPTFTSQY (pp65)	A1	5µg /ml	Glycotope Biotechnology, Heidelberg
FLKGGGGFV (neg. control)	A2	5µg /ml	Glycotope Biotechnology, Heidelberg

MHC class I tetramers used in this study are listed in Table 3. All tetramers were obtained from Glycotope Biotechnology (Heidelberg).

Table 3: MHC class I tetramers used for quantification of CMV specific CD8 T cells.

MHC class I allele	Peptide sequence	HCMV epitope	Fluorochrome	Volume [µl] per test
A*0101	YSEHPTFTSQY	HCMV pp65 363-373	PE	4
A*0101	VTEHDTLLY	HCMV pp50 245-253	PE	2.5
A*0201	NLVPMVATV	HCMV pp65 495-504	PE	2.5

B*0702	TPRVTGGGAM	HCMV pp65 417-426	PE	4
A*0201	FLKGGGGFV	Negative control	PE	2.5

2.1.7 Antibodies

All antibodies used in this thesis are listed in Table 4. All fluorochrome conjugated antibodies were used for flow cytometric analysis of T cells. All unconjugated antibodies were used to block inhibitory signalling pathways in CFDA-SE proliferation assays. All antibodies are directed against human antigens.

Table 4: List of antibodies.

Target antigen	Fluorochrome	Clone	Ig-class	Origin	Producer
CD3	APC	SK7	IgG1 k	mouse	BD Biosciences
CD4	APC	SK3	IgG1 k	mouse	BD Biosciences
CD4	FITC	13B8.2	IgG1	mouse	Beckmann Coulter
CD4	PE-Cy7 APC-H7	SK3	IgG1 k	mouse	BD Biosciences
CD8	PerCP, V500	SK1	IgG1	mouse	BD Biosciences
CD25	PE-Cy7	M-A251	IgG1 k	mouse	BD Biosciences
CD27	FITC	L128	IgG1 k	mouse	BD Biosciences
CD27	V450	M-T271	IgG1 k	mouse	BD Biosciences
CD28	PerCP-Cy5.5	L293	IgG1 k	mouse	BD Biosciences
CD45 RA	FITC	HI100	IgG2b k	mouse	BD Biosciences
CD45 RO	PE-Cy7	UCHL-1	IgG2a k	mouse	BD Biosciences
CD62L	PE	DREG-56	IgG1 k	mouse	BD Biosciences
CD69	PerCP	L78	IgG1 k	mouse	BD Biosciences
CD127	AlexaFluor647	HIL-7R-M21	IgG1 k	mouse	BD Biosciences
CD152 (CTLA-4)	PE, APC	BNI3	IgG2a k	mouse	BD Biosciences
CD279 (PD-1)	APC	MIH4	IgG1 k	mouse	BD Biosciences
IFNγ	FITC	4S.B3	IgG1 k	mouse	BD Biosciences
IL-2	PE	MQ1-17H12	IgG2a	mouse	BD Biosciences

Target antigen	Fluorochrome	Clone	Ig-class	Origin	Producer
TIM-3	PE	344823	IgG2a	mouse	R&D Systems
TNFα	APC	MAb 11	IgG1	rat	BD Biosciences
bPD-L1	-	MIH1	IgG1 k	mouse	eBiosciences
bPD-L2	-	MIH18	IgG1 k	mouse	eBiosciences
bTIM-3	-	F38-2E2	IgG1 k	mouse	Biozol
bCTLA-4	-	BNI3	IgG2a k	mouse	BD Biosciences
Isotype (bPD-L)	-	P3	IgG1 k	mouse	eBiosciences
Isotype (bTIM-3)	-	MOPC-21	IgG1 k	mouse	Biozol
Isotype (bCTLA-4)	-	G155-178	IgG2a k	mouse	BD Biosciences

"b" denotes blocking antibodies.

2.2 Patients and controls

Different patient and control groups were recruited for the individual parts of this thesis. Individuals were either analysed cross-sectionally or longitudinally over time. Characteristics of these groups of individuals are specified below. All studies were approved by the local ethics committee and all participants gave informed consent.

2.2.1 Individuals to characterise CMV associated and CMV specific immunity

A total of 93 healthy controls (40.58±13.33 years; 36 CMV seronegative, 57 CMV seropositive), 67 hemodialysis patients (54.41±14.29 years; 12 CMV seronegative, 55 CMV seropositive), and 81 renal transplant recipients (55.07±14.11 years; 19 CMV seronegative, 62 CMV seropositive) were analysed in a cross-sectional manner. Among seropositive transplant recipients, 20 patients did not show any reactivation, and 42 were analysed at the time of reactivation. In addition, 12 transplant recipients (53.75±16.54 years) were included at the time of primary viremia.

Longitudinal analyses comprised 46 CMV seropositive transplant recipients with (n=35) and without (n=11) CMV reactivation, and 10 patients with CMV primary infection. Transplant recipients received basiliximab induction and a standard immunosuppressive triple drug-regimen consisting of mycophenolate mofetile, tacrolimus and steroids. T cell analyses were

performed in parallel with viral load at the day of transplantation until week 16 (weekly during the first six weeks and biweekly thereafter). Furthermore, patients were stratified into non-viremic patients with no detectable CMV load during the four months observation period (n=11), patients with low level viremia (with a single positive CMV load between 500 and 800 copies/ml, or all patients with multiple low level CMV loads of ≤450; n=21) and high level viremia (at least one CMV load >800 copies/ml or multiple CMV loads >450 copies/ml; n=14). Antiviral therapy with valganciclovir was administered at the discretion of the clinicians in 13/14 (93%) patients with high level viremia, 6/21 (29%) patients with low level viremia, and 3/11 (27%) of patients without viremia. The rationale to start therapy was either ≥2 consecutive positive viral loads and/or mild clinical symptoms where CMV infection was a differential diagnosis.

2.2.2 Individuals to characterise inhibitory receptor blockade on proliferation and cytokine expression

Multiplex cytokine profiling was performed in a cross-sectional study design among 10 kidney transplant recipients who were selected from a previous study that showed an anti-PD-L1/L2-mediated restoration of proliferative responses after CMV specific stimulation of peripheral blood mononuclear cells (PBMC) from patients with a high percentage of PD-1 positive CMV specific T cells (SESTER et al., 2008b). This study was now specifically performed to assess the cytokines involved in increased proliferative responses in such patients. As such, it is a cross-sectional study where subgroups of patients were explicitly enrolled with respect to high and low PD-1 expression on CMV specific CD4 T cells, respectively. All patients were seropositive for CMV and received standard double or triple immunosuppression. Patients with high levels of PD-1 were defined as patients with >50% of PD-1 expressing CMV specific T cells. These patients were viremic at the time of analysis (n=3 with 450, 450, and 900 copies/mL, respectively) or within the last 2 months (n=2). Two of three viremic patients received valganciclovir at the time of blood sampling. Patients with low PD-1 expression did not show any evidence of viremia and served as controls. None of the patients had CMV disease. Three healthy non-immunosuppressed CMV seropositive persons were analysed as additional controls. Further characteristics of the study participants including drug treatment are shown in Table 5.

Table 5: Characteristics of healthy controls and transplant recipients.

	Healthy controls	Kidney transplant recipients	
	PD-1 low n=3	PD-1 low n=5	PD-1 high n=5
Years of age, mean±SD	39.4±3.5	58.7±8.8	57.1±9.6
Gender, m/f	2/1	4/1	4/1
years after transplantation, median (range)	n.a.	6.2 (1.3-21.9)	0.7 (0.4-19.7)*
Immunosuppression	n.a.	5	5
- Tacrolimus or cyclosporine		4	4
- Mycophenolate mofetil or azathioprine		4	4
- Methylprednisone		5	5
Valganciclovir		0	2

SD, standard deviation; *the three viremic patients were 0.40, 0.43 and 2.1 years after transplantation.

2.2.3 Individuals to analyse CMV specific T cells using HLA-peptide-tetramers

For assessment CD8 T cell responses using HLA-peptide-tetramers, individuals included were chosen based on positive CMV serostatus and positivity of at least one of the indicated HLA-molecules A2, A1 and B7. We cross-sectionally analysed 17 healthy controls (mean age 36.25±25.19 years) and a total of 72 immunocompromised individuals, which included 15 patients on hemodialysis (mean age 59.58±11.98 years), 43 patients after kidney transplantation (mean age 58.08±12.34 years) and 14 after stem cell transplantation (mean age 47.76±14.15 years). Kidney transplant recipients were on a similar immunosuppressive regimen as those described in section 2.2.1 and patient stratification according to episodes of viremia was done as outlined in section 2.2.1.

2.2.4 Individuals to characterise *M. tuberculosis* specific T cells

Similar to a previous study on IFNγ/IL-2 cytokine profiling (SESTER et al., 2011b), we analysed samples from 18 patients with confirmed active tuberculosis (mean age 55.41±19.89 years), 25 patients with a history of successfully treated tuberculosis (mean age 51.85±16.08 years), 108 patients with latent M. tuberculosis infection (mean age 58.33±16.69 years) and 137 individuals with immunity consistent with BCG vaccination or NTM infection (mean age

49.48±19.15 years). All patients diagnosed with active tuberculosis had positive cultures for *M. tuberculosis* or acid fast bacilli from one or more clinical specimens, or clinical and radiologic features highly suggestive of tuberculosis together with a good response to anti-tuberculosis treatment. Patients with successfully treated tuberculosis were studied after treatment completion and did not show any signs or symptoms compatible with active tuberculosis at the time of analysis. Latent *M. tuberculosis* infection was defined by T cell reactivity towards both PPD and towards ESAT-6 and/or CFP-10 proteins. A positive immune response towards PPD in the absence of specific immunity towards ESAT-6 and CFP-10 is consistent with either BCG vaccination status or NTM infection, although BCG vaccination status was not consistently known and this does not firmly exclude latent infection.

2.2.5 Individuals to characterise functional and phenotypical properties of specific immunity towards influenza vaccines

This part of the study was conducted among 19 immunocompetent individuals in October/November 2009. Among them, ten persons (mean age 43.64±10.05 years) had been pre-vaccinated against seasonal influenza (Begrivac 2009/2010, Novartis, Marburg, Germany) about 3 weeks before (mean 18.9±7.9 days), whereas nine (mean age 37.8±15.9 years) had not. All individuals did not have any signs or symptoms of prior contact with pandemic influenza A/H1N1. They received a routine vaccination using one standard dose of the pdmH1N1 vaccine (Pandemrix, GlaxoSmithKline Biologicals s.a., Rixensart, Belgium). Whole blood samples were drawn prior to vaccination with Pandemrix as well as 1, 2, and 10 weeks thereafter. To characterize cellular immunity in acute infection, additional whole blood samples of seven immunocompetent individuals (mean age 49.28±11.07 years) who were newly diagnosed with influenza A/H1N1 infection on a clinical/epidemiological or virological basis were analysed. All except one individual were hospitalized due to influenza-related symptoms and samples were collected within a median of 5 days (range 1–18 days) of symptom onset.

2.2.6 HIV infected and non-infected individuals to quantify CMV and *M. tuberculosis* specific immunity

This part of the study was conducted among 60 HIV infected individuals from Homburg, Germany (low tuberculosis endemic country), and 39 HIV infected patients from Mbeya, Tanzania (high tuberculosis endemic country). Immunocompetent HIV seronegative

individuals from both countries served as controls (Table 6) and were recruited from the same geographic regions as HIV positive patients. All individuals from Germany were German whites except for two HIV infected individuals of African origin. All individuals from Tanzania were of African ethnicity. Study participants had no signs or symptoms of active tuberculosis during the study period. Furthermore, a group of 34 HIV infected patients with active tuberculosis from Tanzania were recruited. Active disease was diagnosed by clinical symptoms and sputum smear testing. Bacillus Calmette-Guerin vaccination status, CD4 cell counts and antiretroviral therapy (ART) status was not consistently available for all study participants (Table 6). Patients in Tanzania were recruited and analysed by Dr. Alexandra Schütz and data were provided for combined analysis with German individuals.

Table 6: Demographic and clinical characteristics of HIV-positive patients and healthy controls.

	low tuberculosis endemicity (Germany)[a]		high tuberculosis endemicity (Tanzania)[b]		
	controls	HIV^+ patients	controls	HIV^+ patients	HIV^+ patients
	n=144	n=60	n=31	n=39	n=34
active tuberculosis	no	no	no	no	yes
age [years]	43.2±14.6	43.0±11.7	23.0±4.2	42.3±8.9 (n=24)	33.2±7.2 (n=16)
male/female [n] (%)	50/94 (35%/65%)	46/14 (77%/23%)	21/10 (67%/33%)	9/19 (32%/68%)	18/16 (53%/47%)
HIV viral load [copies/ml]	n.a.	7109±29498 (n=55)	n.a.	19537±49285 (n=25)	176648±153079 (n=16)
CD4 counts [cells/µl]	n.a.	525±335 (n=57)	n.a.	506±177 (n=25)	214±156 (n=28)
Number (%) of patients <200 CD4 T cells/µl	n.a.	5/57 (9%)	n.a.	0/25 (0%)	17/28 (60.7%)
Number (%) of patients on ART	n.a.	49/57 (86%)	n.a.	16 (41%)	0/18 (0%)

[a]TB prevalence 5.9 (1.9-10)/100000 individuals. [b]TB prevalence 183 (87-281)/100000 individuals; ranges represent uncertainty intervals (WHO). Some parameters were not determined/available for all individuals; in this situation the number of individuals wherein respective values were available is indicated.

2.3 Methods

2.3.1 Flow cytometry

Flow cytometry is a high throughput method, which allows sequential analysis of up to 10.000 cellular events/second and at the same time enables characterisation of multiple antigens on a single cell level. Flow cytometric analysis of up to four antigens was performed on FACS Calibur and FACS Canto A flow cytometers, whereas the FACS Canto II cytometer was used for simultaneous staining of up to eight parameters. Flow cytometric data were acquired and analysed by Cellquest Pro, FACS DIVA 6 (both BD Biosciences), or FlowJo software (Tree Star, Ashland, USA).

2.3.2 Antigen specific stimulation of T cells from whole blood samples and subsequent intracellular cytokine staining

Heparinised blood samples can be stimulated in vitro with either specific peptides or whole protein preparations. Whereas peptides are directly integrated in and presented by the appropriate human leucocyte antigen molecules (MHC class I or II), proteins have to be first taken up and processed by antigen presenting cells. External uptake and processing leads to predominant MHC class II associated antigen presentation. Stimulation time may range from 6 to 24 hours, depending on the antigens used and the cytokines analysed for readout.

The stimulation assay used in this thesis was performed as described before (SCHMIDT, SESTER, 2013). Subject to the numbers of cells needed for meaningful analyses, between 150µl and 300µl blood per staining reaction was stimulated as specified below. Heparinised blood samples were stimulated with specific antigens in the presence of 1µg/ml anti-CD28 and anti-CD49d costimulatory antibodies for a total of 6 hours (37°C, 6% CO_2). After 2 hours, brefeldin A (BFA) was added as a blocker of vesicular transport, which leads to intracellular accumulation of cytokines. After stimulation, blood cells were treated with 2mM ethylenediaminetetraacetic acid (EDTA) to disrupt cell-cell interactions. Thereafter, red blood cells were lysed and white blood cells were fixed for 10 min using BD-lysing solution according to the manufacturer´s instruction. After a washing step with FACS buffer and pre-treatment with FACS buffer containing 0.1% saponin, cells were stained intracellularly with fluorochrome labelled antibodies against stimulation induced cytokines. After staining, cells were either analysed directly or stored overnight in 1% PFA. One notable exception from our

published protocol was the staining protocol that included PD-1 and TIM-3 (see Table 7). As antibodies towards PD-1 and TIM-3 do recognise antigens after cellular fixation, both antibodies had to be stained directly after EDTA treatment before the fixation step.

2.3.2.1 Stimulation of CMV specific CD4 T cells

For CMV specific stimulation, a lysate of CMV infected fibroblasts was used as stimulus (CMV antigen, 32 µL/ml). A lysate of non-infected fibroblasts was used as negative control (control antigen, 32 µl/ml) and *Staphylococcus aureus* Enterotoxin B (SEB, 2.5µg/ml) as positive control, respectively (Table 2). For stimulation with CMV antigen and SEB, 600µl whole blood each was stimulated to enable three subsequent staining reactions (Staining I-III, Table 7). For each reaction, ≥15.000 CD4 T cells were acquired for analysis. For negative control stimulations, stimulation of 450µl whole blood was generally sufficient, as staining I was usually omitted to save antibodies, and as the results from staining II and III could be used to asses and subtract unspecific T cell reactivity. The detection limit for defining a positive T cell response was 0.05% CD69/IFNγ co-producing CD4 T cells as previously established (SESTER et al., 2001). The combination of antibodies used in the three staining reactions including volumes of reagents per sample after titration are summarised in Table 7. Analyses were performed on a FACS Canto II (Staining I-III) and/or FACS Canto A (Staining II).

Table 7: Staining reactions for characterisation of CMV specific T cells.

Staining I A (surface)		
Antibody/ Reagent	Fluoro-chrome	Volume [µl]
CD4	PE-Cy7	0.50
PD-1	APC	5.00
TIM-3	PE	10.00
Total volume		15.5
Staining I B (intracellular)		
IFNγ	FITC	0.50
CD69	PerCP	2.00
Saponin (5%)		1.00
FACS buffer		46.50
Total volume		50.00

Staining II (intracellular)				**Staining III (intracellular)**		
Antibody/ Reagent	Fluoro-chrome	Volume [µl]		Antibody/ Reagent	Fluoro-chrome	Volume [µl]
CD4	APC	0.50		CD4	PE-Cy7	0.50
CD69	PerCP	2.00		CD69	PerCP	2.00
IFNγ	FITC	0.50		IFNγ	FITC	0.50
IL-2	PE	2.00		TNFα	PE	0.30
				CTLA-4	APC	1.00

Saponin 5%		1.00		Saponin 5%		1.00
FACS buffer		44.00		FACS buffer		44.70
Total volume		50.00		Total volume		50.00

2.3.2.2 Stimulation of M. tuberculosis specific CD4 T cells

Titered amounts of PPD (7.32 µg/ml, Tuberkulin for in vitro use (RT-50)), recombinant ESAT-6 and CFP-10 (10 µg/ml each) were used as stimuli (see Table 2). As negative control, cells were stimulated with diluent (phosphate buffered saline). To provide enough T cells (≥15.000) for two separate staining reactions, 450µl whole blood was stimulated with each antigen. The staining reaction performed on all samples was Staining I (Table 8). Staining II, III, or IV (Table 8) was performed in addition as specified in the results section. Although reactivity in the control stimulations was largely negligible, the percentage of specific T cells was calculated by subtracting the frequency obtained by the control stimulation. The lower limit of detection for all antigen preparations was 0.05% CD69/IFNγ co-producing CD4 T cells as previously established (SESTER et al., 2001).

Table 8: Staining reactions for characterization of *M. tuberculosis* specific T cells.

Staining I (intracellular)				**Staining II (intracellular)**		
Antibody/ Reagent	Fluorochrome	Volume [µl]		Antibody/ Reagent	Fluorochrome	Volume [µl]
CD4	APC	0.50		CD4	APC	0.50
CD69	PerCP	2.00				
IFNγ	FITC	0.50		IFNγ	FITC	0.50
IL-2	PE	2.00		CTLA-4	PE	2.00
SAP 5%		1.00		SAP 5%		1.00
FACS buffer		44.00		FACS buffer		46.00
Total volume		50.00		Total volume		50.00
Staining III (intracellular)				**Staining IV (intracellular)**		
Antibody/Reagent	Fluorochrome	Volume [µl]		Antibody/ Reagent	Fluorochrome	Volume [µl]
CD4	APC	0.50		CD4	PE-Cy7	0.50
CD69	PerCP	2.00		CD69	PerCP	2.00
IFNγ	FITC	0.50		IFNγ	FITC	0.50
CD27	PE	2.00		CD27	PE	2.00
				CTLA-4	APC	2.00
SAP 5%		1.00		SAP 5%		1.00
FACS buffer		44.00		FACS buffer		42.00
Total volume		50.00		Total volume		50.00

2.3.2.3 Peptide stimulation for assessment of CMV specific CD8 T cell responses

For stimulation of CMV specific CD8 T cells peptides comprising 8-11 amino acids with binding affinity to HLA-A1, HLA-A2 and HLA-B7 were used (Table 2). Cells were stimulated with 5µg/ml peptides in the presence of 1 µg/ml anti-CD28 and anti-CD49d antibodies. Mock stimulation and stimulation with 2.5 µg/mL SEB were performed as negative and positive controls, respectively. For each peptide, 200µl heparinised whole blood was used and stained post-stimulation as outlined in Table 9.

Table 9: Staining protocol for CMV specific CD8 T cells after peptide stimulation

Staining I A (surface)				**Staining I B (intracellular)**		
Antibody/ Reagent	Fluorochrome	Volume [µl]		Antibody/ Reagent	Fluorochrome	Volume [µl]
CD8	PerCP	0.50		IFNγ	FITC	0.50
PD-1	APC	5.00		Saponin 5%		1.00
				FACS buffer		48.50
Total volume		5.5		Total volume		50.00

2.3.3 HLA-tetramer staining for stimulation-independent assessment of CMV specific CD8 T cell responses

Depending on the individual HLA-type, one or more of the CMV specific MHC class I tetramers as well as the negative control tetramer were added together with 10µl PD-1 APC antibody to separate FACS-tubes pre-filled with 200µl whole blood. After 30 minutes of incubation, cells were lysed with BD lysing solution for 10 minutes and washed with 2ml PBS. After centrifugation, cells were incubated for an additional 20 minutes with 70µl staining mix (10µl CD8 FITC, 10µl CD3 PerCP, 50µl PBS). After staining, cells were washed with 3ml PBS and resuspended in 1% PFA. Cells were analysed immediately or stored at 4°C until analysis.

2.3.4 Cell surface staining and phenotypical characterisation of CMV associated CD4 T cells

Cell-surface expression of CD4, CD28 and PD-1 was simultaneously analysed by staining 100µl of heparinized whole blood for 20 min in the dark (Staining I and II, Table 10). Subsequently, cells were treated with BD lysing solution and washed using FACS buffer. Thereafter cells were permeabilised for 10 minutes with saponin buffer and subsequently stained for 30 min in the dark using anti-CD27 and CTLA-4 antibodies (staining I, Table 10). CD27 may also be stained directly on the cell surface, although the presence of soluble CD27 in whole blood from patients with chronic renal failure reduces staining efficacy

(unpublished observations). Samples were kept in 1% PFA at 4°C until flow cytometric analysis. $CD28^-CD27^-$ cells were identified among CD4 T cells and analysed for co-expression of PD-1 and CTLA-4, which was quantified as mean fluorescence intensity (MFI). Absolute numbers of $CD28^-CD27^-$ CD4 T cells were calculated from differential blood-counts. In selected experiments, $CD28^-CD27^-$ CD4 T cells were co-stained for CD62L and CD45RO to determine their differentiation status (staining II, Table 10).

Table 10: Staining protocol for cell surface identification of CMV asscociated CD4 T cells

Staining I A (surface)				**Staining II A (surface)**		
Antibody/ Reagent	Fluorochrome	Volume [µl]		Antibody/ Reagent	Fluorochrome	Volume [µl]
CD4	APC-H7	0.50		CD4		0.50
PD-1	APC	5.00		CD45 RO		1.25
CD28	PerCP-C5.5	5.00		CD62L		8.00
CD25	PE-Cy7	1.00		CD28		5.00
Total volume		11.5		CD27		2.50
Staining I B (intracellular)				CD8		0.50
CD27	FITC	4.00		CD45 RA		10.00
CTLA-4	PE	2.00				
Saponine 5%		1.00				
FACS buffer		43.00				
Total volume		50.00		Total volume		27.75

2.3.5 Isolation of peripheral blood mononuclear cells

For proliferation analyses, peripheral blood mononuclear cells (PBMC) were isolated from whole blood samples via density gradient centrifugation using Ficoll. In short, up to 10ml heparinised whole blood was distributed into a 50ml tube, filled up to 35ml with PBS 0.02% EDTA and gently mixed. Next, samples were underlayed with 13ml Ficoll and centrifuged for 20 minutes (1120g, no brake). The intermediate phase containing PBMC was then carefully transferred to a new 50ml tube using a pipette. This was followed by two washing steps with PBS (centrifugation at 580g and 260g, respectively). Finally cells were resuspended in 2ml PBS (0.1% HSA).

2.3.6 Cell proliferation analyses using CFDA-SE dilution assay

PBMC were prepared from 20ml heparinised whole blood via ficoll separation. Between $1\text{-}50*10^6$ PBMC were resuspended in 1ml PBS (0.1% HSA) and stained with 1ml CFDA-SE labelling solution for 10 minutes at 37°C on a MACSmix tube rotator. Labelling was stopped by addition of 13ml CFDA-SE washing solution. After a centrifugation step (300g, 7min), cells were incubated twice with CFDA-SE washing solution on a MACSmix tube rotator for 5min

and centrifuged after each step. Cells were resuspended in CFDA-SE culture medium at a final concentration of $1*10^4$ cells/µl. 600.000 cells/well were seeded into a 96-well round bottom culture plate and stimulated with 32µl/ml CMV antigen, 32µl/ml CMV negative control (Bio Whittaker) or 2.5µg/ml SEB. For each stimulus, the following blocking antibodies were added in separate wells: PD-L1+PD-L2 (10µg/ml each), CTLA-4 (50µg/ml), TIM-3 (10µg/ml), PD-L isotype control, CTLA-4 isotype control, and TIM-3 isotype control. In addition double blockade with TIM-3 and PD-L-1/2 and triple blockade with TIM-3, PD-L-1/2 and CTLA-4 was performed. Cells were incubated for a total of 3 (SEB reactions) or 5 days (CMV antigen and CMV control reactions) at 37°C and 6% CO_2. After 16-20 hours of stimulation, 240µl CFDA-SE culture medium was added to each well. After the end of the respective incubation period, 150µl supernatant of each well was sampled and frozen at -20°C for later analysis of secreted cytokines. The cells were transferred to FACS-tubes for subsequent flow cytometric staining. Cells were treated with 2mM EDTA for 15 minutes to disrupt cell-cell interactions, followed by washing with 2ml FACS buffer (300g, 7min). Staining was performed as indicated in Table 11 and cells were stored in 150µl 1%PFA at 4°C until flow cytometric analysis.

Table 11: Staining protocol for stimulated CFSE labelled PBMC

Antibody/Reagent	Fluorochrome	Volume [µl]
CD4	PE	4.00
CD8	PerCP	6.00
CD3	APC	1.00
FACS buffer		39.00
Total volume		50.00

2.3.7 Viral load analysis

CMV DNA was determined based on current recommendations for pre-emptive monitoring (KOTTON et al., 2013b) and quantified from whole blood using the Cobas-Amplicor-assay (Roche Diagnostics). All analyses were performed as part of the clinical routine by the diagnostics department of Virology. The clinically relevant detection limit was 450 copies/ml.

2.3.8 IgG-ELISA for detection of cytomegalovirus specific antibodies

CMV specific antibodies were determined using an IgG ELISA (Euroimmun-AG). Titers <16 RE/ml were classified as negative.

2.3.9 Differential blood-counts

Differential blood counts were determined at the central laboratory of Saarland University Medical center according to standard procedures. The absolute numbers of lymphocytes/µl whole blood obtained via differential blood-counts was used for enumeration of absolute numbers of T cells and their subpopulations based on frequencies determined using flow cytometry.

2.3.10 Multiplex assay for multiple cytokine analyses

Simultaneous analysis of 27 cytokines was performed from stimulated PBMC using Luminex technology according to the manufacturer's instructions (BioRad 27-plex, Reinach, Switzerland). These analyses were performed by Dr. Adrian Egli and Prof. Hans H. Hirsch as a collaborative project (Basel, Switzerland).

2.3.11 Statistical analyses

Statistical analysis was performed using GraphPad Prism-V5.02 (San Diego, CA). Mann Whitney-testing was used for comparisons of a single parameter between two patient groups. Wilcoxon matched-pairs testing was used to compare a single parameter in the same patient group at two different time points/clinical situations or in cellular subpopulations. Kruskall-Wallis test with Dunn´s post-test was used for comparison of a single parameter between three or more patient groups. For longitudinal analysis of a patient group at several time points Friedman test with Dunn´s post-test was used. Correlation of distinct cell populations or parameters was performed according to Spearman. Receiver operator characteristics (ROC) analysis was performed to analyse sensitivities and specificities.

2.3.12 Data management

Patient characteristics, clinical data, diagnostic results as well as parts of the measured data were managed in a specifically designed Microsoft Access database and pre-processed for further analysis by individual queries. These were further managed and pre-processed for statistical analyses by Pivot charts using Microsoft Excel software.

3 Results

3.1 CMV immunomonitoring in transplant recipients

Current immune monitoring approaches which largely focus on quantitative changes in CMV specific T cell responses are applicable to estimate the risk of CMV disease, but only of limited value to predict CMV reactivation episodes (LISBOA et al., 2012; MANUEL et al., 2013; SESTER et al., 2001). We therefore designed a panel of phenotypical and functional markers and elucidated if changes in expression of these markers allow for prediction of CMV reactivation and relapse in longitudinally monitored transplant patients.

3.1.1 Functional characterisation after antigen specific stimulation

In this part of the thesis, analysis of a total of 28 CMV seropositive healthy controls, 51 seropositive hemodialysis patients and 62 CMV infected kidney transplant recipients was performed. The kidney transplant recipients (KTR) were further stratified according to CMV infection status in patients with (n=38) and without (n=12) reactivation episode as well as patients with primary CMV infection (n=12). For all patients CMV specific CD4 T cells were quantified by determining the frequency of IFNγ/CD69 co-expressing cells and their functionality was further characterised by monitoring the co-expression of the cytokines TNFα and IL-2. To elucidate if the CMV specific CD4 T cells show signs of anergy, we analysed the expression of three inhibitory T cell receptors, PD-1, CTLA-4 and TIM-3.

3.1.1.1 Sole quantification of CMV specific CD4 T cells is valuable in patients after primary CMV infection, but of limited use in detecting CMV reactivation periods

To elucidate if quantitative changes in CMV specific CD4 T cell frequencies correlate with CMV infection status, we compared CMV seropositive patients with and without reactivation episode and patients with primary viremia to patients on hemodialysis and healthy controls. Kidney transplant recipients during primary CMV infection showed significantly lower frequencies compared to hemodialysis patients and transplant recipients during CMV reactivation (Figure 3, p=0.0002), whereas no difference was observed between kidney transplant recipients with and without reactivation, hemodialysis patients and healthy controls.

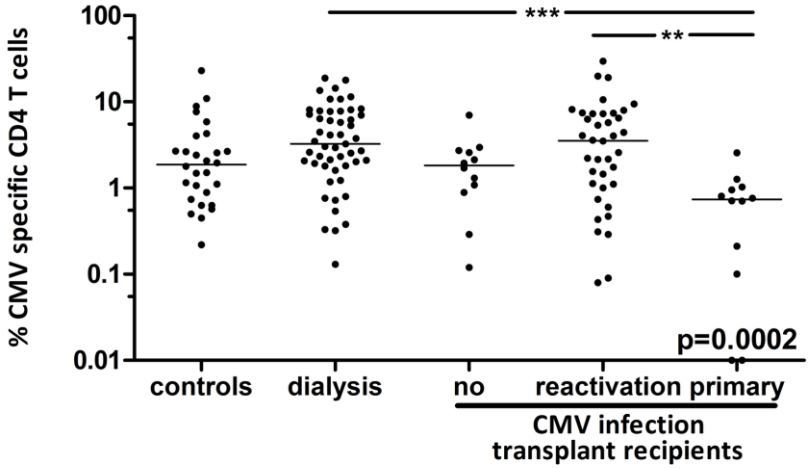


Figure 3: Frequencies of CMV specific CD4 T cells after antigen specific stimulation in transplant patients and controls. Frequencies of CMV specific CD4 T cells were compared between healthy controls, dialysis patients and kidney transplant recipients, who were stratified according to their CMV infection status. No difference in frequencies were observed, except for transplant recipients during primary CMV infection, who showed significantly lower frequencies than hemodialysis patients and transplant recipients with CMV reactivation ($p<0.001$ and $p<0.01$, respectively). If more than one data set per individual existed, mean values were calculated and displayed as a single value.

3.1.1.2 Episodes of viral replication are paralleled by a loss in multifunctionality of CMV specific CD4 T cells

Next, we wanted to assess if there are changes in T cell functionality depending on transplantation and CMV infection status. Whereas CMV specific CD4 T cells in healthy controls, dialysis patients and KTR without reactivation episodes were dominated by IFNγ-TNFα co-expressing cells (median>85.00% in all groups), a striking loss in IFNγ/TNFα co-expression was observed in patients with CMV primary infection (median 32.25%, $p<0.0001$, Figure 4A). This loss was mainly caused by a shift towards IFNγ single producing cells. Patients with reactivation showed only a slight loss in IFNγ/TNFα co-expression compared to dialysis patients ($p<0.05$). Levels of IFNγ/IL2 co-expressing cells were generally lower (median<35.00% in all groups, Figure 4B), again resulting from a shift towards IFNγ single producing cells. In contrast to IFNγ/TNFα co-expression, we observed a prominent additional loss in IFNγ/IL2 co-expressing cells during both reactivation (median=11.55%) and primary infection (median=6.76%), compared to healthy controls and dialysis patients ($p<0.0001$, Figure 4B).

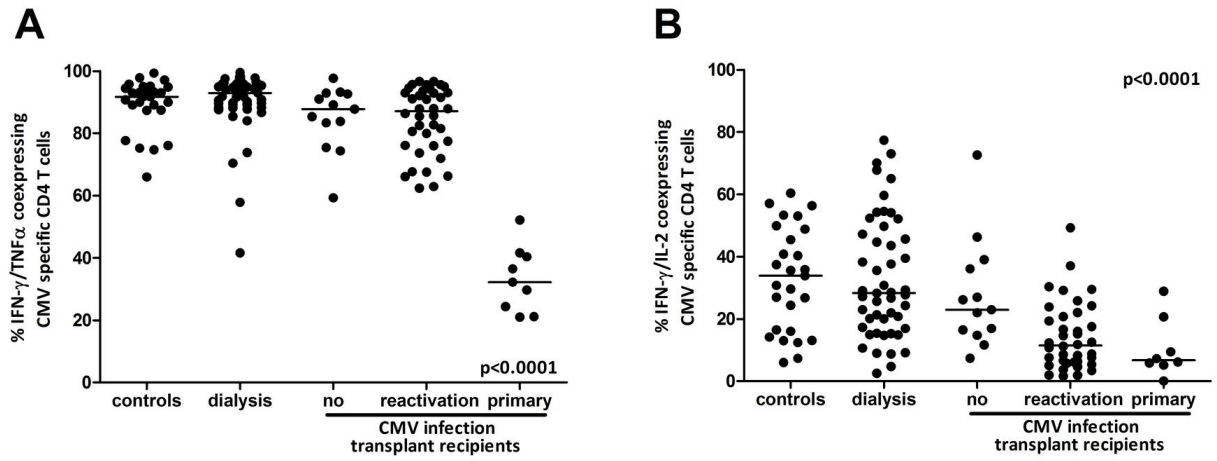


Figure 4: Changes in cytokine profiles according to CMV infection status. Frequencies of dual cytokine expressing cells IFNγ/TNFα (**A**) and IFNγ/IL-2 (**B**) co-expressing cells were determined among CMV reactive CD4 T cells. (**A**) IFNγ/TNFα co-expressing cells were strikingly reduced in patients with primary infection (p<0.0001). (**B**) IFNγ/IL-2 co-expressing cells where reduced in both KTR during reactivation and primary infection (p<0.0001). If more than one data set per individual existed, mean values were calculated.

3.1.1.3 Longitudinal analysis reveals a viremia associated and partially reversible loss of CD4 T cell multifunctionality after kidney transplantation

A total of 46 CMV seropositive kidney transplant recipients were longitudinally monitored pre-transplant and during the first 4 months after transplantation. Among those, 35 (76%) had detectable CMV viremia after transplantation with high level viremia in 14 (40%) cases. High level viremia was defined by at least one viral load >800 copies/ml, or >500 copies/ml on multiple time points and is used as a parameter to initiate antiviral therapy. Patients with low level viremia were only treated if additional clinical signs of CMV infection were present. Whereas IFNγ/TNFα profiles in patients without reactivation and those with low level viremia was stable over time, a significant decrease in IFNγ/TNFα co-expressing cells between month two and three post-transplant was observed in patients with high level viremia (p=0.001, Figure 5A). This loss was followed by a subsequent increase towards the end of the observation period. For IFNγ/IL2 co-expression, a trend towards reduced frequencies was observed in patients without reactivation, yet this decrease was significantly more pronounced in patients with high and low level viremia (p=0.09, p<0.0001, p<0.0001, respectively, Figure 5B). In contrast to IFNγ/TNFα co-expression, levels of IFNγ/IL2 co-expressing cells remained low throughout the end of the observation period.

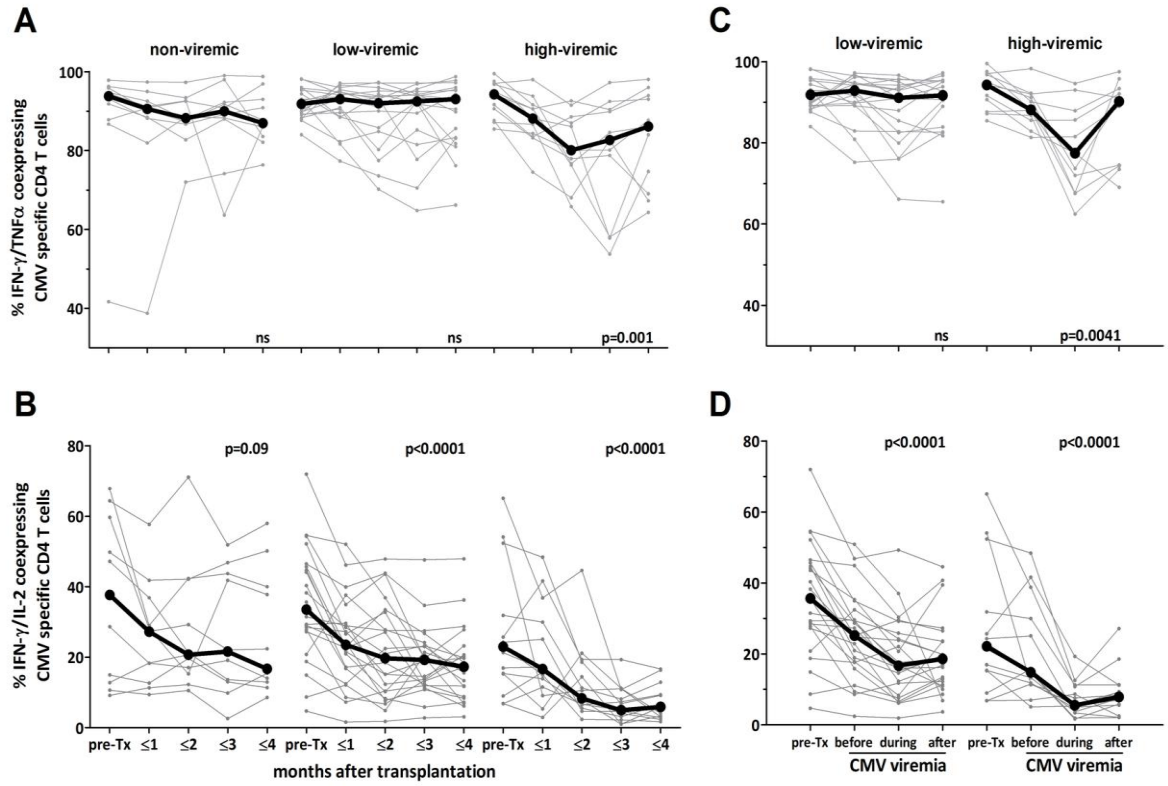


Figure 5: Longitudinal analysis reveals changes in cytokine profiles after transplantation and in relation to CMV viremia. CMV seropositive kidney transplant recipients were longitudinally analysed before (pre-Tx) and during the first four months after transplantation. Patients were stratified according to time from transplantation (**A+B**) and time from viremia (**C+D**). (**A**) Significant decrease in IFNγ/TNFα co-expressing cells during the first two months after transplantation in patients with high level viremia but not in those with no or low level viremia. (**B**) Significant decrease in IFNγ/IL2 co-expressing cells after transplantation in patients with high level and low level viremia. (**C**) Loss of IFNγ/TNFα co-expression occurs only during high level viremic episodes and reverses thereafter. (**D**) Loss of IFNγ/IL-2 co-expression occurs during high level and low level viremic episodes with only partial reversion. "Before viremia" refers to the analysis before the first CMV-DNA was positive, and "after viremia" denotes all analyses >28 days after the last positive viral load. If more than one data set existed, mean values were calculated. Median viral load in patients with low and high level viremia was 450 (IQR 50) and 2800 (IQR 6500) copies/ml, respectively.

To elucidate if the observed dynamics in frequencies of multifunctional cells correlated with time of viral reactivation, results were stratified according to temporal proximity to the reactivation period (Figure 5C and D). In patients with high level reactivation a significant loss in IFNγ/TNFα co-expressing cells was observed during the reactivation period, which rapidly reverted thereafter (p=0.0041, Figure 5C). In low level viremic patients IFNγ/TNFα co-expression remained stable even during the reactivation period (n.s., Figure 5C). Both patients with low and high level viremia showed a significant loss in IFNγ/IL2 co-expression during reactivation with only a slight reversion thereafter (p<0.0001 for both groups, Figure 5D). This indicates that the ability of IFNγ producing CMV specific CD4 T cells to co-produce

IL-2 is sensitive to even low level viral replication and mostly irreversible, whereas TNF-α co-production is more stable and correlates closely with time of viremia.

3.1.1.4 Increased expression of inhibitory T cell receptors in patients during CMV primary infection and reactivation

To assess if the observed changes in CD4 T cell function during CMV primary infection and reactivation are associated with phenotypical signs of anergy, the expression of the three inhibitory T cell receptors PD-1, TIM-3 and CTLA-4 was analysed. The expression strength of these receptors was determined by their mean fluorescence intensity (MFI) on CMV specific IFNγ producing CD4 T cells, as illustrated in representative examples in Figure 6A-C. For all three receptors no difference in expression was observed between healthy controls, dialysis patients and kidney transplant recipients without CMV viremic episodes, yet patients with CMV reactivation and primary infection showed significantly increased expression levels ($p<0.0001$ each, Figure 6D-F). Expression during primary infection was generally higher than during reactivation, reaching significance for TIM-3 and CTLA-4, but not PD-1 expression.

3.1.1.5 Close correlation between increased expression of inhibitory T cell receptors and episodes of viremia in longitudinally monitored transplant patients

To investigate the temporal association between CMV replication and the expression of the three inhibitory T cell receptors, we longitudinally analysed 46 CMV seropositive kidney transplant recipients and 10 patients with primary CMV infection before and for a minimum of 4 months after transplantation. As illustrated in a representative patient without CMV reactivation (Figure 7, left panel) both frequencies of CMV specific CD4 T cells and MFI of the three inhibitory receptors remained stable over time. In a patient with reactivation (Figure 7, middle panel), expression of all three inhibitory receptors rapidly increased at onset of viremia and slowly decreased thereafter. A decrease in CMV specific CD4 T cell frequencies was also observed, although this succeeded the onset of viremia by several days. In a patient with primary infection, CMV specific CD4 T cells were detectable a few days after onset of viremia (Figure 7, right panel) and demonstrated a highly anergic phenotype. The expression of the inhibitory receptors decreased after cessation of primary viremia but increased again at onset of a secondary reactivation period, whereas a temporary decrease in frequency was observed not until several days later (Figure 7, right panel).

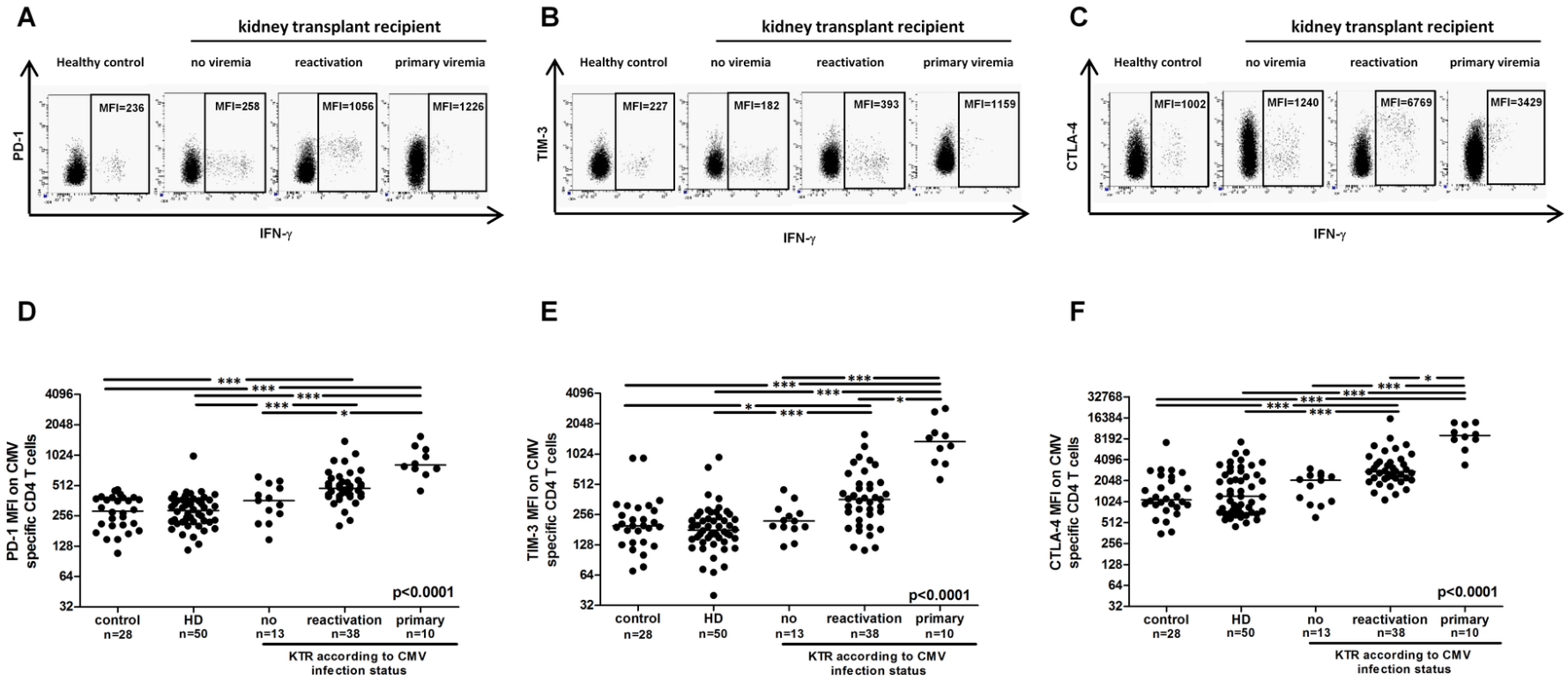


Figure 6: Increased expression of inhibitory T cell receptors during viremic episodes. Representative dotplots for the expression of PD-1 (**A**), TIM-3 (**B**) and CTLA-4 (**C**) are shown for healthy controls and kidney transplant recipients (KTR) stratified according to viremia. (**D-F**) Expression of all three inhibitory T cell receptors is significantly increased during both, CMV reactivation and primary infection. If more than one data set per individual existed, mean values were calculated.

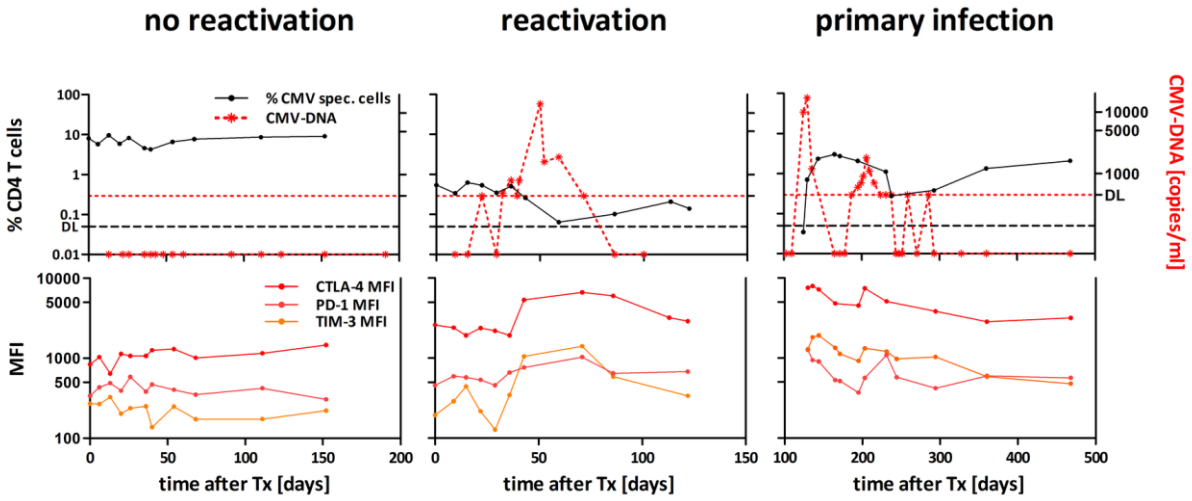


Figure 7: Dynamic changes in CMV specific immunity correlate with viral load. Typical examples of longitudinal data of each a CMV seropositive transplant recipient without and with reactivation episode and a patient with primary CMV infection are shown. Frequencies of CMV specific CD4 T cells and viral load [copies/ml] are depicted in the upper panel. DL denotes the detection limit for CMV specific CD4 T cells and CMV-DNA, respectively. Expression of the inhibitory T cell receptors PD-1, TIM-3 and CTLA-4 at each time point are depicted in the lower panel.

Longitudinal data for all 46 seropositive kidney transplant recipients were analysed in fixed time intervals after transplantation (Figure 8A-C) and in association with viremia (Figure 8D-F). For PD-1 expression, an initial increase in the first month after transplant was observed in patients with and without reactivation episode, followed by a further increase in patients with high and low level viremia, whereas PD-1 expression decreased thereafter in patients without reactivation ($p=0.0003$, $p<0.0001$, $p=0.009$, respectively, Figure 8A). TIM-3 expression was stable over time in patients with low level and without reactivation, but increased significantly in those with high level reactivation ($p=0.0008$, Figure 8B). For CTLA-4, expression remained stable in non-reactivating patients, yet significantly increased in low level and high level reactivating patients ($p<0.0001$, $p<0.0001$, respectively, Figure 8C).

To elucidate if the observed increases in anergy marker expression correlated with viremia, the data were analysed in temporal association with viremia (Figure 8D-F). An increase in PD-1 expression was already observed prior to viremia in both low and high level reactivating patients ($p<0.0001$ each, Figure 8D). In high level reactivating patients, we observed a further increase during viremia followed by a decrease thereafter. TIM-3 expression was found to be significantly upregulated during viremia in high level viremic patients ($p<0.0001$), whereas this did not reach statistical significance in low level reactivators (Figure 8E). In contrast to PD-1, there was no increase in expression prior to

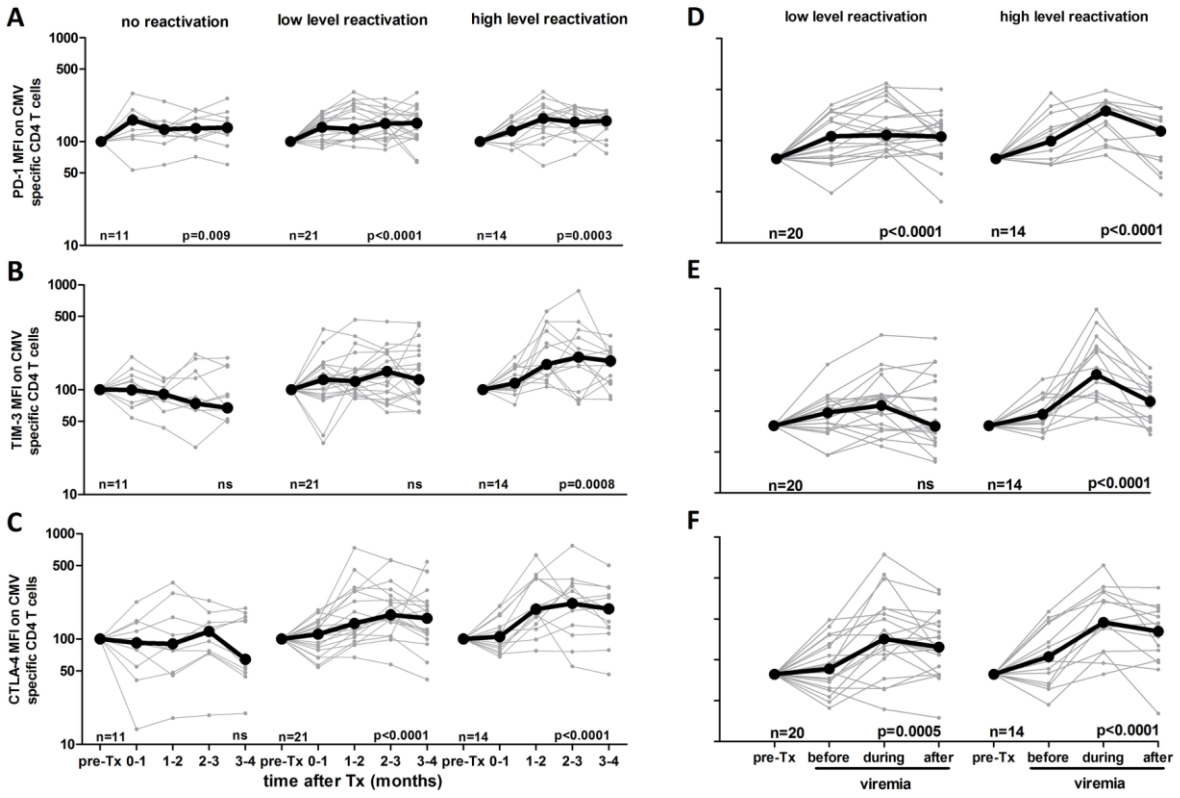


Figure 8: Expression of inhibitory T cell receptors correlates with time of viremia. CMV seropositive transplant recipients were longitudinally analysed before (pre-Tx) and during the first four months after transplantation. Patients were stratified according to time from transplantation (**A-C**) or time from viremia (**D-F**). (**A**) Increase in PD-1 expression in all patient groups, with highest increase in patients with high level viremia during the second month after transplantation. (**B**) Significant increase in TIM-3 expression in patients with high level viremia peaks during second month after transplantation. (**C**) Significant increase in CTLA-4 expression in patients with high level and low level viremia peaks between second and third month after transplantation. (**D-F**) Upregulation of inhibitory T cell receptors precedes onset of viremia and peaks during viremic episodes in both patients with high level and low level reactivation with statistical significance for all but TIM-3 expression in low level viremic patients. "Before viremia" refers to the analysis before the first CMV-DNA was positive, and "after viremia" denotes all analyses >28 days after the last positive viral load. If more than one data set existed, mean values were calculated. Median viral load in patients with low and high level viremia was 450 (IQR 50) and 2800 (IQR 6500) copies/ml, respectively.

viremia. CTLA-4 significantly increased during viremia in both groups, but this increase did not precede viremia (p<0.0001, p=0.0005, Figure 8F). Taken together; TIM-3 discriminates best between high level and low level viremic patients, whereas PD-1 is the only marker which is significantly upregulated already during the pre-viremic episode.

3.1.1.6 TIM-3 either alone or in combination with PD-1 and CTLA-4 predicts episodes of CMV reactivation

To test the potential of the three markers to detect viremia in patients, we calculated the fold increase in anergy marker expression at onset of viremia (time of first positive viral load

5.8±2.4 weeks after transplantation) compared to pre-transplant. For patients without reactivation, the mean expression between week 5 and 8 after transplantation was compared to that pre-transplant. As illustrated in Figure 9, fold increase in anergy marker expression discriminated between non-viremic and highly viremic patients (PD-1: AUC=0.74, p=0.022; CTLA-4: AUC=0.84, p=0.032; TIM-3: AUC=0.95, p=0.004; Figure 9A-C). Of the three markers, TIM-3 discriminated best with a sensitivity of 78.57% and a specificity of 100%. Next we assessed, if combined analysis of all three markers may further improve the diagnostic power and summed up the respective fold increases in expression. These combined analyses allowed good discrimination similar to, but not better, than the best marker TIM-3 individually (AUC=0.92, p=0.003, Figure 9D). As the ultimate goal of T cell monitoring is not to detect viremic episodes but to predict them, the same analysis was performed with the fold increase between baseline and the last measurement before onset of viremia (median 7 (IQR 4.8-12) days before onset of viremia). We now no longer saw significant differences in the increase in PD-1 and CTLA-4 expression between patients with high level viremia and without viremia (data not shown). In contrast a >1.3-fold increase in TIM-3 expression predicted viremia with 61.54% sensitivity and 100% specificity (AUC=0.81, p=0.009, Figure 9E). The combined increase of all three markers by >4-fold led to a higher sensitivity of 85.71% but lower specificity of 81.82% (AUC=0.85, p=0.006, Figure 9F). Thus, our data indicate that expression of all three inhibitory T cell receptors on CMV specific CD4 T cells correlates closely with episodes of viremia. Analysis of TIM-3, either alone or in combination with PD-1 and CTLA-4 has substantial potential to predict viremia.

3.1.2 Tetramer staining as a stimulation independent approach to characterise CMV specific CD8 T cells

As demonstrated in the preceding chapter and in several publications by our group and others (KOTTON et al., 2013b; MANUEL et al., 2013; SESTER et al., 2001; SESTER et al., 2008b; WIDMANN et al., 2008), CMV antigen specific stimulation is well suited for quantitation, phenotypical and functional characterisation of CMV specific immunity. Yet a technical disadvantage is the relatively long assay time and the prerequisite of live and fully functional T cells. One approach to limit assay time is the use of peptide-HLA-multimers (BROOIMANS et al., 2008; GRATAMA et al., 2001). In the following experiments we used

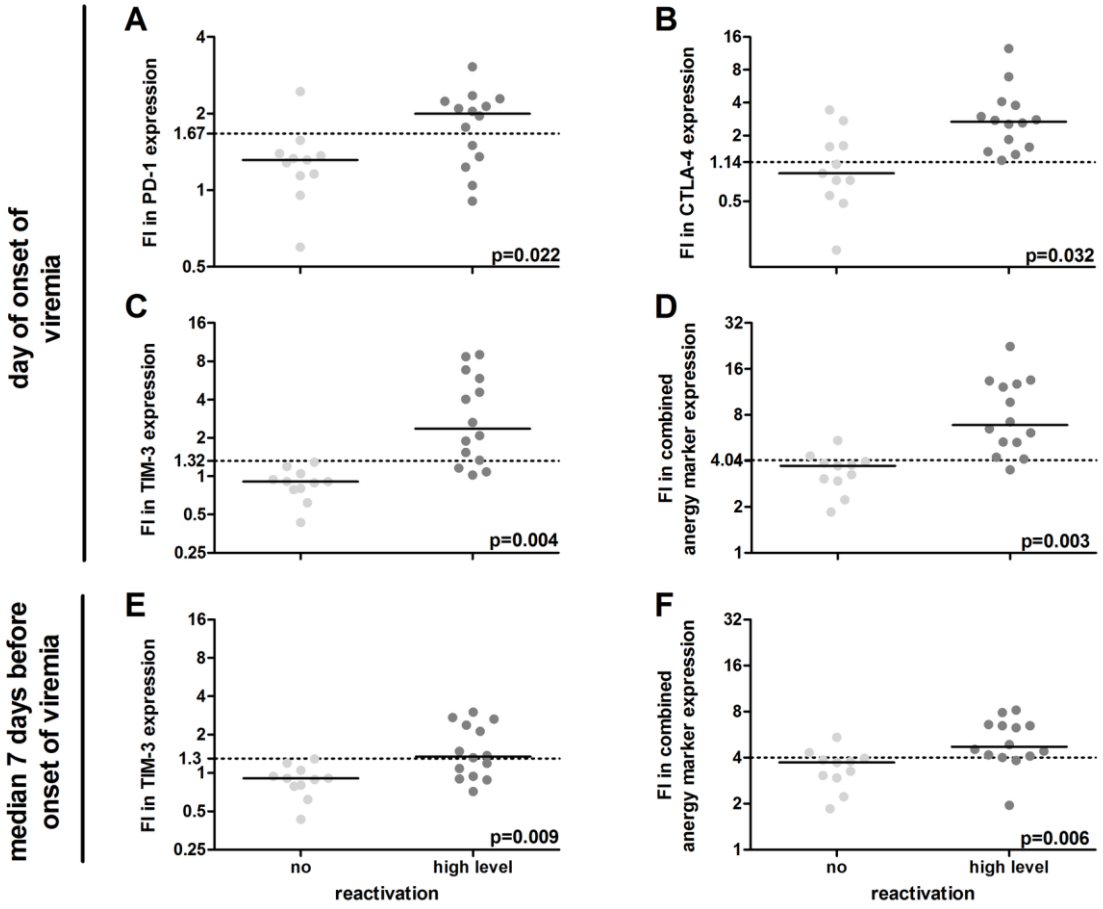


Figure 9: Evaluation of the diagnostic potential of inhibitory T cell receptors to detect patients at risk for CMV reactivation. Fold increase (FI) in expression of inhibitory T cell receptors is calculated between time of onset of viremia and pre-Tx. For patients without reactivation, the mean expression between week 5 and 8 post-transplant is divided by the expression pre-transplant. ROC analysis was performed to find the threshold discriminating best between transplant recipients with high level reactivation and those without reactivation. (**A-C**) Of the three inhibitory T cell receptors, TIM-3 discriminates best between KTR with and without reactivation (PD-1: AUC=0.74, sensitivity=64.29%, specificity=90.91; CTLA-4: AUC=0.84, sensitivity=100%, specificity=63.64%; TIM-3: AUC=0.95, sensitivity=78.57%, specificity=100%). (**D**) Combined analysis of all three inhibitory T cell receptors does not further enhance diagnostic power (AUC=0.92, sensitivity=92.86%, specificity=81.82%). (**E+F**) Fold increase in expression was calculated between the last measurement before onset of viremia (median 7 (IQR: 4.8-12) days before viremia) and pre-transplant. TIM-3 and the combination of all three markers discriminated well between patients with and without viremic episode (AUC=0.81, sensitivity=61.54%, specificity=100% and AUC=0.85, sensitivity=85.71, specificity=81.82%). The threshold discriminating best between patients with and without reactivation is indicated by a stippled line.

fluorochrome-labeled peptide-HLA-tetramers for ex vivo stimulation-independent analysis of CMV specific CD8 T cells.

3.1.2.1 Tetramer CD8 T cell frequencies correlate with the peptide specific immune response after stimulation but do not differ between patient groups

To elucidate if CMV specific peptide-HLA-tetramers are suitable to assess the CMV specific immunity, we analysed 17 healthy controls and a total of 72 immunocompromised individuals, which included 15 patients on hemodialysis, 43 patients after kidney transplantation and 14 after stem cell transplantation. Individuals were chosen based on positive CMV serostatus and positivity of at least one of the indicated HLA-molecules A2, A1 and B7. This precluded a priori ~40% of CMV seropositive individuals, which were negative for all three HLA molecules included in the tetramers used (RAHMEL, 2013).

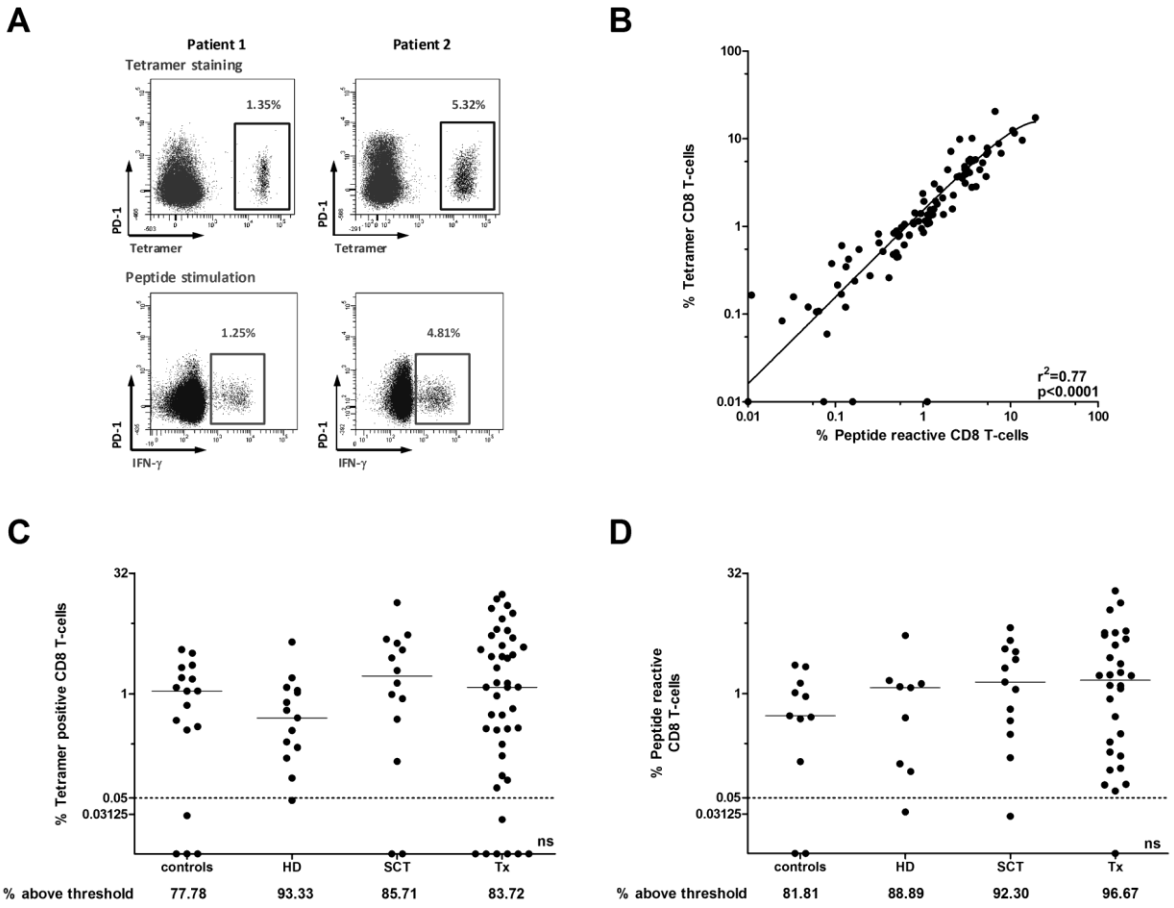


Figure 10: Tetramer positive CD8 T cells correlate with peptide reactive CD8 T cells and their frequencies do not differ between patient groups. (**A-B**) Close correlation between tetramer positive and peptide reactive CD8 T cell frequencies, determined via positive staining for tetramer-PE and IFNγ-FITC, respectively. (**C-D**) Low sensitivity for detection of CMV specific CD8 T cells after both, tetramer staining and peptide stimulation, but no significant differences in frequencies between healthy controls (HC), hemodialysis patients (HD), stem cell transplants (SCT) and kidney transplants (Tx).

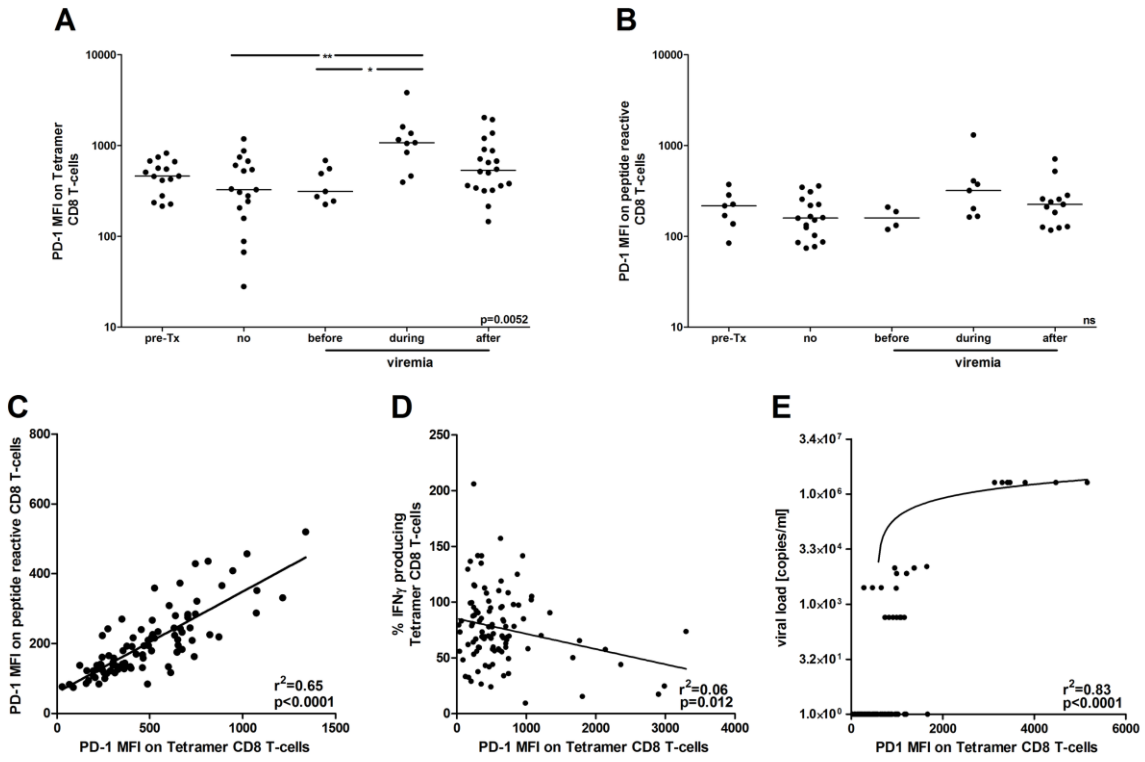


Figure 11: PD-1 expression on tetramer cells correlates with viral load and reactivation episodes after transplantation. (**A**) PD-1 expression on tetramer positive CD8 T cells is increased during viremia. (**B**) Peptide reactive CD8 T cells show an insignificant trend towards higher PD-1 expression during viremia. (**C-E**) Frequencies of PD-1 expressing tetramer positive CD8 T cells correlate with viral load (**C**), PD-1 expression on peptide reactive CD8 T cells (**D**) and inversely with their functionality (**E**).

Fourty-four out of 64 individuals (68.75%) showed cells reactive to HLA-A*0201 NLVPMVATV CMV pp65, 10/14 (71.43%) to HLA-A*0101 VTEHDTLLY CMV pp50, 12/15 (80%) to HLA-A*0101 YSEHPTFTSQY CMV pp65 and 7/15 (46.66%) to HLA-B*0702 TPRVTGGGM CMV pp65. Thirteen individuals reacted to two or more (14.61%) and 14 (15.73%) to none of the tetramers. In 11 healthy controls and 52 of the immunocompromised patients, peptide specific stimulation was performed in parallel with the same peptides as included in the tetramers. As illustrated in Figure 10A and B, we observed a close correlation between frequencies of tetramer positive cells and peptide reactive cells in our study population. When comparing healthy controls and the three immunocompromised patients groups, no significant differences were observed in the frequencies enumerated by tetramers and those after peptide stimulation (Figure 10C and D). Yet, despite the fact that only seropositive individuals were included, sensitivity was variable, with tetramer positive cells being detectable in 84.27% of the study population and peptide reactive cells in 92.10% (Figure 10C and D).

3.1.2.2 Increased PD-1 expression on tetramer cells during CMV viremia

Similar to analysis of inhibitory T cell receptors on CMV specific CD4 T cells, we tried to elucidate if PD-1 expression on tetramer positive CD8 T cells might be indicative of CMV viremic episodes. Indeed, we observed a specific upregulation of PD-1 expression during viremia, compared to pre-viremic patients and patients without CMV reactivation ($p=0.0052$, Figure 11A). We saw a similar yet non-significant trend for PD-1 expression on peptide reactive CD8 T cells after stimulation (n.s., Figure 11B). This might be due to the smaller sample size, as PD-1 expression on tetramer positive and peptide reactive cells correlated closely ($r^2=0.65$, $p<0.0001$, Figure 11C). The ratio between IFNγ producing CD8 T cells after peptide stimulation and tetramer positive CD8 T cells after surface staining revealed a correlation between high expression of PD-1 and reduced functionality ($r^2=0.06$, $p=0.012$, Figure 11D). In line with that we found a correlation between PD-1 expression levels on tetramer positive cells and level of viral load in patients ($r^2=0.83$, $p<0.0001$, Figure 11E).

3.1.3 $CD28^-CD27^-$ CD4 T cells as a stimulation independent correlate of CMV specific immunity

As currently available CMV specific tetramers allow only for analysis of CD8 T cell immunity, require the knowledge of patient HLA-status and represent only a small fraction of the CMV specific T cell immunity, we looked into alternate stimulation independent approaches to characterize CMV specific T cells. It is known that one prominent effect of CMV infection on the T cell repertoire is the loss of CD28 and CD27 expression. Whereas CD28 and/or CD27 negative CD8 T cell populations exist in both CMV seropositive and CMV seronegative individuals, over 99% of the CD4 T cell population in seronegative individuals are CD28 and/or CD27 positive ((DERHOVANESSIAN et al., 2011; VAN DE BERG et al., 2008; VAN LEEUWEN et al., 2004) and own unpublished data). Therefore we explored if analysis of $CD28^-CD27^-$ CD4 T cells might serve as a reliable stimulation independent correlate of CMV specific immunity.

3.1.3.1 $CD28^-CD27^-$ CD4 T cells serve as a reliable marker to define the CMV infection status

To address whether the occurrence and frequency of $CD28^-CD27^-$ CD4 T cells may represent an estimate of CMV specific immune responses, $CD28^-CD27^-$ CD4 T cells were quantified from a total of 316 samples from CMV seropositive and seronegative non-symptomatic healthy controls and immunocompromised patients, and compared with CD4 T cell frequencies identified using intracellular cytokine staining after CMV specific stimulation. Representative dotplots of each a CMV seropositive and seronegative healthy control person and kidney transplant recipient are shown in Figure 12A. As shown in a representative example, $CD28^-CD27^-$ CD4 T cells after surface staining were largely $CD62L^-CD45RO^+$ effector memory cells (Figure 12B; 60.1%, IQR 37.2%, n=17) and their frequencies correlated closely with those of CMV reactive CD4 T cells after CMV antigen specific stimulation (Figure 12C, $r=0.73$, $p<0.0001$).

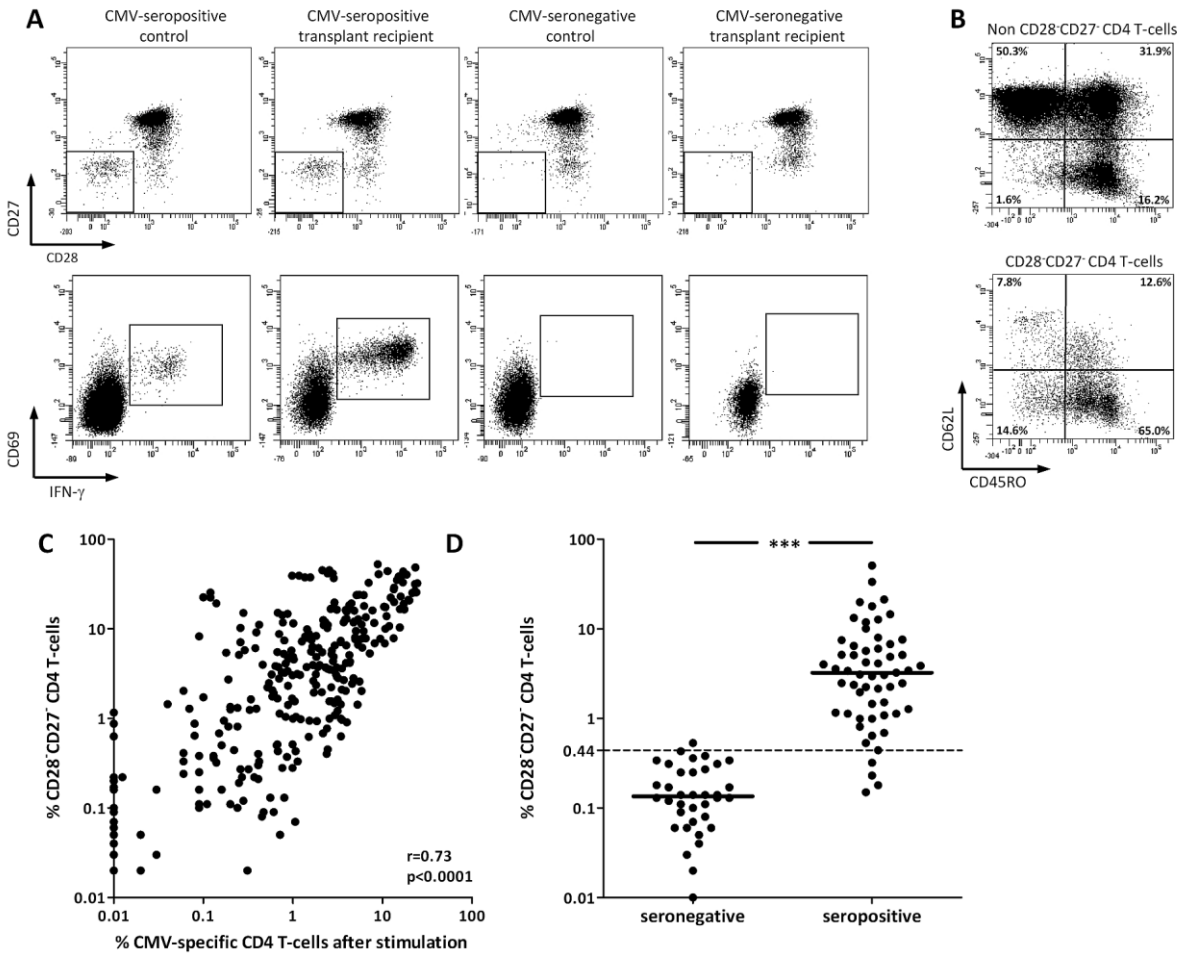


Figure 12: Close correlation between frequencies of CD28$^-$CD27$^-$ and stimulation induced CMV specific CD4 T cells. (**A**) Representative examples of CD28$^-$CD27$^-$ and CMV-specific CD4 T cell frequencies in each, a CMV seropositive and seronegative healthy control and renal transplant recipient. (**B**) Representative dotplots of CD62L and CD45RO expression patterns on CD28$^-$CD27$^-$ -CD4 T cells and non CD28-CD27- CD4 T cells. The majority of CD28$^-$CD27$^-$ CD4 T cells show an effector memory phenotype (60.1%, IQR 37.2%, 8 controls and 9 transplant recipients, data not shown), which holds true for both controls and patients. In comparison with non CD28$^-$CD27$^-$ CD4 T cells, not only effector memory but also terminally differentiated memory populations are enriched among CD28$^-$CD27$^-$ CD4 T cells (3.1-fold and 20.8-fold enrichment, respectively). (**C**) Frequencies of CD28$^-$CD27$^-$ and CMV specific CD4 T cells in samples assessed in parallel correlate closely (r=0.73, p<0.0001). CD28$^-$CD27$^-$ CD4 T cells were assessed in 57 CMV seropositive and 36 seronegative healthy controls. (**D**) Frequencies were significantly higher in the seropositive (median 3.23%, IQR 5.45%) compared to the seronegative cohort (0.14%, IQR 0.19%; p<0.0001). ROC analysis revealed a detection limit of ≥0.44% CD28-CD28$^-$CD27$^-$ CD4 T cells to discriminate with a sensitivity of 93% and a specificity of 97% between CMV seropositive and seronegative individuals (AUC 0.98; p<0.0001). We did not consider CD28$^-$CD27$^-$ CD8 T cells in our study, as a substantial proportion of these cells were also found in CMV seronegative individuals (median 14.7%, from 3.8-27.1%, n=6).

To analyse whether the presence of CD28$^-$CD27$^-$ CD4 T cells may serve as a reliable marker for CMV-seropositivity, results from the 57 CMV seropositive and 36 seronegative healthy controls were compared using ROC analysis. As shown in Figure 12D, CD28$^-$CD27$^-$ CD4 T cell frequencies were significantly lower in seronegative individuals (p<0.0001), and a percentage of ≥0.44% CD28$^-$CD27$^-$ CD4 T cells discriminates between CMV seropositive and

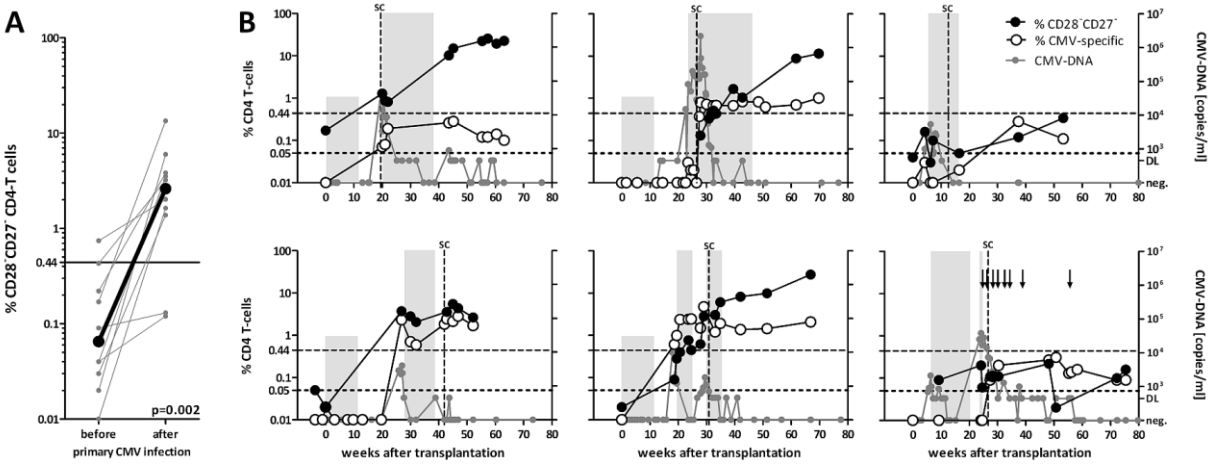


Figure 13: CD28$^-$CD27$^-$ and stimulation induced CMV specific CD4 T cells develop after primary CMV infection. (**A**) Significant increase in CD28$^-$CD27$^-$ CD4 T cell frequencies after CMV primary infection in CMV seronegative recipients of a CMV seropositive transplant (p<0.002, n=10). If more than one data set existed, mean values were calculated for each patient. (**B**) Typical examples of the time course of CD28$^-$CD27$^-$ CD4 T cells and CMV specific CD4 T cells in 6 patients with CMV primary infection. Onset of CD28$^-$CD27$^-$ and CMV reactive CD4 T cells may occur parallel to onset of viremia (left panels). Onset of CMV reactive CD4 T cells may precede onset of CD28$^-$CD27$^-$ CD4 T cells (middle panel). In two patients who did not receive prophylaxis due to misclassification of the donor CMV infection status, development of CMV specific CD4 T cells was delayed and CD28$^-$CD27$^-$ CD4 T cells remained below the cutoff (right panel). CD28$^-$CD27$^-$ and CMV reactive CD4 T cells are indicated by black and white circles and their individual cutoffs by a dotted or stippled horizontal line, respectively. Gray curves depict viral load (right axis, detection limit (DL) 450 copies/ml). Seroconversion (SC) is indicated by a dotted vertical line. Small and large shaded areas indicate duration of antiviral prophylaxis and therapy, respectively, with valganciclovir. Arrows indicate administration of CMV specific immunoglobulins.

seronegative individuals with a sensitivity of 93% and specificity of 97% (AUC 0.98). Likewise, this detection limit also discriminates well between seropositive and seronegative individuals with immunodeficiency such as hemodialysis patients (sensitivity 94%, specificity 100%, AUC=0.98) and transplant recipients (sensitivity 97%, specificity 89%, AUC=0.99; data not shown). Thus, CD28$^-$CD27$^-$ CD4 T cell frequencies ≥0.44% may serve as a reliable marker to define a positive CMV infection status.

3.1.3.2 CD28$^-$CD27$^-$ CD4 T cells are induced after CMV primary infection

As the presence of CD28$^-$CD27$^-$ CD4 T cells was linked to CMV positivity, we comparatively investigated the evolution of CD28$^-$CD27$^-$ CD4 T cells and CMV specific CD4 T cells in the context of primary infection. A total of 10 CMV seronegative recipients of a CMV seropositive graft with CMV primary infection were studied of which only eight had received CMV prophylaxis for the first 12 weeks after transplantation. Median CD28$^-$CD27$^-$ CD4 T cell frequencies showed a significant increase from 0.07% (interquartile range IQR 0.25%) before onset of primary viremia to 2.61% (IQR 3.28%) after its resolution (p=0.002, Figure 13A),

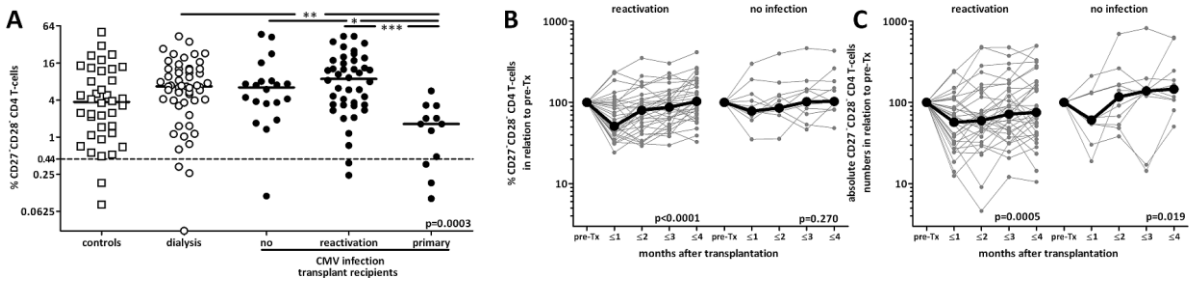


Figure 14: Comparison of CD28$^-$CD27$^-$ CD4 T cell frequencies between patients groups and their dynamics after transplantation. (**A**) Comparable frequencies of CD28$^-$CD27$^-$ CD4 T cells in transplant patients with and without reactivation episode, healthy controls and dialysis patients and markedly lower frequencies in transplant patients during primary CMV-viremia. Mean values are shown if more than one dataset per person existed. For patients with reactivation and primary infection, only datasets during viremic episodes are depicted. (**B**) Kinetics in the percentage of CD28$^-$CD27$^-$ CD4 T cells during the first month after transplantation differ between viremic and non-viremic patients. (C) Absolute numbers of CD28$^-$CD27$^-$ CD4 T cells show significant changes in both viremic and non-viremic patients. Data in panels B and C are normalized to the respective pre-transplant values.

although the time of first detection varied. Individual time courses of CD28$^-$CD27$^-$ CD4 T cells and CMV specific CD4 T cell frequencies in association with CMV load are shown in Figure 13B for 6 typical patients. The two patients in the left panels show a rapid onset of both CMV specific and CD28$^-$CD27$^-$ CD4 T cells directly after onset of primary CMV viremia. In the middle panels, the onset of detectable CMV specific CD4 T cell frequencies preceded that of CD28$^-$CD27$^-$ CD4 T cells. The right panels depict the time courses of the two patients who inadvertently did not receive CMV-prophylaxis. As expected, primary viremia occurred early after transplantation (week 4 and 5, respectively) compared to the other 8 patients (median week 23, from week 18 to 49, data not shown). Interestingly, these two patients developed comparably low levels of CMV specific CD4 T cell frequencies only late after resolution of primary viremia, and CD28$^-$CD27$^-$ CD4 T cells remained below the detection limit during the entire observation period. Taken together, consistent with the acquisition of a positive CMV infection status, CD28$^-$CD27$^-$ CD4 T cells evolve after CMV primary infection in the majority of transplant recipients.

3.1.4 $CD28^-CD27^-$ CD4 T cell frequencies differ in viremic patients with reactivation and primary infection

To analyse whether viremia was associated with quantitative changes in $CD28^-CD27^-$ CD4 T cells, their frequencies were compared between age-matched CMV seropositive healthy controls (n=38), dialysis patients (n=54), and non-viremic transplant recipients (n=20). In addition, viremic transplant recipients at the time of reactivation (n=42) or primary CMV infection were analysed (n=12; A). Of note, patients during CMV reactivation showed similar levels of $CD28^-CD27^-$ CD4 T cells as non-viremic transplant recipients, dialysis patients and healthy controls (n.s., Figure 14A). In contrast, although the $CD28^-CD27^-$ CD4 T cell population evolved in most patients after cessation of primary CMV infection , their median frequencies during the primary infection episode itself were significantly lower (1.63%, IQR 2.55%, p=0.0003, see also Figure 14). To analyse dynamic changes in patients before and after reactivation, we longitudinally analysed a total of 46 CMV seropositive renal transplant patients before and in the first four months after transplantation and relative and absolute numbers of $CD28^-CD27^-$ CD4 T cells were plotted against the pre-transplant values (Figure 14B and C). Among those, 35 developed CMV viremia, whereas 11 remained CMV-DNA negative throughout this period. Of note, while kinetics in relative percentages of $CD28^-CD27^-$ CD4 T cell frequencies exhibited significant changes in viremic patients only (Figure 14B, $p<0.0001$), absolute numbers showed some initial decline in both viremic and non-viremic patients (Figure 14C). This indicates that the mere quantitation of $CD28^-CD27^-$ CD4 T cells and in particular their dynamics in absolute numbers are inappropriate to distinguish between patients with and without reactivation.

3.1.4.1 Increased expression of PD-1 on $CD28^-CD27^-$ CD4 T cells in patients with viremia

To elucidate whether increased expression levels of PD-1 and CTLA-4 on $CD28^-CD27^-$ CD4 T cells are associated with viremia in CMV seropositive individuals, we analysed $CD28^-CD27^-$ CD4 T cells for co-expression of PD-1 and CTLA-4 in 28 healthy controls, 51 hemodialysis-patients and 69 renal transplant recipients. The latter were stratified into patients without viremic episodes (n=19), patients with CMV reactivation (n=41) and patients with primary CMV infection (n=9). Representative histograms of PD-1 and CTLA-4 expression on $CD28^-CD27^-$ CD4 T cells as compared to remaining CD4 T cells in a control, in a patient without viremia, and viremic patients at the time of reactivation or primary infection are

shown in figure Figure 15A and B, respectively. When compared to healthy controls and hemodialysis-patients, both patients with reactivation and CMV primary infection showed a significantly higher PD-1 expression on $CD28^-CD27^-$ CD4 T cells (median MFI 767, IQR 569; and 863, IQR 533 respectively, $p<0.0001$, Figure 15C). In addition, their PD-1 expression levels correlated with viral load ($r=0.24$, $p=0.0007$, data not shown). In contrast, whilst CTLA-4 expression was significantly higher in individuals during primary CMV infection (median MFI 595, IQR 207; $p=0.03$), this was not the case in those with reactivation (MFI 483, IQR 220; Figure 15D).

3.1.4.2 An increase in PD-1 expression on $CD28^-CD27^-$ CD4 T cells correlates with periods of viremia in longitudinally monitored transplant recipients

To analyse dynamics of PD-1 and CTLA-4 expression on a single patient basis, we longitudinally analysed kidney transplant recipients with regard to their PD-1 and CTLA-4 expression levels before and after transplantation. To allow for PD-1 analysis independent of local flow cytometer settings and of potential PD-1 expression on non-$CD28^-CD27^-$ CD4 T cells, we calculated a PD-1 expression score as a ratio of PD-1 MFI on $CD28^-CD27^-$ CD4 T cells divided by the PD-1 MFI of non-$CD28^-CD27^-$ CD4 T cells, and data were plotted in relation to the pre-transplant values. Meaningful analysis of PD-1 and CTLA-4 co-expression was precluded in one patient with and one without reactivation, as their $CD28^-CD27^-$ CD4 T cell frequencies remained under the threshold of 0.44% (see Figure 15B).

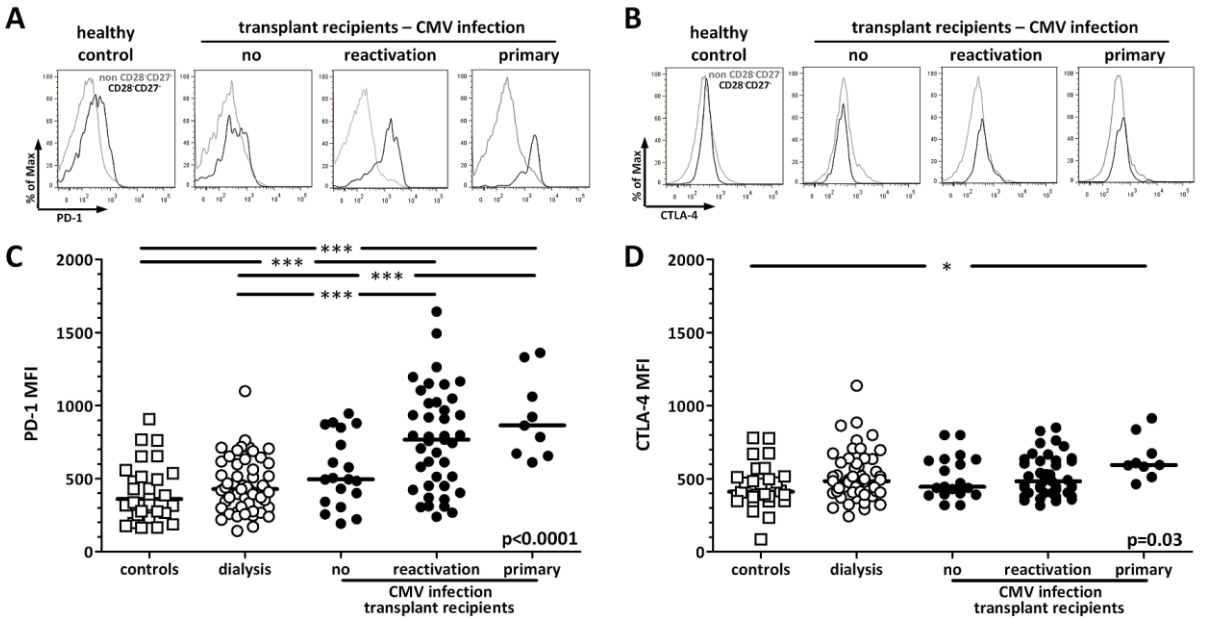


Figure 15: Expression profiles of PD-1 and CTLA-4 on CD28$^-$CD27$^-$ CD4 T cells in association with CMV-infection status. Representative examples of PD-1 (**A**) and CTLA-4 (**B**) expression on CD28$^-$CD27$^-$ CD4 T cells (black line) in each, a CMV seropositive healthy control, a renal transplant-recipient without viremia, with CMV reactivation or CMV primary infection. Respective expression levels on non CD28$^-$CD27$^-$ CD4 T cells are depicted as gray lines. (**C**) Both transplant recipients with CMV primary infection and CMV reactivation expressed significantly higher levels of PD-1 on CD28$^-$CD27$^-$ CD4 T cells compared to healthy controls ($p<0.001$) and dialysis patients ($p<0.001$) and showed a trend towards higher expression compared to transplant recipients without viremic episodes. (**D**) CTLA-4 expression was significantly higher in patients with CMV primary infection compared to healthy controls ($p<0.01$). Bars indicate median expression levels. Each patient is depicted once, apart from some of the dialysis patients who underwent kidney transplantation and were assigned to the dialysis and to the respective transplant group according to viremia. If more than one data set existed, mean values were calculated for each patient.

Of note, in patients with reactivation, a significant increase in PD-1 expression was observed during the first two months after transplantation, which decreased thereafter ($p<0.0001$, Figure 16A). In contrast, PD-1 expression in non-viremic patients only slightly increased, but changes did not reach statistical significance. The CTLA-4 expression score also showed significant changes over time, but this held true for both viremic and non-viremic patients (Figure 16B). The subgroups of low and high level viremic patients were additionally analysed in temporal association with viremia, i.e. before, during and after resolution of viremia (Figure 16C and D). Compared to pre-transplant, there was a significant increase in the PD-1 expression score during the reactivation episode for both high- and low level viremic patients, which was preceded by a strong increase prior to reactivation and followed by a decrease after resolution of viremia (Figure 16C, $p<0.0001$ respectively). In contrast, increases in CTLA-4 expression were less pronounced (Figure 16D).

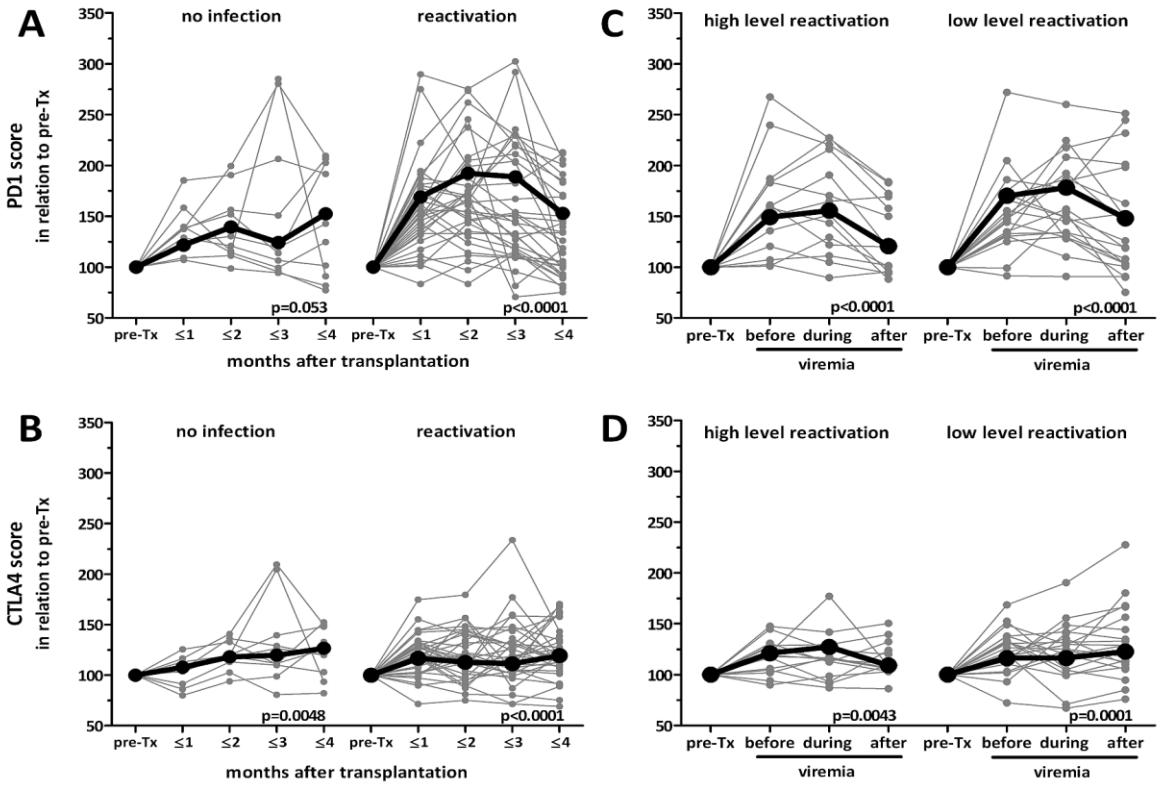


Figure 16: PD-1 and CTLA-4 expression on $CD28^{-}CD27^{-}$ CD4 T cells in association with viremia in prospectively monitored transplant recipients. (**A**) Significant increase in PD-1 expression in the first two months after transplantation in patients with, but not those without CMV reactivation episode. (**B**) Significant changes in CTLA-4 expression in the first month post-transplant in both patients with and without reactivation episodes. (**C**) PD-1 upregulation precedes onset of viremia and peaks during viremic episodes in both patients with high level and low level reactivation. (**D**) Significant yet less pronounced upregulation of CTLA-4 expression before and during CMV reactivation episodes. "Before viremia" refers to the analysis before the first CMV-DNA was positive, and "after viremia" denotes all analyses >28 days after the last positive viral load. If more than one data set existed, mean values were calculated. Median viral load in patients with low and high level viremia was 450 (IQR 50) and 2800 (IQR 6500) copies/ml, respectively. Data are normalized to the respective pre-transplant values.

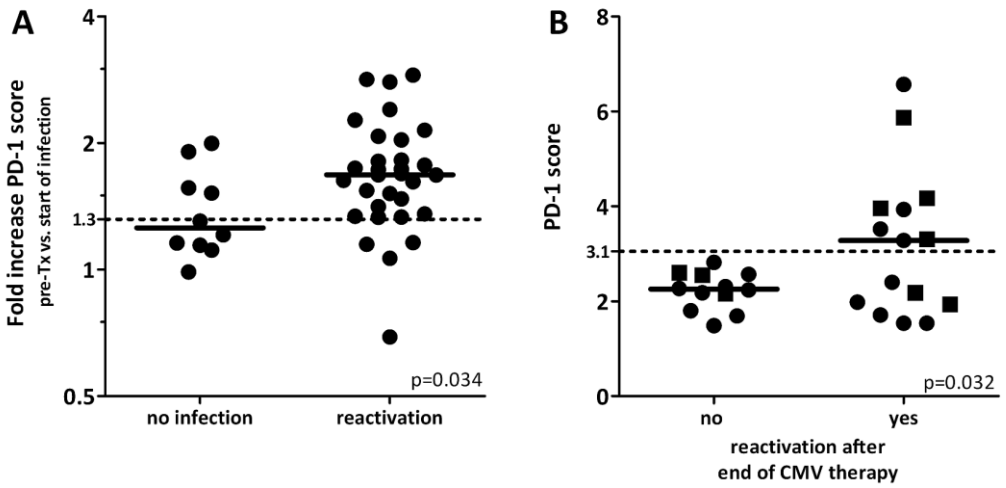


Figure 17: Evaluation of the diagnostic potential for measuring PD-1 expression on $CD28^-CD27^-$ CD4 T cells. (**A**) The increase in PD-1 expression is significantly higher in CMV-seropositive transplant-recipients with reactivation compared to patients without reactivation. ROC analysis revealed a cutoff value ≥1.3 to be best predictive of CMV-viremia (AUC=0.73, sensitivity 87%, specificity 60%). Fold increase in PD-1 expression was determined as the ratio between PD-1 score at time of first positive viral load and PD-1 score pre-transplant. For patients without viremic episode mean PD-1 score during week 5 and 8 post-transplant was divided by the PD-1 score pre-transplant. (**B**) PD-1 score ≥3.1 at time of therapy cessation is specific for patients with recurrent CMV-viremia. ROC analysis revealed a PD-1 score ≥3.1 to predict recurrence of CMV-viremia with 100% specificity and 53% sensitivity (AUC=0.66). Transplant recipients with therapy indicated CMV reactivation (circles) or primary infection (squares) were analysed for PD-1 expression at the end of therapy. The PD-1 score

3.1.4.3 Evaluation of the diagnostic potential of PD-1 analysis on $CD28^-CD27^-$ CD4 T cells

Although PD-1 expression dynamics may have been confounded by antiviral therapy, we evaluated the diagnostic potential of monitoring PD-1 expression on $CD28^-CD27^-$ CD4 T cells to identify patients with reactivation among CMV seropositive transplant recipients. The increase in PD-1 expression at the time of viremia was calculated by the ratio of the respective PD-1 expression score at the day of first detectable viral load (5.8±2.4 weeks after transplantation) and pre-transplant. For patients without reactivation, the mean score between week 5 and 8 after transplantation was compared to that pre-transplant. ROC analysis revealed a ≥1.3-fold increase to be indicative of viremia with a sensitivity of 87% and a specificity of 60% (AUC=0.73, p=0.034, Figure 17A). Assessment of high level viremia by an increase of ≥1.6 was less sensitive (75%), but specificity was higher (80%, AUC=0.80, p=0.018, data not shown).

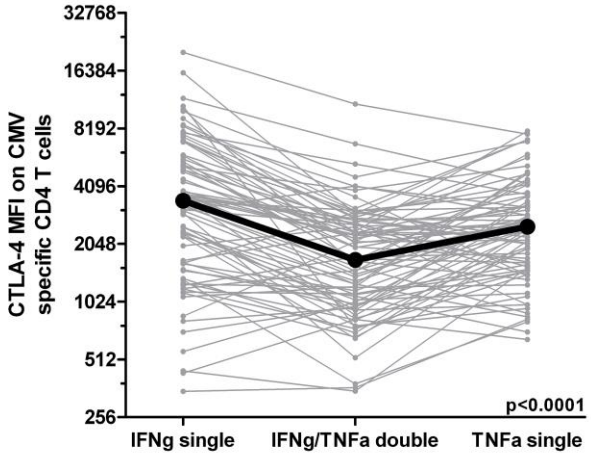


Figure 18: CTLA-4 expression is increased on TNFα and IFNγ single producing CD4 T cells. Combined data from healthy controls and immunocompromised patients with and without viremic episodes reveals increased expression of CTLA-4 on TNFα and IFNγ single producing CD4 T cells (p<0.0001). Compartments of TNFα and IFNγ single and co-producing cells were flow cytometrically determined after CMV specific stimulation with subsequent ICS and analysed for CTLA-4 expression by MFI.

We next analysed whether the PD-1 expression score after the end of therapy may predict additional episodes of viremia. In our center antiviral therapy is typically terminated after four weeks with at least two consecutive negative viral load determinations. A total of 27 patients (18 after CMV reactivation, 9 after primary CMV viremia) were included in this analysis of which 15 experienced a second episode of viremia. As shown in Figure 17B, the PD-1 expression score at the end of treatment was significantly higher in individuals experiencing subsequent viremia. Interestingly, although sensitivity was low (53%), a PD-1 score of ≥3.1 had a 100% specificity to predict a second viremic episode (AUC=0.66, p=0.032).

3.1.5 Functional T cell anergy during viremic episodes is marked by an altered cytokine expression pattern and can be reversed by blocking inhibitory T cell receptors

In the previous chapters of this thesis, both changes in inhibitory T cell receptor expression and cytokine profiles were shown to be associated with episodes of viremia after transplantation. Therefore, we analysed if inhibitory T cell receptor expression was associated with changes in cytokine profiles. Furthermore, we performed CFSE dilution assays with and without blockade of inhibitory pathways to elucidate the effect of inhibitory T cell receptors on CMV specific proliferation. In addition, multiplex cytokine analysis was performed from the supernatants to explore potential alterations in cytokine expression and to analyse if altered cytokine expression may be reverted by blockade of inhibitory pathways.

3.1.5.1 Polyfunctional T cells show lower grade of anergy compared to IFNγ or TNFα -only producing T cells

Cells from healthy controls and immunocompromised patients with and without viremic episodes were co-stained with the cytokines IFNγ and TNFα and the inhibitory T cell receptor CTLA-4 after CMV specific stimulation. As illustrated in Figure 18, TNFα and especially IFNγ single positive cells expressed significantly higher levels of CTLA-4 than IFNγ/TNFα co-expressing cells ($p<0.0001$). This indicates that high expression of CTLA-4 might play a role in the loss of T cell multifunctionality.

3.1.5.2 Patients with high expression levels of inhibitory T cell receptors show a reduced proliferative capacity, which can be in part restored by blockade in vitro

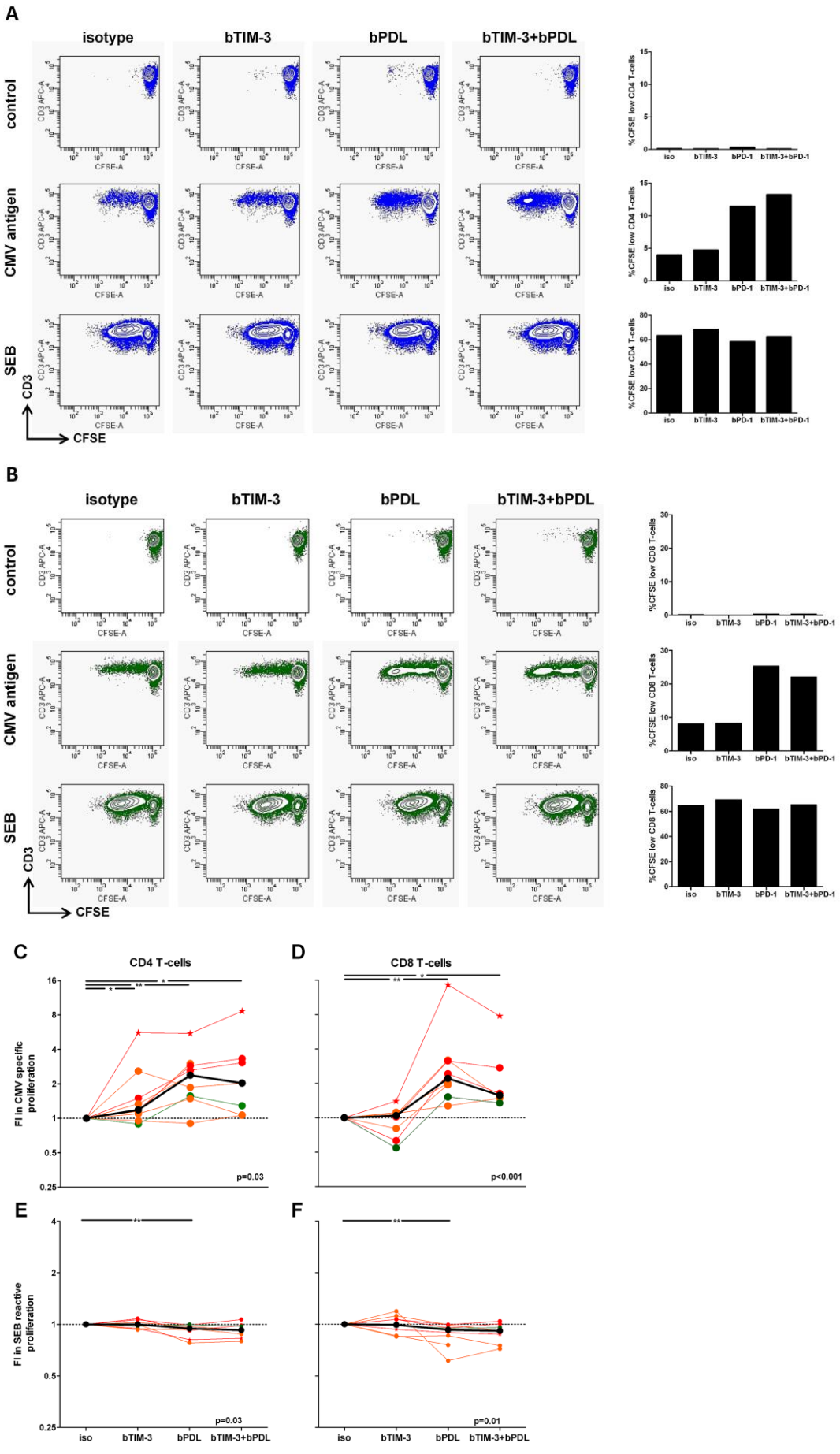
As high expression of inhibitory T cell receptors was associated with a functional loss in cytokine production, we now assessed to which extent these receptors also negatively impact the proliferative capacity and if this can be reverted by blockade of the inhibitory pathways. Therefore we intentionally chose three transplant patients with high level expression of all three inhibitory receptors (PD-1, TIM-3, and CTLA-4) on their CMV specific CD4 T cells (MFI>median of the CMV reactivating group for each marker), five with medium levels (MFI<median of the CMV reactivating group for at least two of the markers) and a healthy control with low levels (MFI<median of the healthy control group for all markers, see Figure 6). The median levels of proliferating CD4 T cells were 0.95% (range 0.25-2.5%) in the high level group, 6.86% (1.51-37.14%) in patients with medium levels and 20.02% for the healthy control after 5 days stimulation with CMV antigen (data not shown). Proliferative responses towards SEB were similar in all individuals tested (data not shown). To assess the effect of inhibitory receptor blockade, we additionally performed proliferation assays with antibody mediated blockade of the single inhibitory T cell receptor pathways or their respective isotype controls, and combined blockade of the PD-1 and TIM-3 pathways or all three markers (PD-1, TIM-3, and CTLA-4). Of note, whereas TIM-3 and CTLA-4 were directly targeted by blocking antibodies, PD-1 blockade was achieved indirectly by combined blockade of the ligands PD-L1 and PD-L2. In all samples containing CTLA-4 or the CTLA-4 isotype control antibodies, we observed an unspecific negative impact on proliferation, and both CMV and SEB stimulated samples were affected (data not shown). Therefore these samples were excluded from further analysis. In two patients from the medium level group,

no combined blockade of TIM-3 and PD-L1/2 was done, as cell numbers were not sufficient to perform all stimulatory reactions.

As illustrated in representative dotplots from a patient with high anergy marker expression (Figure 19A), individual blockade of TIM-3 signalling led to a 1.2fold increase in proliferation of CMV specific CD4 T cells (Figure 19C, p=0.027). Individual blockade of PD-L1/2 led to a 2.4fold increase in proliferation (p=0.008), which was not altered after combined blockade of TIM-3 and PD-L1/2 (2fold increase, p=0.016, Figure 19C). As expected, the effect of blockade was higher in patients with high expression levels of anergy markers (see colour code in Figure 19C) and highest in a patient, who was acutely viremic at time of analysis (indicated by asterisks, Figure 19C). Interestingly, blockade of PD-L1/2 alone led to a slight but significant decrease in SEB reactive proliferation of 1.05fold (p=0.004) and a non-significant decrease of 1.07fold in combination with TIM-3 (Figure 19E).

In contrast to CMV specific CD4 T cell proliferation, we observed no significant effect of TIM-3 blockade upon CD8 T cell proliferation (n.s., Figure 19B and D). Blockade of PD-L1/2 alone or in combination with TIM-3 led to 2.2fold and 1.6fold increases in proliferation (p=0.008 and p=0.031, respectively, Figure 19D). As with CD4 T cells, the effect of blockade was higher in patients with high levels of anergy marker expression and highest for the acutely viremic patient (see Figure 19D). Likewise, blockade of PD-L1/2 alone led to a slight but significant decrease in SEB reactive proliferation of 1.07fold (p=0.004), and a non-significant decrease of 1.09fold in combination with TIM-3 (Figure 19F).

Figure 19 (next page): Blockade of inhibitory receptors partly restores proliferative capacity of anergic T cells. CFSE dilution after antigen specific stimulation of freshly isolated PBMC was analysed in three transplant patients with high level expression of all inhibitory receptors on their CMV specific CD4 T cells, five with medium levels and a healthy control with low levels. Mean fluorescence intensities (MFI) >median of the CMV reactivating group for each marker was regarded as high level expression, MFI of <median of the healthy control group as low level expression and MFI<median of the CMV reactivating group for at least two out of three markers as medium expression (see Fig. 4). Representative examples of proliferating CD4 (**A**) and CD8 T cells (**B**) after stimulation with CMV control antigen, CMV antigen or SEB with and without blocking antibodies against PD-L1/2 and TIM-3 or isotype controls are illustrated (left panels) and the respective frequencies of proliferating CFSE-low cells indicated (right panel). Fold increase (FI) in proliferation compared to isotype control proliferation is illustrated for CD4 and CD8 T cells after CMV specific (**C+D**) and SEB stimulation (**E+F**). Patients with high and medium level expression of anergy markers are indicated in red and orange and the healthy control in green respectively. Asterisks indicate the single patient who was viremic at time of analysis. As for two patients with medium anergy marker expression PD-L1/2 single and TIM-3 single blockade but not combinatorial blockade was performed, Wilcoxon matched pairs test was used to test for differences between samples with blocking antibodies and isotype controls and Friedman test excluding those two data sets were performed to test for overall significance. In addition, one individual with medium level anergy marker expression did not show CD8 T cell proliferation in any sample and was hence excluded from analysis. b denotes blocking antibodies.


A
isotype
bTIM-3
bPDL
bTIM-3+bPDL
control
CMV antigen
SEB
CD3
CFSE
CD3 APC-A
CFSE-A
%CFSE low CD4 T-cells
iso
bTIM-3
bPD-1
bTIM-3+bPD-1
B
%CFSE low CD8 T-cells
C
CD4 T-cells
FI in CMV specific proliferation
p=0.03
D
CD8 T-cells
p<0.001
E
FI in SEB reactive proliferation
p=0.03
F
p=0.01
bPDL

3.1.5.3 Reduced proliferative capacity of PD-1 high expressing cells can be reversed via PD-L-blockade

As our data from the previous chapter indicate that PD-1 is the anergy marker with the most pronounced effect on T cell proliferation we next performed proliferation assays with and without blockade of the PD-1 pathway and subsequent cytokine analysis of the respective supernatants. Therefore we specifically chose three healthy controls, five transplant recipients with low and five with high PD-1 expression on CMV specific T cells for this set of experiments. Representative dotplots of PD-1 expression on CMV specific CD4 and CD8 T cells in healthy donors and renal transplant recipients are shown in Figure 20A. Transplant recipients with a high percentage of PD-1 expressing CMV specific T cells (n=5; median 63.9%, range 53.7%-87.0%) were compared to patients with a low percentage of PD-1 expressing cells (n=5; median 28.7%, range from 15.9%-39.2%; Figure 20B, data refer to both CD4 and CD8 T cells), where the PD-1 high group was defined by having more than 50% PD-1 expressing CMV specific T cells We observed a strong correlation between PD-1 expression on total unstimulated CD4 and CD8 T cells and total CD4 and CD8 T cells after stimulation with CMV antigen or SEB (Figure 20D). This excludes an unspecific impact of *in vitro* stimulation on PD-1 expression. Three out of the five patients with high PD-1 expression had concomitant CMV viremia, whereas viral load in all patients with low PD-1 expression was below the limit of detection (<450 copies/mL). The overall frequencies of CMV specific T cells in the two patient groups were not significantly different and corresponded to those found in healthy controls (p=0.59, Figure 20C). However, as shown in Figure 20E and F, the percentage of proliferating T cells after 5 days of CMV specific stimulation was lower in transplant recipients with high PD-1 expression (p=0.03, Figure 20F). We have previously shown that the low proliferative capacities of PD-1 positive CMV specific T cells can be restored in the presence of a combination of antibodies towards PD-L1 and PD-L2 (anti-PD-L) (SESTER et al., 2008b). To assess, whether this is associated with a concomitant restoration of cytokine production, the effect of anti-PD-L blockade on CMV specific cytokine release was analysed. In our group of individuals, anti-PD-L blockade led to a 3-fold increase in CMV specific proliferation in patients with high PD-1 expression, whereas no such effect was observed in controls or patients with low PD-1 expression (p=0.009, Figure 20). Data displayed in Figure 20B-C and F-G represent combined results for CD4 and CD8 T cells to

account for the fact that cytokines subsequently analysed from supernatants of stimulated PBMC cannot be assigned to an individual cell population.

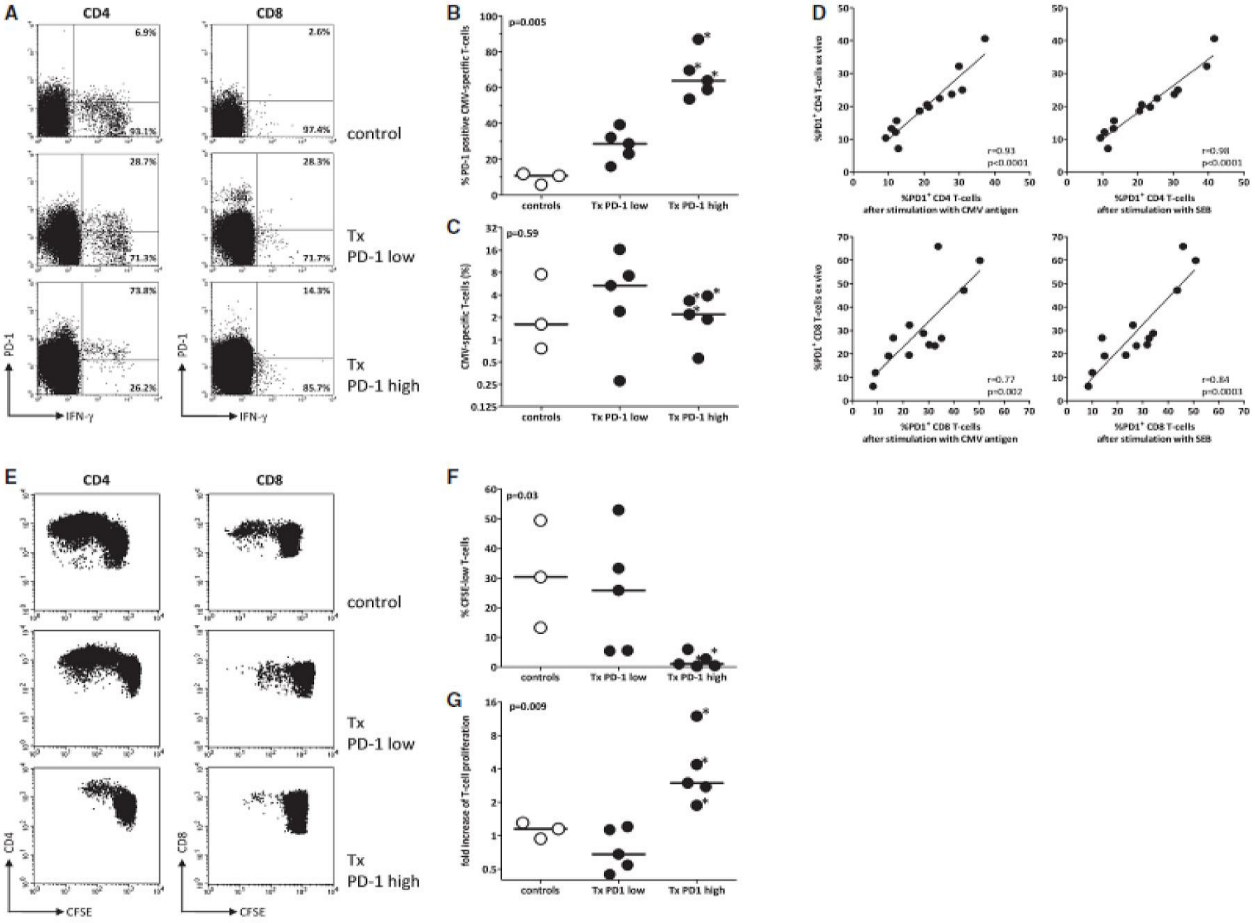


Figure 20: PD-1 as a marker of reversible functional anergy in transplant patients. (**A**) Representative examples of PD-1 expression on CMV specific CD4 and CD8 T cells are shown for a control and transplant recipients with low and high PD-1 expression, respectively. (**B**) The percentage of PD-1 expressing cells among CMV-specific T cells and (**C**) the frequency of CMV-specific T cells were determined after specific stimulation using flow cytometry. As with the percentage of PD-1 positive CMV specific T cells, the mean fluorescence intensity (MFI) of PD-1 on CMV specific T cells was significantly different among controls (median 6.4; range 5.8-19.7), PD-1 low transplant recipients (15.4; 9.8-18.9) and PD-1 high transplant recipients (24.7; 22.3-65.7, p=0.01). (**D**) PD-1 expression on unstimulated CD4 and CD8 T cells correlated with PD-1 expression on CD4 and CD8 T cells after stimulation with CMV antigen or SEB thereby excluding a stimulation bias of PD-1 expression. (**E**) Representative examples of CFSE-assays from CD4 and CD8 T cells after CMV specific stimulation in individuals shown in panel A. (**F**) The proliferative potential of CMV-specific T cells was quantified as the percentage of CFSE-low cells after 5 days of stimulation. (**G**) The increase in proliferation after antibody-mediated blockade of PD-L1 and PD-L2 was expressed as a multiple of the proliferation observed upon incubation with isotype controls. This increase was significantly different between PD-1 high and PD-1 low patients (p=0.009). Data displayed in panels B-C and F-G represent combined results for CD4 and CD8 T cells. Asterisks denote patients that were viremic at the time of analysis.

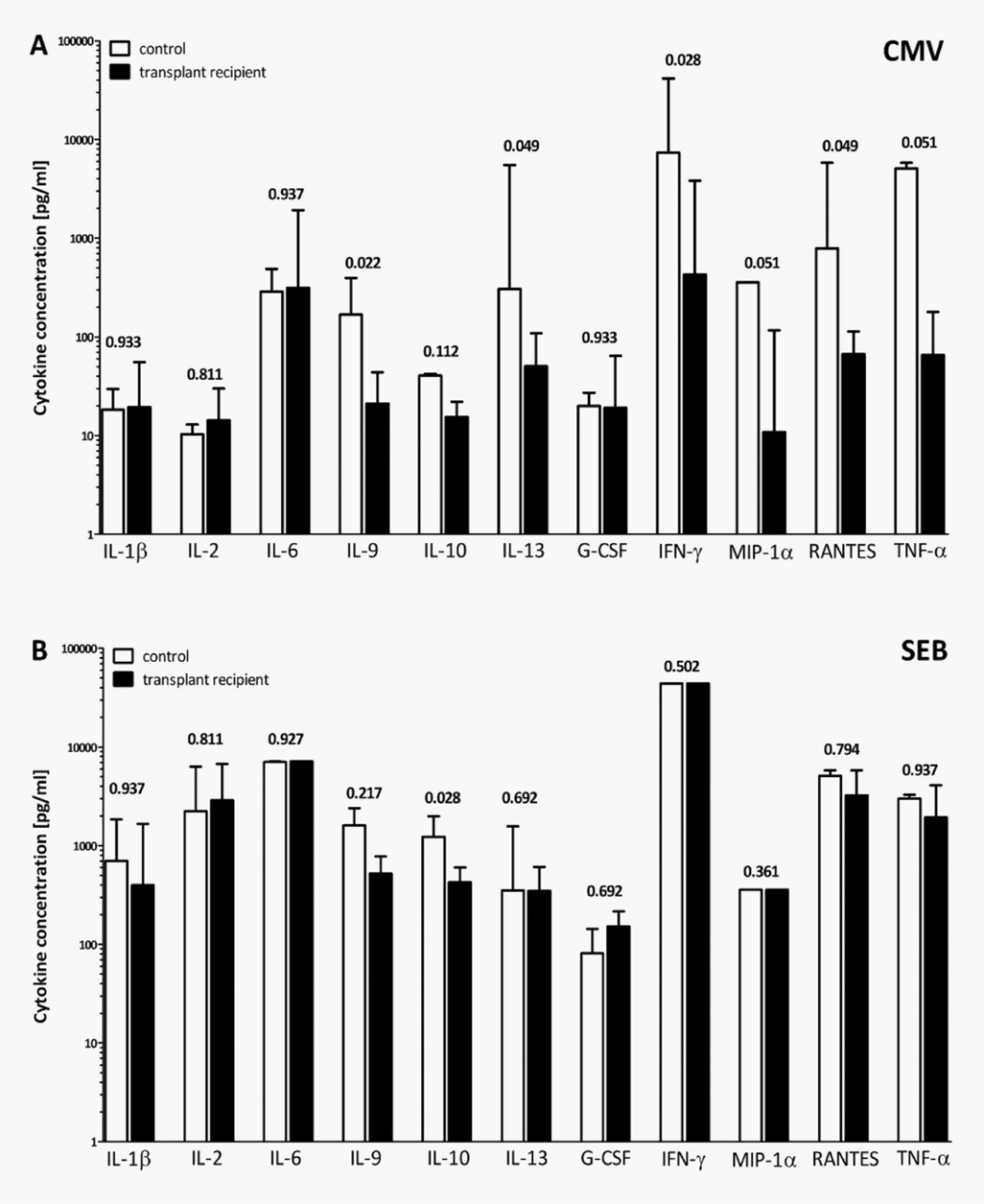


Figure 21: Cytokine release in controls and transplant recipients. Bars show median values and interquartile ranges of cytokines (in pg/mL) in supernatants from (**A**) CMV and (**B**) SEB stimulated cells in controls (n=3, white bars) or transplant recipients (n=10, black bars). The Mann Whitney test was used to compare cytokine expression in the two groups (p-values are indicated).

3.1.5.4 General screening of cytokines

Analyzing the expression levels of 27 cytokines in supernatants of stimulated PBMC, we found that the cytokines IL-5, IL-7, IL-12-p70, IL-17, FGF basic, and GM-CSF did not show any pronounced expression after CMV specific stimulation, as a large percentage of samples were below or close to detection limit. Therefore, no specific role of these cytokines could be attributed to CMV specific cellular immunity. Conversely, the levels of four cytokines (IL-8, IP-10, MCP-1 and MIP-1β) were above the highest cut-off in most samples regardless of PD-1 expression, which precluded further analysis on the effect of PD-1/PD-L blockade (percentages of antigen-stimulated samples above cut-off: IL-8 87.5%, IP-10 68.3%, MCP-1 59.6%, MIP-1β 79.8%). A CMV specific role of IL-8 and MCP-1 is rather unlikely as they were also highly expressed in samples stimulated with control antigens.

As judged by comparative analysis of specific stimulation with the polyclonal T cell stimulus SEB and control antigen, the cytokines IL-1Rα, IL-4, IL-15, eotaxin, PDGF und VEGF were not sufficiently induced by T cells (stimulation index <10 as compared to a median SI of 70.0 for the remaining cytokines). Therefore, 11 of 27 cytokines with specific effects were further analysed.

3.1.5.5 Cytokine profiles in kidney transplant recipients differ from that of healthy control persons

The expression levels of the 11 cytokines in transplant recipients were first compared to those in healthy controls. In supernatants of CMV-stimulated cells, levels from 4/11 cytokines were significantly higher in healthy controls (IL-9, IL-13, IFNγ and RANTES, Figure 21A). As an example, the median CMV-induced IFNγ release in controls was 7369.4 pg/ml compared to 428.1 pg/ml in patients (p=0.028). A trend towards a higher expression was observed for MIP-1α and TNFα (Figure 21A). Overall, cytokine-levels in the supernatants of SEB-stimulated PBMC did not differ between controls and patients. A notable exception was IL-10, where the respective levels were higher in controls (p=0.028, Figure 21B).

3.1.5.6 Influence of PD-1 expression on cytokine profiles in transplant recipients

To further assess the role of PD-1 expression on CMV specific T cells, transplant recipients with high and low PD-1 expression were compared. As shown in Figure 1, PD-1 expression levels did influence the amount of cytokines released after stimulation with CMV antigen, as

patients with high PD-1 expression showed an overall lower cytokine-release compared to patients with low PD-1 expression. The difference in IL-13 expression was significant and IL-9, IFNγ and RANTES showed a trend towards lower expression (Figure 1A). After SEB stimulation, however, cytokine levels were in general higher and the relative difference in their expression levels between both groups was less pronounced (Figure 1B).

3.1.5.7 Changes in cytokine profiles upon anti-PD-L blockade

To mechanistically link PD-1 expression to lower cytokine expression, we investigated the effect of anti-PD-L on cytokine expression. As shown in Table 12, anti-PD-L blockade induced an increase in the levels of a variety of cytokines in CMV-stimulated samples. When the effect of anti-PD-L1/2 blockade was analysed in samples of transplant recipients irrespective of PD-1 baseline expression, cytokines with a pronounced median increase were IL-6, IL-9, and IFNγ (data not shown). Interestingly, in line with proliferative responses the anti-PD-L-mediated increase of CMV specific cytokine-release was strongest for patients with high PD-1 expression compared to patients with low PD-1 expression on CMV specific T cells (Figure 20G; Table 12 and data not shown). Low PD-1 expression was associated with only moderate increase in 10/11 cytokines, whereas high PD-1 expression led to a pronounced increase in the levels of 9/11 cytokines, of which cytokines such as IL-6 and IFNγ were induced by more than 10-fold. Interestingly, unlike CMV specific stimulation, cytokine-release in SEB stimulated PBMCs was largely unaffected by anti-PD-L-blockade (<3fold in all cytokines, Table 12).

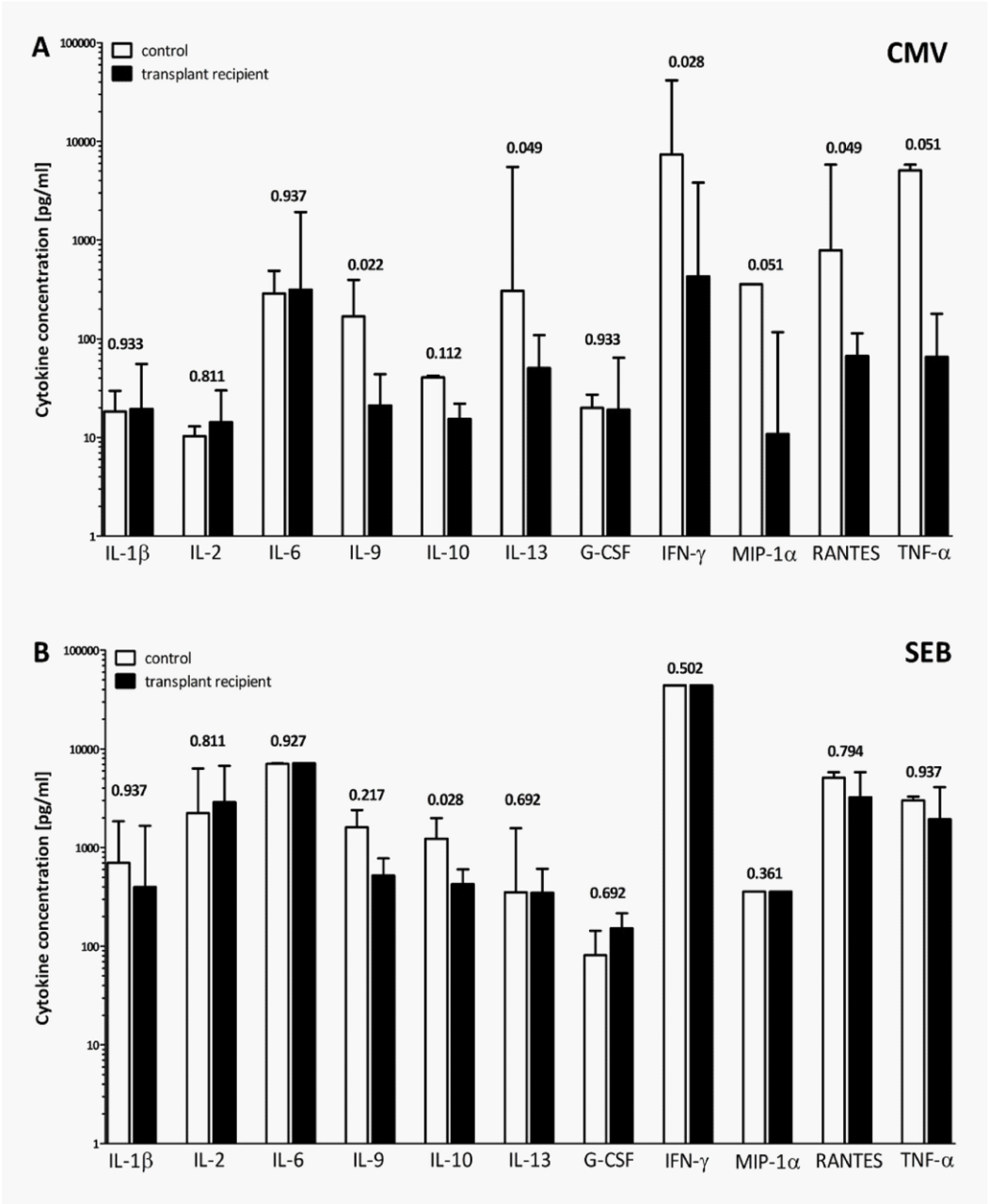


Figure 22: Cytokine release in transplant recipients with high and low PD-1 expression on CMV specific T cells. Bars show median values and interquartile ranges of cytokines (in pg/mL) in supernatants from (**A**) CMV and (**B**) SEB stimulated cells in transplant recipients with low and high PD-1 expression on CMV-specific T cells (white and black bars, respectively, n=5 patients each). CMV and SEB stimulated samples were analysed after 5 and 3 days, respectively. The Mann Whitney test was used to compare cytokine expression in the two groups (p-values are indicated).

Table 12: Restoration of cytokine expression after blockade with anti-PD-L1 and anti-PD-L2 blocking antibodies in kidney transplant patients.

	SEB		CMV antigens	
	PD-1 low	PD-1 high	PD-1 low	PD-1 high
IL-1β	1.7 (18.3)	1.9 (2.0)	1.1 (15.4)	6.4 (2.1)
IL-2	2.0 (13.2)	0.7 (1.7)	1.5 (5.4)	5.3 (1.4)
IL-6	0.8 (19.8)	0.9 (2.3)	1.9 (17.2)	13.9 (2.8)
IL-9	0.7 (4.8)	0.5 (1.7)	1.6 (3.0)	7.4 (2.2)
IL-10	0.5 (2.1)	0.7 (1.8)	1.6 (3.4)	3.4 (1.4)
IL-13	0.9 (1.9)	0.8 (1.5)	1.9 (2.1)	2.2 (2.1)
G-CSF	1.4 (6.0)	1.2 (2.8)	1.5 (14.3)	5.3 (2.2)
IFN-γ	1.1 (4.0)	0.6 (1.2)	3.7 (6.0)	11.8 (1.9)
MIP-1α	1.1 (2.5)	0.7 (1.2)	1.6 (7.5)	3.9 (1.7)
RANTES	1.0 (4.4)	0.8 (1.3)	2.2 (3.7)	2.8 (2.0)
TNF-α	1.6 (4.0)	0.9 (1.2)	2.0 (3.3)	4.6 (2.3)

Numbers show mean fold increases of respective cytokines compared to isotype-treated cell cultures including standard deviation (SD). Increases of 3-4.9-fold; 5-9.9-fold, and more than 10-fold are indicated in yellow, orange, and red, respectively. CMV- and SEB-stimulated samples were analysed after 5 and 3 days, respectively.

When comparing the individual impact of antibody-mediated blockade of either PD-L1 or PD-L2 in separate stimulation experiments, IL-6 was the only cytokine that showed a 3.1-fold induction after blockade with anti-PD-L1 in patients with high PD-1 expression, whereas no other cytokine was induced by individual blockade (data not shown). This indicates that the combined blockade of both PD-L1 and PD-L2 is necessary to mediate sufficient restoration of cytokine expression. The reversibility of PD-1 induced T cell anergy confirms our previous findings (see 3.1.1) that monitoring of inhibitory T cell receptors is a good correlate of CMV specific T cell functionality and hence valuable in identifying patients at increased immunological risk for viremia.

3.2 T cell assays for diagnosis of active *M. tuberculosis* infection

Whereas novel T cell based assays for diagnosis of tuberculosis infection, such as the commercially available ELISA based Quantiferon TB gold in tube assay and ELISPOT based T-SPOT.*TB* assay are able to discriminate between BCG vaccination responses and actual infection with *M. tuberculosis*, they are insufficient to specifically discriminate between active and latent infection (HINKS et al., 2009; LALVANI, 2007; SESTER et al., 2011a). Our aim was therefore to identify phenotypical and functional markers of the *M. tuberculosis* specific T cell response as biomarkers with potential correlation with disease activity *in vivo*.

Based on our studies on cytomegalovirus infection in chapter 3.1.1 of this thesis and evidence from other viral infections (PANTALEO, HARARI, 2006; SESTER et al., 2008b), disease activity correlates with changes in cytokine profiles of the pathogen specific T cells. This approach was applied to improve *M. tuberculosis* diagnostics. Indeed, active *M. tuberculosis* infection (A-TB) was associated with a loss of PPD specific CD4 T cell multifunctionality, which was not seen in successfully treated (T-TB) or latent *M. tuberculosis* infection (L-TB). A frequency of less than 56% of IFNγ/IL-2 cytokine secreting cells identified patients with active tuberculosis with a specificity of 100% and a sensitivity of 70%. These data, where I contributed with experimental analyses and supervision of a medical student are published and described in detail in (SESTER et al., 2011b).

3.2.1 CTLA-4 and CD27 expression on PPD specific CD4 T cells as an indicator of active *M. tuberculosis* replication

In addition to cytokine profiling, a loss of CD27 on *M. tuberculosis* specific CD4 T cells has been shown to correlate with active infection (STREITZ et al., 2007). As we demonstrated in the previous chapters, that expression of inhibitory T cell receptors correlates with disease activity in active CMV infection, we sought to characterise expression of these molecules on antigen-specific T cells of patients with active *M. tuberculosis* infection. Our unpublished preliminary data from a first series of patients indicated CTLA-4 expression on purified protein derivate (PPD) specific CD4 T cells may be more informative than PD-1 expression (data not shown).

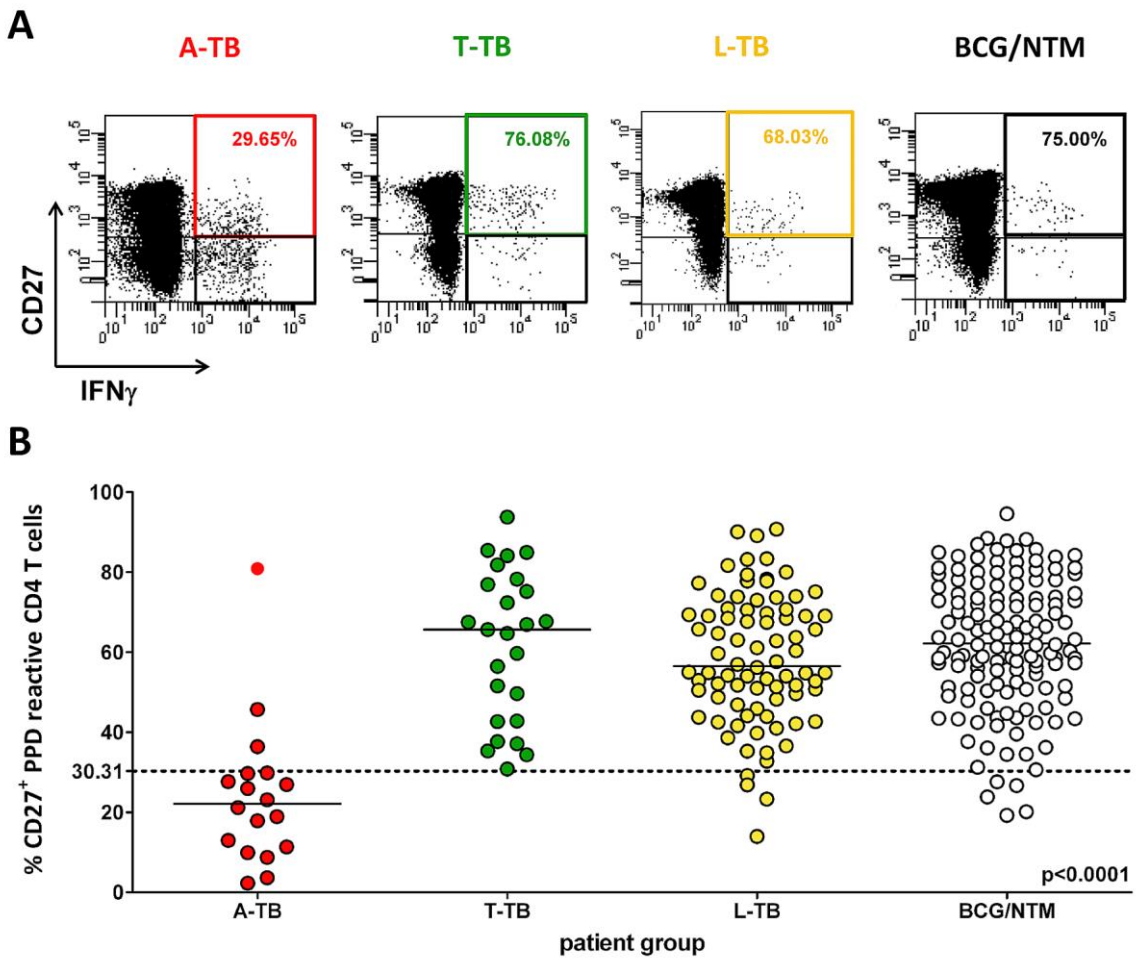


Figure 23: Loss of CD27 expression on PPD specific CD4 T cells is indicative of active tuberculosis infection. Whole blood from all individuals was stimulated with PPD and antigen specific CD4 T cells co-expressing CD69 and IFNγ were quantified and analysed for CD27 expression using flow cytometry. (**A**) Representative examples of CD27 expression on PPD specific CD4 T cells are shown for each patient group. Percentages of CD27 expressing cells among PPD specific CD4 T cells are indicated. (**B**) Patients with A-TB had significantly lower percentages of CD27 expressing cells, than all three non-active states ($p<0.0001$). The stippled line indicates the 30.31% threshold established by ROC analysis.

Therefore we analysed the expression of the biomarkers CTLA-4 and CD27 on PPD specific CD4 T cells, compared their diagnostic power to cytokine profiling and elucidated if combination of the markers might be of added value. As with cytokine profiling, we performed 6h antigen specific stimulation of whole blood samples from 18 patients with confirmed active tuberculosis, 25 patients with successfully treated TB, 108 patients with latent TB infection and 137 with immunity consistent with BCG vaccination or NTM infection. Latent TB infection was defined by reactivity towards PPD concomitant with reactivity towards ESAT-6 and/or CFP-10 proteins. PPD specific CD4 T cells were enumerated via IFNγ/CD69 co-expression and analysed for CD27 expression. A representative CD27 expression pattern for each patient group is illustrated in Figure 23A. Patients with active *M. tuberculosis* infection showed significantly lower frequencies of CD27 expressing PPD specific cells compared to the three non-active states ($p<0.0001$, Figure 23B). Based on receiver operator characteristics (ROC) analysis, a percentage of less than 30.31% CD27

positive PPD specific CD4 T cells discriminated with 100% specificity and 83.33% sensitivity between patients with active TB and successfully treated TB (AUC=0.93, Figure 23B and Table 13). The threshold of <30.31% CD27 positive CD4 T cells was also powerful to discriminate patients with active TB from latently infected and BCG vaccinated/NTM infected individuals (91.67% and 96.35% specificity, 83.33% sensitivity, AUC of 0.91 and 0.93 respectively, Figure 23B and Table 13). Thus, a decrease in CD27 expression on PPD specific CD4 T cells is highly indicative of active *M. tuberculosis* replication.

As with analysis of CD27, expression of CTLA-4 on PPD specific CD4 T cells was characterised in a similar set of patients. A total of 40 patients with confirmed active TB, 9 with successfully treated TB, 45 with latent TB infection and 249 with BCG/NTM related immunity were tested. As illustrated by representative profiles in Figure 24A, patients with active TB showed significantly higher frequencies of CTLA-4 expressing PPD specific CD4 T cells than the three non-active states ($p<0.0001$; Figure 24B). ROC analysis revealed a percentage of >17.5% CTLA-4 expressing PPD specific CD4 T cells to be best in discriminating between active TB and successfully treated TB (specificity 88.89%, sensitivity 72.50%, AUC=0.84, Figure 24B and Table 13). This threshold discriminated tuberculosis patients similarly well from latently infected and BCG vaccinated/NTM infected individuals (latent TB: 91.11% specificity, 72.50% sensitivity, AUC=0.90; BCG/NTM: 92.77% specificity, 72.50% sensitivity, AUC=0.91; Figure 24B and Table 13).

3.2.2 Combination of phenotypical and functional markers might further improve diagnostic power

Compared to our previously published results on cytokine profiling (SESTER et al., 2011b), CD27 seems to be of higher sensitivity in discriminating active from successfully treated TB than the IFNγ/IL-2 profile (83.33% vs. 70.00% sensitivity, respectively), with both being of 100% specificity.

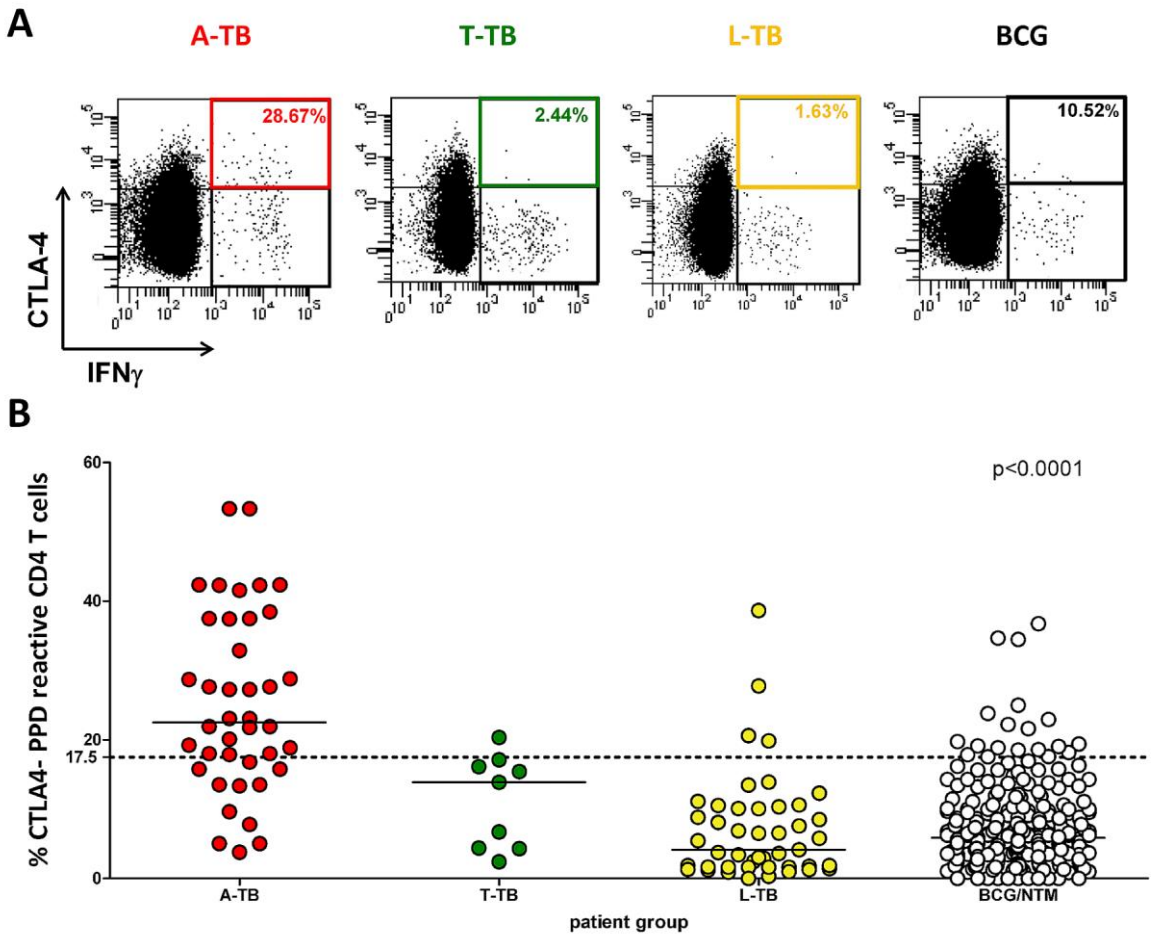


Figure 24: Increase in frequency of CTLA-4 expressing PPD specific CD4 T cells is indicative of active tuberculosis infection. PPD specific CD4 T cells were analysed for CTLA-4 expression using flow cytometry. (**A**) Representative examples of CTLA-4 expression are shown for each patient group. Percentages of CTLA-4 expressing cells among PPD specific CD4 T cells are indicated. (**B**) Frequencies of CTLA-4 expressing cells are significantly higher in patients with A-TB compared to all three non-active states ($p<0.0001$). The stippled line indicates the 17.5% threshold established by ROC analysis.

Table 13: Analysis of CD27 and CTLA-4 allows discrimination between active *M. tuberculosis* infection and non-active states.

Biomarker	Threshold for active TB	A-TB vs. T-TB			A-TB vs. L-TB			A-TB vs. BCG/NTM		
		AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity
CD27	<30.31%	0.93	83.33%	100%	0.91	83.33%	91.67%	0.93	83.33%	96.35%
CTLA-4	>17.50%	0.84	72.75%	88.89%	0.90	72.75%	91.11%	0.91	72.75%	92.77%
IFNγ/ IL-2*	<56.00%	0.84	70.00%	100%	0.87	70.00%	100%	0.87	70.00%	100%

The respective potentials to diagnose active TB infection between the inhibitory T cell receptor CTLA-4, the co-stimulatory receptor CD27 and the IFNγ/IL-2 cytokine profile were compared. Thresholds for diagnosis of active TB were determined via ROC analysis. *The values for the IFNγ/IL-2 cytokine profile are taken from a previous publication (SESTER et al., 2011b). A-TB, active tuberculosis, L-TB, latent infection with *M. tuberculosis,* BCG, Bacille Calmette-Guérin vaccine, NTM, nontuberculous mycobacteria.

In comparison, CTLA-4 is slightly more sensitive but less specific than the IFNγ/IL-2 profile (72.50% and 88.89%). Indeed, CD27 seems to have the best diagnostic power of the three markers in discriminating A-TB from T-TB (AUC=0.93 for CD27 vs. AUC=0.84 for CTLA-4 and IFNγ/IL-2, respectively). To evaluate if the combination of the markers might further increase the diagnostic potential, we performed parallel staining and analysis of CTLA-4, CD27 and IFNγ/IL-2 in 9 patients with clinically proven active and 4 patients with successfully treated *M. tuberculosis* infection. Although sample size was low, the combined analysis of the IFNγ/IL-2 cytokine profile with either CTLA-4 or CD27 did not improve the diagnostic power to discriminate between active and successfully treated TB compared to analysis of single markers (Figure 25, IFNγ/IL-2 profiles <56% are indicated by squares). In contrast, combination of CTLA-4 and CD27 led to a strong increase in diagnostic power. Whereas CD27 and CTLA-4 alone discriminated with 100% specificity but rather low sensitivity (66% and 77%, respectively, Figure 25) between A-TB and T-TB in this subgroup of patients, the combination of both markers retained 100% specificity and increased sensitivity to 100%. Likewise the negative predictive value (NPV) increased to 100%, compared to 57% for CD27 and 66% for CTLA-4 analysis only. Even though limited by the small number of patients included, the combination of these two markers may have potential as a powerful tool to improve immune-based diagnosis of active tuberculosis.

3.2.3 The immunological profile in patients with latent *M. tuberculosis* infection at risk for progression to tuberculosis

Up to now there is neither a measure to estimate the individual risk for progression from latent to active *M. tuberculosis* infection with sufficiently high positive predictive value nor to discriminate a *de novo* infection from a previous immunological well contained infection. As demonstrated above, all three of our analysed markers are able to discriminate between active and non-active states of *M. tuberculosis* infection. We therefore used the combined analysis of these markers to elucidate, whether alterations in the expression of the markers may also be observed in asymptomatic individuals at increased risk for active TB.

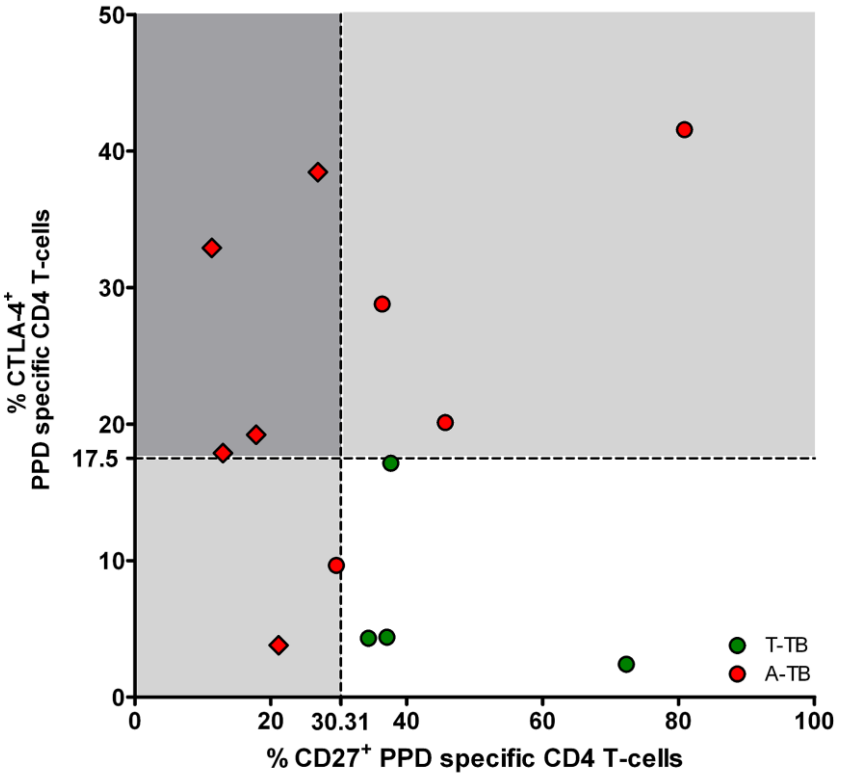


Figure 25: Combination of phenotypical and functional markers might further increase diagnostic potential. In a subset of 9 patients with A-TB and 4 with T-TB staining and analysis of CD27, CTLA-4 and the IFNγ/IL-2 cytokine profile were performed in parallel. Combination of CD27 and CTLA-4 analysis improves discrimination between A-TB and T-TB to 100% sensitivity and specificity each. Stippled lines indicate the thresholds of <30.31% for CD27 and >17.5% for CTLA-4. Round dots indicate patients with >56%, square dots patients with <56% IFNγ/IL-2 co-producing cells. A-TB patients are indicated by red and patients with T-TB by green colour.

A total of 25 ESAT-6 and/or CFP-10 confirmed latently infected individuals with increased exposition risk or clinical suspicion of TB infection were recruited. Thirteen of those individuals were analysed in the context of TB contact tracing, 3 because of increased exposure risk, 3 in the context of immunosuppression and 6 due to radiological or clinical signs compatible with tuberculosis. Of the individuals analysed, 13/25 (52%) were below the threshold for active disease for all three markers, 8/25 (32%) showed a shift in either IFNγ/IL-2 profile (n=7) or in CD27 expression, and 4/25 (16%) showed a shift in two of the markers (Figure 26). Among these, two showed a shift in their IFNγ/IL-2 profile and CD27 expression, one a shift in IFNγ/IL2 and CTLA-4 and one a shift in both CD27 and CTLA-4 (Figure 26, IFNγ/IL-2 profiles <56% are indicated by squares). Of note, the patient who showed a shift in CD27 only displayed 57% IFNγ/IL-2 double positive CD4 T cells which is close to the threshold of 56%.

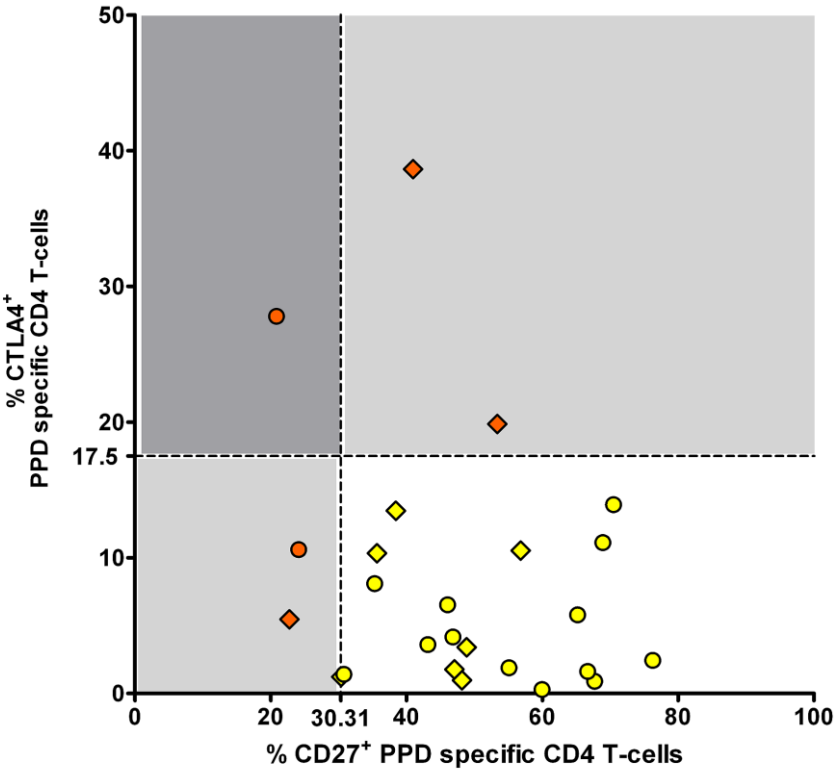


Figure 26: Immunological profile in TB contacts. 25 ESAT-6/CFP-10 confirmed L-TB patients with increased exposition risk or clinical suspicion of TB infection were analysed for expression of CD27, CTLA-4 and the cytokines IFNγ/IL-2. 4/25 (16%) patients showed a shift towards an A-TB associated profile in 2 of the three markers and 8/25 (32%) in one marker. Stippled lines indicate the thresholds of <30.31% for CD27 and >17.5% for CTLA-4. Round dots indicate patients with >56%, square dots patients with <56% IFNγ/IL-2 co-producing cells.

3.3 Phenotype and functionality of CD4 T cell immunity induced by pandemic H1N1 vaccination differs from active influenza infection

As described in detail in our recent publication (SCHMIDT et al., 2012a), we analysed the induction of antigen-specific T cells in 19 individuals before and after vaccination against the pandemic H1N1 strain (pdmH1N1). Part of this thesis focussed on the comparison of the vaccine induced phenotype and functionality of the pdmH1N1 specific immune response with that observed after natural H1N1 infection. As illustrated in Figure 27A and C we found that the pdmH1N1 specific CD4 T cells one week after vaccination consisted mainly of IFNγ/IL-2 co-producing as well as IFNγ single producing cells and lower numbers of IL-2 single producing cells. Already at week two after vaccination the functional profile changed to a predominance of IFNγ/IL-2 co-producing cells, followed by cells producing IL-2 and IFNγ only. This phenotype seems to be stable over time, as suggested by a similar profile 10 weeks after vaccination (Figure 27A and C). In contrast, acute infection with pdmH1N1 led to a functional profile dominated by IFNγ single producing cells, with markedly less IFNγ/IL-2 co-producers and an almost complete absence of IL-2 single producers (Figure 27B and D).

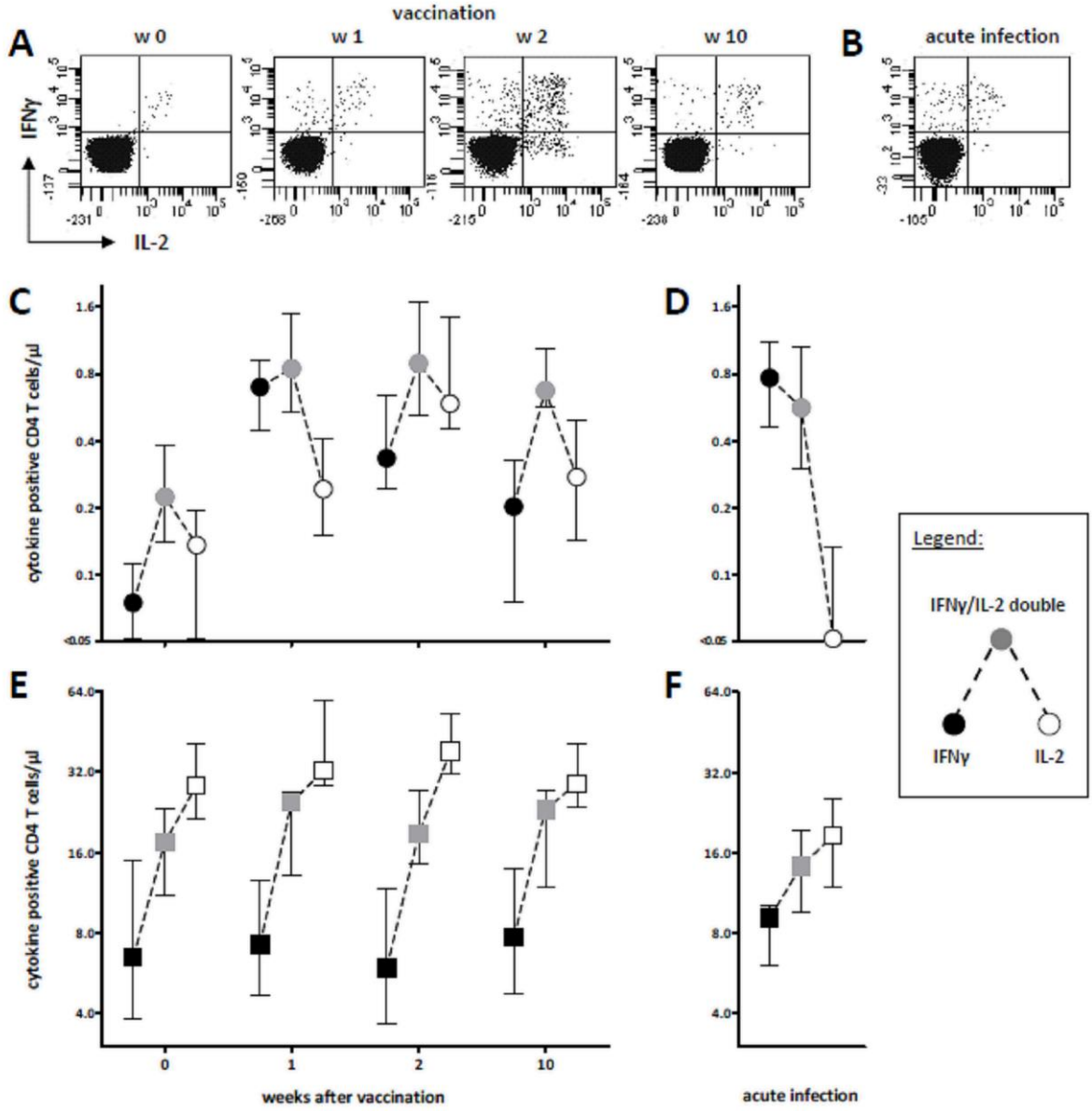


Figure 27: Influenza-specific CD4 T cells induced after vaccination differ in the cytokine expression profile from T cells in acute infection. Influenza-specific CD4 T cells induced after vaccination differ in the cytokine expression profile from T cells in acute infection. Representative dotplots of the flow cytometric analysis of IFNγ and IL-2 expression in CD4 T cells after stimulation with pdmH1N1 antigen in blood samples of (**A**) one healthy individual (not prevaccinated against seasonal influenza) before and after vaccination with pdmH1N1 and (B) one patient with acute pandemic H1N1 influenza infection within 1 week after onset of symptoms. IFNγ/IL-2 cytokine profiling was performed in pdmH1N1-specific CD4 T cells of (C) all 19 individuals after immunization with pdmH1N1 over time and (**D**) of seven patients with acute pandemic H1N1 influenza infection. Shown are the median values and IQR of absolute numbers of IFNγ single positive (black circles), IFNγ/IL-2 double positive (grey circles), and IL-2 single positive (white circles) CD4 T cells. Respective values after stimulation with SEB (positive control) are depicted for (**E**) the vaccination cohort (n=19) and (**F**) the acutely infected patients (n=7). Stimulation with the seasonal vaccine yielded the same results (data not shown).

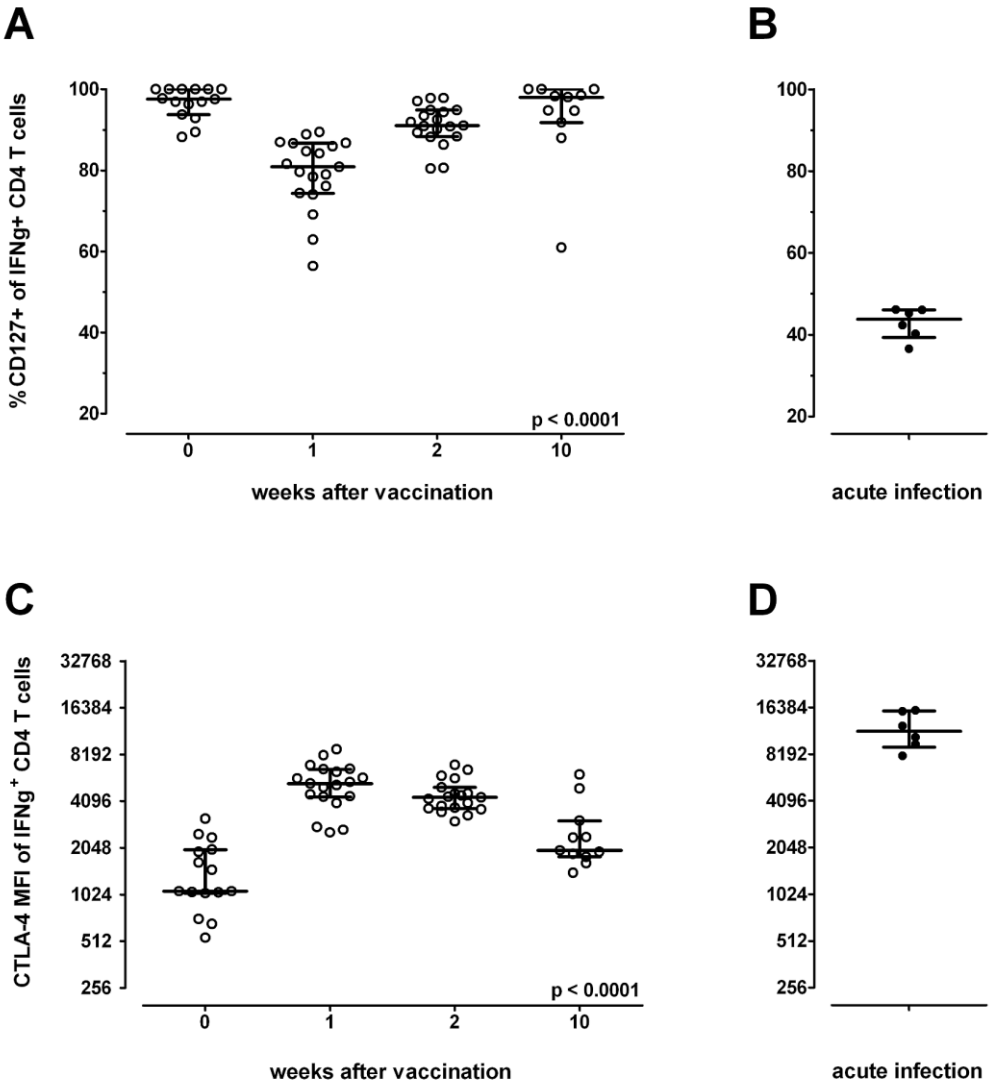


Figure 28: Differences in CD4 T cell phenotype after pdmH1N1 vaccination and acute infection. Induction of pdmH1N1 specific CD4 T cells after vaccination is associated with changes in phenotype, which are clearly distinct from phenotype after acute infection. Percentages of CD127 expressing IFNγ positive CD4 T cells after stimulation with the pdmH1N1 antigen of all tested individuals are depicted over time (**A**) and in patients during acute pdmH1N1 infection (**B**). CTLA-4 mean fluorescence intensities (MFI) of pdmH1N1 specific CD4 T cells of all tested individuals are depicted over time (**C**) and in patients during acute pdmH1N1 infection (**D**). Each symbol represents data from one individual. Analysis was performed in all 7 patients with acute infection and in all 19 individuals before vaccination, and after 1 and 2 weeks. In 4 of 19 individuals, the percentage of reactive T cells before vaccination was too low to perform phenotypical analysis. Analysis after 10 weeks was performed in 11 individuals only. Bars indicate median values and IQR. Statistical analysis was performed using the Kruskal-Wallis test.

To assess the phenotype of the induced responses, we analysed in a second staining reaction the expression of the T cell memory marker CD127 and the T cell inhibitory receptor CTLA-4. Because of low pdmH1N1 specific responses, this analysis could only be performed in 15/19 individuals pre-vaccination (Figure 28).

In line with a memory T cell phenotype in the absence of recent antigen encounter, almost all pdmH1N1 specific CD4 T cells expressed CD127 (median 97.6%, IQR 5%, Figure 28A). The newly generated pdmH1N1 specific CD4 T cells at week one post-vaccination, consisted to a higher percentage of CD127 negative effector phenotype like CD4 T cells, resulting in a reduced proportion of CD127 positive cells (median 80.0%, IQR 12.1%, $p<0.0001$, Figure 28A). This shift in memory/effector phenotype was only transient, as at week two already 91.1% (12.3%) of pdmH1N1 specific cells (re-)expressed CD127 and reached pre-vaccination levels at week ten (median 98.0%, IQR 6%, Figure 28A). CTLA-4 mean fluorescence intensities (MFI) on pdmH1N1 specific CD4 T cells showed an inverse relationship, as they increased from pre-vaccination levels to peak levels at week one (median 5311, IQR 2204) and declined thereafter to almost pre-vaccination levels at week ten (Figure 28C, $p<0.0001$). In contrast to this peak, CTLA-4 expression levels during acute pdmH1N1 infection were about 2.2fold higher than peak levels post-vaccination (median 11479, IQR 6405, Figure 28D, $p=0.0005$). Likewise CD127 expressing cells were significantly reduced to 43.8% (6.79) compared to pre-vaccination ($p=0.0003$, Figure 28B).

Similar to our results on CMV and *M. tuberculosis* infection, we observed a pro-inflammatory effector response in acute pdmH1N1 infection, whereas vaccination with pdmH1N1 antigens led to a protective multifunctional response of predominantly memory phenotype.

3.4 Pathogen prevalence may determine maintenance of antigen specific T cell responses after HIV infection

One serious issue in HIV infection is the weakened immune response to numerous pathogens, resulting in an increase in opportunistic infections, reduced vaccination responses and low sensitivity in T cell based diagnostic assays. In the following part of the thesis, we assessed whether the increased turnover of CD4 T cells in HIV infection favours the maintenance of immune responses towards antigens that are regularly encountered over those with no or only limited antigen rechallenge and might therefore be linked to antigen prevalence.

To model differences in antigen rechallenge we analysed specific immunity in latent *M. tuberculosis* infection in healthy controls and HIV patients in a high (Tanzania) and a low prevalence country (Germany). We compared this with specific immunity in CMV infection, where continuous endogenous antigen rechallenge is present.

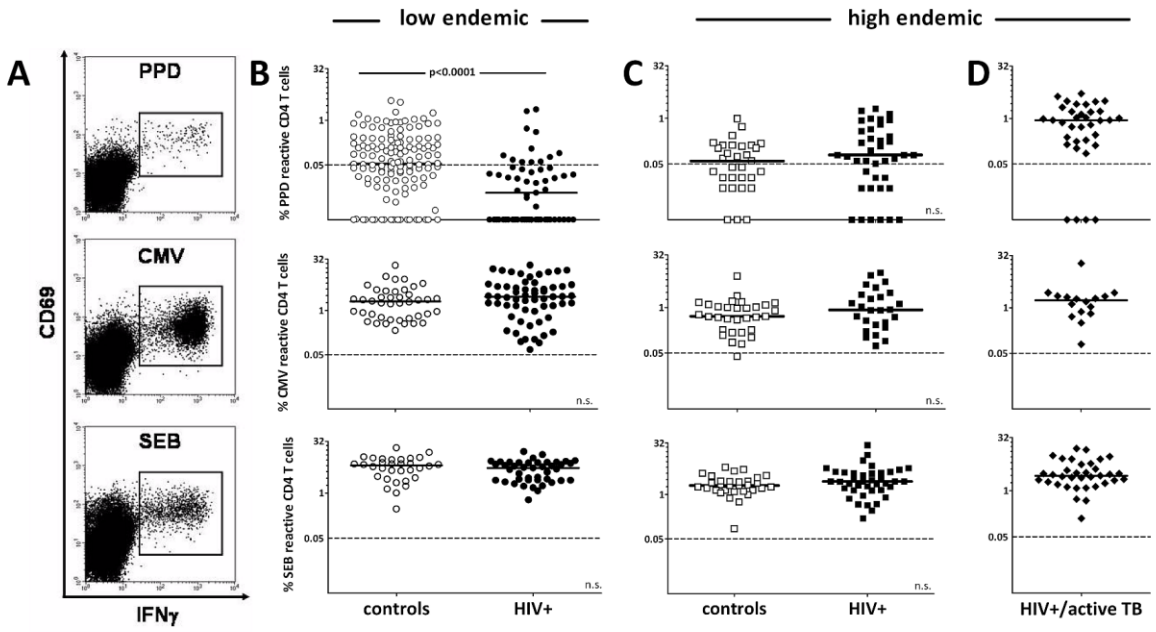


Figure 29: Decreased prevalence and frequency of PPD reactive CD4 T cells in HIV$^+$ patients from Germany. (**A**) Representative dot plots after purified protein derivative (PPD), cytomegalovirus (CMV) and SEB stimulation, where antigen-reactive CD4 T cells are defined as CD69 and IFNγ coexpressing cells. PPD, CMV and SEB reactive CD4 T cells were assessed in healthy controls and HIV positive patients without active tuberculosis from (**B**) Germany and (**C**) Tanzania. (**D**) In addition, HIV positive patients with active tuberculosis are shown. They are from the same endemic region as individuals in panel (C), who serve as controls for this group. Median frequencies are indicated by bold lines, the dotted line represents the detection limit for antigen-reactive CD4 T cells (0.05%). CMV specific T cell frequencies are displayed for CMV seropositive individuals. Significant differences were observed between PPD reactive T cell frequencies in Tanzanian HIV infected individuals with and without active tuberculosis (p<0.0001, compare panel C and D), whereas CMV or SEB reactive T cell frequencies did not differ (p=n.s.). TB, tuberculosis.

Data from Tanzanian patients were provided by Dr. Alexandra Schütz and Prof. Dr. Andreas Meyerhans who collaborated in this part of the project.

3.4.1 A low prevalence and frequency of PPD specific immunity is a particular feature of HIV related immunosuppression in tuberculosis low endemic regions

To test if HIV related immunodeficiency may affect cellular immunity toward mycobacterial antigens, PPD reactive CD4 T cells were assessed in 60 HIV infected individuals and 144 age matched HIV seronegative healthy controls from Germany. Typical dot plots of antigen specific CD4 T cells are shown in Figure 29A. The percentage of individuals with detectable T cell immunity towards PPD was significantly lower in HIV positive patients (20%) as compared to healthy controls (52.7%, P<0.0001, Figure 29B). In line with these results, HIV positive patients showed a significantly lower frequency of PPD specific CD4 T cells (median 0.008%, ≤0.001–2.08%) as compared to immunocompetent individuals (median 0.05%, ≤0.001–3.76%; p<0.001). Interestingly, this was not due to a general loss of immunity

towards recall antigens, as both groups did not differ in CMV reactive or SEB reactive T cell frequencies (p=0.20 and p=0.30, respectively, Figure 29B).

3.4.2 A high antigen prevalence is associated with a sustained PPD specific immunity in HIV infected individuals from a tuberculosis high prevalence region

The observed loss in PPD specific immunity in HIV-positive individuals may be due to a considerably low antigenic challenge in a low endemic region such as Germany. To assess the effect of a higher extent of exogenous challenge with *M. tuberculosis*, PPD reactive T cells were analysed in a group of 39 HIV infected patients from a high prevalence country. Notably, in this setting, the percentage of patients with detectable PPD specific immunity was higher (64.1%) as compared to HIV positive patients in a low endemic region and indistinguishable to HIV seronegative healthy controls (51.6%, p=0.34). Likewise, median frequencies of PPD reactive CD4 T cells did not differ between HIV positive individuals and controls from endemic regions (0.09%, ≤0.001–1.91% and 0.06%, ≤0.001–0.99%, respectively, p=0.29, Figure 29C). Moreover, the two groups did not differ in their frequencies of reactive T cells towards CMV and SEB (p=0.22 and p=0.17, Figure 29C). Together this indicates that an increased extent of mycobacterial exposure in a high endemic area may contribute to the maintenance of specific immunity in HIV infected individuals. Of note, differences in sex did not have any confounding effect on our results observed in low and high endemic regions. In Germany, the percentages of both male and female HIV infected individuals with positive PPD responses was lower as respective controls (45.7% female controls vs. 14.3% female HIV infected individuals, p=0.04; 66.0% male controls vs. 21.7% male HIV infected individuals, p<0.0001). Likewise, overall results in Tanzania were not confounded by sex (60.0% female controls vs. 63.2% female HIV infected individuals, p=1.00; 47.6% male controls vs. 77.8% male HIV infected individuals, p=0.23). Moreover, there was no significant difference in the percentage of PPD positive responses in patients with and without antiretroviral therapy (ART) (Germany: p=1.00, Tanzania: p=0.74). To assess the effect of active tuberculosis on PPD specific immunity in HIV infected individuals, a total of 34 patients with active tuberculosis were recruited from the same high endemic region. In this group, both the prevalence of a specific response (88.2%) as well as their median frequencies (0.9%, ≤0.001–5.2%) were significantly higher as compared to HIV infected patients or healthy controls without active tuberculosis (P<0.001, Figure 29D). PPD specific T cell frequencies did not

show any correlation with CD4 T cell counts/ml (r=0.03, p=0.89) or viral load (r=0.02, p=0.96). Likewise, although most patients with tuberculosis were treatment naive, their PPD specific T cell frequencies were still higher as compared to respective HIV infected patients without tuberculosis (p=0.017). Thus, differences in these parameters are unlikely to contribute to the increased frequency of PPD reactive T cells in patients with active tuberculosis. As expected, tuberculosis in HIV infected individuals did not have any effect on the frequencies of CMV reactive or SEB reactive CD4 T cells (p=0.10). Notably, however, CMV reactive T cell levels in patients with active tuberculosis were slightly higher as compared to HIV seronegative controls (p=0.03).

4 Discussion

Adaptive immunity is important for pathogen control and its loss results in uncontrolled pathogen replication and disease. In this thesis, phenotypical and functional characterisation of specific immunity towards clinically relevant persistent pathogens such as CMV and *M. tuberculosis* as well as vaccine antigens was performed. A more terminal effector memory differentiation state was found to be a common determinant for pathogen chronicity, which was indicated by the loss of CD27 on PPD specific and a parallel loss of CD28 and CD27 on CMV specific T cells. Furthermore, a loss of multifunctionality and an increase in the expression of anergy markers such as PD-1, TIM-3 and CTLA-4 was found to correlate with disease activity. We postulate that a meaningful combination of these biomarkers might help to assess the risk of pathogen and disease reactivation and progression and may in future play an important role in guidance of therapy initiation and duration.

4.1 CMV immunomonitoring in transplant recipients

Cytomegalovirus is a clinically relevant persisting pathogen, which is immunologically well controlled in immunocompetent individuals, yet may cause severe clinical symptoms in immunocompromised patients (FISHMAN, 2007; FISHMAN, RUBIN, 1998; GANDHI, KHANNA, 2004). It is well known that the risk of CMV complications after solid organ transplantation is determined by the level of immunosuppression and the transplanted organ (FISHMAN, RUBIN, 1998; SMITH et al., 2001; SPEICH, VAN DER BIJ, 2001). CMV complications in renal transplant recipients are largely restricted to the first three month post-transplant, whereas heart and lung transplant recipients are at prolonged risk to suffer from infectious complications (FISHMAN, RUBIN, 1998; LOWANCE et al., 1999; ZAMORA, 2004). Furthermore, seronegative recipients of a seropositive organ are at increased risk of infectious complications, as they have to establish a protective immune response under the influence of high levels of immunosuppressive drugs (SMILEY et al., 1985). CMV specific T cells have been proven critical for CMV control and we and others have shown that a quantitative loss of CMV specific immunity correlates with CMV disease in transplant patients (KOTTON et al., 2013a; SESTER et al., 2001; WALKER et al., 2007; WESTALL et al., 2008).

Most immune-based assays to analyse cell-mediated immunity towards CMV such as the commercially available IFNγ release assays based on ELISA or ELISPOT assays are able to quantify immune responses but do not assess T cell phenotype and multifunctionality. Whereas this might be helpful to determine the onset of a CMV specific immune response in CMV seronegative recipients of a seropositive organ (MANUEL et al., 2013), these assays are not well suited to predict episodes of viral reactivation or CMV-relapse after cessation of antiviral therapy in transplant patients with pre-existing CMV specific immunity (KOTTON et al., 2013b).

In this thesis functional and phenotypical changes of CMV specific T cells were shown to correlate with clinical events and the occurrence of CMV reactivation episodes in longitudinally monitored kidney transplant patients. Furthermore, the diagnostic potential of the identified markers of T cell anergy to predict the risk of viral reactivation was evaluated.

4.1.1 Function and phenotype of CMV specific immune responses are interrelated and correlate closely with episodes of viremia after transplantation

Previous studies by our group and others indicated that quantitative changes in CMV specific T cell frequencies correlate with CMV disease but not with CMV reactivation per se (SESTER et al., 2001; WALKER et al., 2007; WESTALL et al., 2008). This was supported by data from this thesis, as we observed no quantitative differences in CMV specific T cell frequencies between patients with and without reactivation episodes but reduced frequencies in patients with acute primary CMV infection, which is in line with the newly initiated induction of CMV specific immunity (Figure 3). In contrast to this, the presented data clearly demonstrate a loss of multifunctionality of CMV specific CD4 T cells not only in patients with CMV primary infection but also in those with reactivation episodes. CMV specific T cells lose the ability to co-produce the cytokines IL-2, TNFα, and IFNγ and show an impaired proliferative capacity. Interestingly, co-production of IFNγ and TNFα seems to be far more robust than IFNγ/IL-2 co-production. IFNγ/TNFα co-production is only lost during high level viremic episodes and recovers rapidly after resolution of viremia. In contrast, co-production of IFNγ and IL-2 is lost during high and low level viremic episodes, does not recover rapidly after resolution of viremia, and even tends to be lost in immunocompromised patients without reactivation (Figure 4 and Figure 5). This loss of multifunctionality was paralleled by phenotypical changes, as the expression of the inhibitory T cell receptors PD-1, TIM-3 and

CTLA-4 was significantly increased during viremic episodes (Figure 6 and Figure 8). Combined analysis of CTLA-4, IFNγ and TNFα in the same staining reaction indicated that increased CTLA-4 expression was directly linked to a loss in T cell multifunctionality (Figure 18).

Our results are in line with those on other chronic viral infections such as HIV, HCV and HBV, where insufficient control of viral replication was marked by loss of T cell multifunctionality and an increase in inhibitory receptor expression (D'SOUZA et al., 2007; DAY et al., 2006; KASPROWICZ et al., 2008; KAUFMANN et al., 2007; PENG et al., 2008; PETROVAS et al., 2006; RADZIEWICZ et al., 2007; TRAUTMANN et al., 2006; URBANI et al., 2008). Likewise, we recently showed that a loss in T cell multifunctionality was also observed in renal transplant recipients with clinically relevant BK polyomavirus replication (SCHMIDT et al., 2014). Of note, increased inhibitory T cell receptor expression and dominance of IFNγ-only producing cells during primary infection per se should not necessarily be mistaken as a sign of impaired immunity, but rather a physiological response to down-regulate the primary immune response (BARBER et al., 2006; PANTALEO, HARARI, 2006). This is also emphasised by results derived from our vaccination study for pandemic H1N1 influenza, where we observed an increased CTLA-4 expression and dominance of IFNγ-only producing cells not only in patients with acute pandemic influenza, but also in the time period of acute induction of immune response to vaccination (SCHMIDT et al., 2012a). Yet, in vaccinated individuals, this anergic phenotype was only temporary, as CTLA-4 expression decreased and multifunctionality was fully restored at week ten after vaccination (SCHMIDT et al., 2012a). In contrast to vaccination and self-limiting infections, the anergic phenotype is sustained in chronic uncontrolled infection. This is illustrated in this thesis by longitudinal data from a patient with primary CMV viremia who maintained high levels of anergy marker expression for about 200 days post-infection and suffered from recurrent viremia during this period (Figure 7).

Another feature of the observed anergic phenotype is a decreased CMV specific proliferation capacity (Figure 19 and Figure 20). Interestingly, it has been demonstrated for other chronic viral infections before that in vitro blockade of inhibitory pathways can at least partly reverse anergy (BARBER et al., 2006; BLACKBURN et al., 2009; DAY et al., 2006; KAUFMANN et al., 2007; SAKUISHI et al., 2010; TRAUTMANN et al., 2006; URBANI et al., 2008). Previous work from our group has shown that PD-1 mediated reversion of functional anergy also

occurred in CMV viremic patients (DIRKS, 2008; SESTER et al., 2008b). This thesis now further analysed the influence of in vitro blockade of other inhibitory pathways on proliferation and cytokine production. It was shown that blockade of PD-1, and to a lesser extent TIM-3 signalling pathways enhanced proliferative capacity (Figure 19), similarly as demonstrated before in antigen-specific T cells from patients with chronic HIV and HCV infection (JONES et al., 2008; MCMAHAN et al., 2010). In addition to an increase in proliferation, multiplex analysis revealed that in vitro blockade of PD-1 signalling in peripheral blood mononuclear cells from patients with high levels of PD-1 on CMV specific T cells restored the production of cytokines such as IL-2, TNFα, IFNγ, IL-6, IL-9 and IL-10 (DIRKS et al., 2013). As our assay also comprised cells from the myeloid lineage, blocking of PD-1 signalling additionally led to a recovery of the cytokines IL-1β, G-CSF, or MIP-1α (DIRKS et al., 2013).

Therapeutic use of blocking antibodies towards inhibitory T cell receptors to restore anergic T cells in vivo is already accomplished in cancer therapy, with so far promising results (ARANDA et al., 2014; HAMID et al., 2013; RIBAS, 2012; TARHINI, IQBAL, 2010; TOPALIAN et al., 2012). Direct therapeutic use to treat chronic viral infections in vivo has not been explored in humans, as inhibitory receptor blockade may have severe side effects and might trigger a variety of autoimmune responses (CRANMER, HERSH, 2007; MOVVA, VERSCHRAEGEN, 2009). Yet, inhibitory receptor blockade might be an option to improve current protocols for in vitro expansion of antigen specific T cells for adoptive transfer (BLYTH et al., 2013; EINSELE et al., 2002; GERDEMANN et al., 2011; HAMID et al., 2013; RIBAS, 2012; TOPALIAN et al., 2012). This may be of particular practical relevance, as adoptive transfer of in vitro expanded T cells is presently used mainly as rescue therapy for severe CMV infections in organ or stem cell transplant recipients. As autologous T cells may likely be functionally anergic in this situation, blockade of inhibitory receptors during in vitro expansion may represent means to improve cell yield for therapeutic infusion.

4.1.2 Features and applicability of T cell based assays for monitoring of CMV infection after organ transplantation

Despite the clinical utility of the above described approach, the ultimate goal of CMV immune monitoring would be not only to detect viremia but also to predict its occurrence. As the expression of the inhibitory receptors PD-1, TIM-3 and CTLA-4 correlated with a decreased functionality and directly with episodes of viremia, the diagnostic potential of the

three markers to detect and to predict episodes of viremia was assessed. Therefore, we performed receiver operator characteristics (ROC) analyses of the fold increase in anergy marker expression at onset of viremia versus pre-transplant. All three markers discriminated well between patients with high level viremia and patients without viremic episodes, with TIM-3 showing the highest diagnostic power (Figure 9). The combined analyses of all three markers led to an increase in sensitivity at the cost of lower specificity. Of note, our analysis revealed that TIM-3 alone, or combined analyses of all three markers was able to predict viremic episodes at a median of 7 days (IQR: 4.8-12) before onset of viremia. Although sensitivity was rather low, this is to our knowledge the first study in the field of CMV immune diagnostics, which provides evidence that analysis of phenotypical changes in CMV specific immunity can actually predict episodes of viremia in CMV seropositive patients post-transplant.

Based on current recommendations, high level viremia is clearly indicative for initiation of antiviral therapy. In contrast, there is currently no consensus on how to deal with low level viremia (KOTTON et al., 2013b). Thresholds for defining high level viremia differ significantly between individual centers, as do therapeutic decisions based on low level viremia. Therefore, initiation of antiviral therapy is highly variable from center to center. Results from this thesis indicate that the analysis of inhibitory receptor expression and/or T cell functionality may have potential to early identify patients with viremia and to more specifically target antiviral therapy to patients at risk for uncontrolled viremia. Phenotypical T cell monitoring could therefore be of significant clinical value, as it may contribute to reduction of both direct and indirect effects of CMV infection on patient health and graft outcome (SAGEDAL et al., 2004). Monitoring CMV specific T cell anergy has meanwhile already been included in the pre-emptive monitoring regimen in our transplant center where decisions on antiviral therapy are based on both viral load as well as on analysis of inhibitory T cell receptor expression on CMV specific T cells.

Current pre-emptive therapy approaches based on viral load monitoring only have been shown to be valuable in reducing treatment costs and potential side effects of antiviral therapy (BALFOUR, 1999; HELLEMANS et al., 2013). We postulate that efficacy of pre-emptive monitoring can be further improved by guiding initiation and duration of antiviral

therapy via assessment of the quality and functionality of patients CMV specific immunity (SESTER et al., 2008a).

4.1.3 Stimulation-independent approaches for CMV immunomonitoring

As described above, stimulation based assays offer the unique opportunity to combine functional and phenotypical characterisation of antigen specific T cell responses and can be performed within one working day. Yet, one limitation of these assays is the need of live and fully functional T cells. Fresh blood samples have to be kept refrigerated and should not be stored longer than 24-32 hours before processing, thereby complicating sample shipment and batched analyses of stored sampled. Pre-treatment of blood samples with reagents like T cell Xtend (Oxford Immunotec) might also increase storage time for use in ELISPOT assays for up to 32 hours. Isolation and subsequent freezing of peripheral blood mononuclear cells (PBMC) allows even longer storage time but is more laborious (MILSON, KELLER, 1982). Furthermore, freezing and re-thawing of samples leads to a quantitative loss in antigen reactive T cells or functional and phenotypical changes and hence is unsuitable for clinical decision making.

One approach to limit both the blood volume needed and sample processing time, is the use of HLA-peptide-multimers (ALTMAN et al., 1996; DAVIS et al., 2011). As they allow direct analysis of antigen specific T cells and can be combined with additional phenotypical characterisation, they might be a promising alternative to stimulation based assays (BROOIMANS et al., 2008; LA ROSA et al., 2008). Our results in kidney and stem cell transplant recipients showed that frequencies of CMV specific CD8 T cells after tetramer staining correlated with those observed after stimulation with the respective peptides and do not differ between healthy controls and immunosuppressed patient groups (Figure 10). We observed that PD-1 expression on CMV specific tetramer positive cells was increased during viremic episodes and correlated with both T cell functionality and viral load (Figure 11), which is in line with results observed in liver transplant recipients (LA ROSA et al., 2008). Analysis of PD-1 or other inhibitory T cell receptors on tetramer positive cells might therefore be a rapid and elegant means to monitor CMV specific T cell anergy after transplantation. Despite its simplicity, the tetramer approach has several drawbacks. Tetramer staining requires the knowledge of patients MHC-status and the availability of suitable MHC-peptide combinations matched for every patient. For CMV immunomonitoring,

this precludes a priori patients with unknown or uncommon HLA antigens, as only the most common HLA-peptide combinations are commercially available. This is illustrated by the fact that with the combination of tetramers against HLA-A1, -A2 and -B7 used in this study (Glycotype Biotechnology) only about ~60% of CMV seropositive individuals could be analysed (RAHMEL, 2013). The tetramer approach is further limited by the fact that T cell responses towards a given epitope differ widely between patients. Therefore, a response against a single epitope is not necessarily representative of the total CMV specific immune response (WIDMANN et al., 2008). Indeed, 15.73% of the patients analysed in this thesis did not react to the available HLA-peptide-combination, despite being positive for the respective HLA subtype (Figure 10).

Available literature and our own preliminary data suggested a specific loss of CD28 and CD27 expression on CD4 T cells of CMV seropositive individuals (FLETCHER et al., 2005; LOONEY et al., 1999; POURGHEYSARI et al., 2007; VAN DE BERG et al., 2008; VAN LEEUWEN et al., 2006; VAN LEEUWEN et al., 2004). Thus, one aim of this thesis was to evaluate if $CD28^{-}CD27^{-}$ CD4 T cells can be used as a stimulation independent surrogate for CMV specific T cell immunity in transplant recipients, and if an increase in PD-1 and CTLA-4 expression on these cells over time was a specific indicator of viremia. Based on a cross-sectional analysis of healthy controls, hemodialysis-patients and renal transplant recipients, frequencies of more than 0.44% of $CD28^{-}CD27^{-}$ CD4 T cells were tightly associated with CMV-seropositivity and quantitatively correlate with the frequency of CMV specific CD4 T cells identified based on cytokine induction after specific stimulation (Figure 12). Association with CMV is in line with previous studies showing that a large fraction of $CD28^{-}CD27^{-}$ CD4 T cells is able to produce cytokines and proliferate after CMV specific stimulation, but not after incubation with stimuli unrelated to CMV such as purified protein derivative (PPD), tetanus toxoid, or antigens from herpes simplex, Epstein Barr or varicella-zoster virus (DERHOVANESSIAN et al., 2011; FLETCHER et al., 2005; VAN LEEUWEN et al., 2006; VAN LEEUWEN et al., 2004). We additionally showed that $CD28^{-}CD27^{-}$ CD4 T cells were rapidly induced in 8 out of 10 patients with CMV primary infection, and their induction kinetics were largely comparable to those of CMV specific T cells. The two patients who did not develop $CD28^{-}CD27^{-}$ CD4 T cells during the observation period were exceptional in that they inadvertently did not receive antiviral prophylaxis and experienced CMV primary infection very early after transplantation (Figure 13). Of note, these patients also showed a delayed induction of CMV specific CD4 T cells that

remained at considerably low frequencies. Together with earlier work in patients with graft-related primary infection (RENTENAAR et al., 2000; SESTER et al., 2002c), this further emphasizes that primary infection early after transplantation may adversely affect the induction of sufficient CMV specific CD4 T cell immunity, which may directly be related to higher levels of immunosuppressive drugs.

Unlike dynamics in relative and absolute numbers of $CD28^{-}CD27^{-}$ CD4 T cells, an increased PD-1 expression on this T cell subset was associated with viremia in both CMV primary infection and reactivation, which is in line with our results in CMV specific CD4 T cells identified after stimulation (Figure 6 and (SESTER et al., 2008b)) or in CMV specific CD8 T cells after MHC-multimer staining (Figure 11 and (LA ROSA et al., 2008)). It is interesting to note that PD-1 expression also showed a slight but insignificant increase on $CD28^{-}CD27^{-}$ CD4 T cells in non-viremic patients, although the increase was less pronounced and kinetics were different from those in viremic patients. Whether this is due to a lower sample size of non-reactivating patients deserves further study. It is unlikely that this is directly mediated by immunosuppressive drugs, as PD-1 expression was not generally increased on bulk CD4 T cells. Rather, the initial increase in PD-1 expression may result from subclinical viral reactivation events in response to immunosuppressive drugs acting early after transplantation. As with other chronically persisting pathogens (DAY et al., 2006; TRAUTMANN et al., 2006; URBANI et al., 2006), a further increase in CMV-replication may lead to a more pronounced increase in PD-1 expression on specific T cells in a subset of patients. It thus seems that PD-1 expression dynamics over time show a direct association with the level of viremia, which is supported by a correlation between PD-1 expression-levels and viral load. Among patients with reactivation, there was no obvious difference in PD-1 expression dynamics between patients with low and high viral loads and symptoms compatible with CMV infection were only mild or absent. This may be due to the fact that viral load generally remained rather low in both groups due to a stringent implementation of pre-emptive therapy based on viral load monitoring and to variable length of antiviral therapy. It is thus tempting to speculate whether the increase in PD-1 expression would be more pronounced in symptomatic patients with higher viral loads in the absence or delay of antiviral therapy, although this was not possible to address in the context of this study.

In contrast to our results after antigen specific stimulation, no differences in CTLA-4 expression on $CD28^-CD27^-$ CD4 T cells were found between viremic and non-viremic patients. One possible explanation might be a more transient cell surface expression of CTLA-4 compared to PD-1 or TIM-3. In fact, CTLA-4 expression is up-regulated upon antigen stimulation and rapidly internalized thereafter by utilizing a mechanism of secretory lysosomes (IIDA et al., 2000). In line with this, we found that 6 hour stimulation with CMV or SEB antigen led to 1.25 or 2.60-fold increases, respectively, in CTLA-4 expression on total CD4 T cells compared to stimulation with control antigen (data not shown). In contrast, PD-1 or TIM-3 on total CD4 T cells did not increase following stimulation (Figure 20D and data not shown). Even though not included in this thesis, we have preliminary evidence indicating that TIM-3 expression on $CD28^-CD27^-$ CD4 T cells is up-regulated during viremic episodes in a similar fashion as on CMV specific T cells detected after stimulation. As with our results after antigen specific stimulation, an increase in PD-1 expression preceded the onset of viremia in longitudinally monitored patients, thus making it a possible tool to identify patients at risk for reactivation and/or to identify patients where the T cell anergy is too high to control viremia in the absence of antiviral therapy. Interestingly, an elevated PD-1 expression-level at the end of therapy correlated with recurrent episodes of viremia. Inability to control viremia was apparently reached, when the PD-1 expression on $CD28^-CD27^-$ CD4 T cells was 3.1-fold higher as compared to the remaining CD4 T cell subset (Figure 17B). Thus, this may be explored further as a highly specific parameter to identify recurrent episodes of viremia.

Determination of $CD28^-CD27^-$ CD4 T cells as surrogate for CMV specific T cells has a number of operational advantages compared to the currently used assays for analysis of CMV specific immunity, such as intracellular cytokine staining, ELISPOT assay or the ELISA-based QuantiFERON-CMV assay, which require higher blood volumes and necessitate antigen-specific stimulation for at least 6 to 20 hours (KOTTON et al., 2013a; SESTER et al., 2008a). Hence, these methods are more time consuming and require a complex panel of reagents. In contrast, PD-1 expression on $CD28^-CD27^-$ CD4 T cells may be determined directly from as little as 100µl of whole blood within 1 hour. Although we have used a sequential procedure for staining of molecules on the cell-surface and intracellularly in this study, the assay may in the future be simplified to a mere one-step surface staining procedure, as CTLA-4 expression was rather unrelated to viremia. Based on preliminary evidence, the cellular phenotype is

stable even after 2 days of sample storage which makes this technique amenable to shipment due to less critical pre-analytic time.

Despite these operational advantages, this approach also has some limitations. Although it had a high sensitivity, the specificity of PD-1 expression for viremia in an individual patient at a given time point was limited by the fact that non-viremic patients also showed some slight increase. In addition, PD-1 expression dynamics may have been confounded by antiviral therapy or by infections other than CMV, but we did not find any evidence for differences in PD-1 expression dynamics on $CD28^-CD27^-$ CD4 T cells in patients with and without BKV-related or other herpesviral infections (data not shown). Clearly, as with other assays for analysis of cellular immunity, it is too early to recommend monitoring of cellular immunity as the only monitoring tool to guide antiviral therapy (KOTTON et al., 2013a).

In conclusion, we have evaluated the clinical utility to directly analyse $CD28^-CD27^-$ CD4 T cells as a surrogate of CMV specific CD4 T cells in patients after renal transplantation. Co-staining of PD-1 holds promise as a simple approach to specifically identify patients who may benefit from prolonged therapy.

4.1.4 Comparison of available assays for CMV immunomonitoring post-tranplant and potential areas of clinical application

Apart from flow cytometry, other stimulation-based interferon gamma release assays such as the ELISA technique or the ELISPOT assay exist that are in part available as commercial assay format and therefore better standardised. Nevertheless, these assays are not suited to predict the risk of subclinical reactivation or relapse in CMV seropositive transplant recipients (KOTTON et al., 2013b; SESTER et al., 2008a). This thesis provides evidence that functional and phenotypical analysis of CMV specific CD4 T cells after stimulation, as well as analysis of PD-1 on HLA-peptide-tetramers positive CD8 or $CD28^-CD27^-$ CD4 T cells correlates with episodes of viremia post-transplant and might be used to identify patients at risk of CMV reactivation or relapse. Together with evidence discussed above, analysis of inhibitory T cell receptors on $CD28^-CD27^-$ CD4 T cells might be best suited for implementation in clinical routine monitoring, as it is quick, easy to perform, does not rely on patients HLA status and is more likely to be representative of the overall CMV specific T cell response of an individual patient than T cell frequencies determined by HLA-peptide multimers. One limitation of $CD28^-CD27^-$ CD4 T cells might be, that this population is not entirely CMV specific, as there is

to some degree a bystander effect in the CMV induced and IFNα mediated differentiation process of this cell lineage (FLETCHER et al., 2005). Yet, this lower specificity might be negligible, as we observed a close correlation between frequencies of $CD28^{-}CD27^{-}$ and CMV specific CD4 T cells determined after stimulation and similar PD-1 expression dynamics on both cell-populations post-transplant. Due to its technical ease and low sample volume, this assay may also be explored as a tool to identify CMV specific immunity in pediatric transplant recipients, or to determine the time of CMV specific T cell reconstitution in patients after stem-cell transplantation. The use of this approach for monitoring of CMV complications in pediatric transplant recipients is currently addressed in a collaborative project with Prof. Jutta Preiksaitis in Edmonton (Canada), who has complementary expertise in viral load monitoring of children in the setting of transplantation (PANG et al., 2003; PREIKSAITIS et al., 2005; PREIKSAITIS et al., 1988; PREIKSAITIS et al., 1997; VAUDRY et al., 2010).

An additional area of application for cell-mediated immunity assays is to correctly assign the CMV infection status in individuals where serology might be confounded by passively transferred antibodies, such as in children under 18 months of age or in patients after plasma transfusion. We have demonstrated before, that our stimulation based assay correlates closely with CMV serology in both healthy and immunocompromised individuals. As T cells are not transferred from mother to child during pregnancy or by plasma transfusion, we postulated that assessing CMV specific CD4 T cells will indicate the true infection status in individuals with passively transferred antibodies. A medical thesis by Marion Ritter in our department, to which I contributed in supervising and study design, revealed a perfect agreement between serostatus and CMV specific CD4 T cells in children over 18 months of age. In contrast, 16.7% of children under 18 months were antibody positive but T cell negative, indicating passively transferred immunity by their seropositive mothers (RITTER et al., 2013). Similarly, the case of a CMV seronegative kidney transplant recipient of a seropositive donor, who received human immunglobulins pre-transplant, revealed the dynamics of CMV specific IgG levels and CD4 T cells after plasma transfusion and primary CMV infection. Plasma transfusion led to a transient low-titer seroconversion in the first weeks post-transplant, whereas CMV specific CD4 T cells and CMV-DNA remained undetectable. At the time of CMV primary infection (week 25 post-transplant), CMV specific IgG were decreased to undetectable levels. CMV specific immunity after primary infection

was established by the successive onset of CMV specific T cells and IgG (SCHMIDT et al., 2012b). This indicates that analysis of CMV specific T cells might be a valuable tool to correctly assign the CMV infection status in patients with presumed passively transferred CMV specific antibodies (EMERY, 2013; KOTTON et al., 2013b).

If the results from this thesis were to be confirmed in larger studies, monitoring of inhibitory T cell receptor expression on $CD28^{-}CD27^{-}$ and/or CMV specific CD4 T cells after stimulation might be a potent tool to assign the individual infection state, to personalise antiviral therapy post-transplant and to determine individual monitoring intervals during follow up.

4.2 Immune-based assays for diagnosis of active *M. tuberculosis* infection

Cell-mediated immunity assays such as the commercially available ELISA based QuantiFERON TB gold in tube test and the ELISPOT based T-SPOT.*TB* assay are superior to the classical tuberculin skin test by their ability to discriminate between BCG vaccination response and actual infection with *M. tuberculosis* bacteria (HINKS et al., 2009; LALVANI, 2007; SESTER et al., 2011a). Yet at present, they are not able to distinguish between patients with active *M. tuberculosis* infection and those with latent or successfully treated infection (SESTER et al., 2011a). We and others demonstrated before that a shift in cytokine profiling and a loss of CD27 expression on *M. tuberculosis* specific T cells correlate with disease activity (HARARI et al., 2011; NEMETH et al., 2012; SCHUETZ et al., 2011; SESTER et al., 2011b; STREITZ et al., 2012; STREITZ et al., 2007). As the expression of inhibitory T cell receptors correlates closely with disease activity in CMV and other chronic viral infections, we compared cytokine profiling and CD27 expression analysis with analysis of CTLA-4 expression on *M. tuberculosis* specific T cells. Indeed, similar to our observations in CMV viremic patients, we observed an increased CTLA-4 expression in patients with active disease.

ROC analyses indicate that determination of CD27 expression alone is superior to cytokine profiling or CTLA-4 analysis for diagnosing active *M. tuberculosis* infection (Table 13). Of note, the combined analysis of CD27 with CTLA-4 but not with cytokine profiling further increased diagnostic power (Table 13). To our knowledge, we are the first to show that an increased CTLA-4 expression is indicative of active *M. tuberculosis* infection. Even though being limited by small patient numbers, our data indicate that the combined analysis of CD27 and CTLA-4 expression may serve as a potent diagnostic marker to identify patients with active *M. tuberculosis* infection with notably higher diagnostic power than other biomarkers

reported before (HARARI et al., 2011; NEMETH et al., 2012; SCHUETZ et al., 2011; SESTER et al., 2011b; STREITZ et al., 2012; STREITZ et al., 2007). The combinatorial effect might be in part explained by the different physiological roles of both markers. Whereas CD27 is a co-stimulatory receptor that is needed for the activation of naïve or central memory T cells, it is lost on cells with an effector-memory phenotype (HASHIMOTO-OKADA et al., 2009; KOBATA et al., 1994; MAHNKE et al., 2013). Interestingly, studies in mice and men showed that CD27 expression on *M. tuberculosis* specific T cells was lost at the site of infection (KAPINA et al., 2007; LYADOVA et al., 2004; NEMETH et al., 2012; NIKITINA et al., 2012). Whereas CD27 negative *M. tuberculosis* specific T cells remain in the lungs in controlled infection, they are detectable in peripheral blood in active disease and their frequency correlates with the degree of tissue destruction (NIKITINA et al., 2012). Unlike CD27, CTLA-4 as an inhibitory T cell receptor has the physiological role to down-regulate T cell responses after antigen encounter (KRUMMEL, ALLISON, 1995; ODORIZZI, WHERRY, 2012). CTLA-4 expression seems to be induced at higher levels in primary immune responses compared to boosting of pre-existing immunity in response to reactivations, as was also shown for CMV primary infection and reactivation. Hence, one might speculate that CTLA-4 expression in patients with primary *M. tuberculosis* infection may be higher as compared to patients suffering from reactivation of a latent infection. Interestingly, among the patients with active *M. tuberculosis* infection analysed during this thesis, there was only one seven-year-old child who was assumed to have contracted *M. tuberculosis* by its uncle diagnosed with active tuberculosis before. This child was distinct, as its PPD-reactive T cells not only expressed high levels of CTLA-4 but also of CD27, which might be due to a primary infection with *M. tuberculosis*. In contrast, reactivation from latent infection and more chronic infection stages might rather be characterised by a loss in CD27 expression. It is thus tempting to explore the use of CTLA-4 as a marker of primary infection in combination with CD27 as a marker of disease chronicity. The benefit of combined analyses of CTLA-4 and CD27 expression on PPD-reactive CD4 T cells would need to be addressed by future studies, where the time of infection can be determined precisely and pre-existing immunity is taken into account. However, as it is generally difficult to determine the exact time point of primary *M. tuberculosis* infection in humans, one might have to look at alternative approaches. One possible approach could be to longitudinally monitor the induction of primary immune responses in bladder cancer patients before and after treatment with live *bacillus Calmette-*

Guérin (BCG) instillation. The antitumor effect of BCG instillation is mediated by a strong innate immune response in the bladder, followed by infiltration of T cells (BOHLE, BRANDAU, 2003; BRANDAU, SUTTMANN, 2007; HIGUCHI et al., 2009). Although the therapeutic activity is restricted to the bladder, mouse models indicate that live bacilli enter bladder-draining lymph nodes where T cell priming is initiated (BIOT et al., 2012). This suggests that local BCG-instillation may be associated with a systemic induction of specific T cells and serve as a model to study the induction of mycobacterial immunity after defined antigen contact in humans. Indeed, our group recently demonstrated that therapy-induced PPD specific CD4 T cells in patients with and without pre-existing immunity showed functional differences. Patients without pre-existing immunity which may be considered as individuals with BCG-primary infection showed a dominance of IL-2 single producing over IFNγ single producing cells, whereas this relation was inverted in patients with pre-existing immunity (ELSASSER et al., 2013). It is tempting to speculate, whether this difference in cytokine profiles is also associated with differences in CD27 and CTLA-4 expression and if those markers correlate with progression to active disease.

As the progression to active *M. tuberculosis* infection only occurs in 5 to 10 percent of individuals with latent infection, chemoprophylaxis and chemoprevention of latent infection are only recommended in situations of increased risk, such as close contact to a patient with active disease or relevant degree of immunosuppression (DIEL et al., 2009; DIEL et al., 2011). Up to now, current measures to estimate the individual risk of a patient to progress from latent infection to active tuberculosis only have considerably low positive predictive values, and therefore it is unclear who will specifically benefit from preventive chemotherapy. In tuberculosis contact tracing it is recommended to treat every contact person with immunity towards M. tuberculosis specific antigens, even though in many cases the measured immune response might not represent a de novo infection but rather a pre-existing immune response towards an immunological well contained previous infection. This suggests that currently considerably more patients are treated than actually needed. Indeed, a study by Diel et al showed that in TB contact tracing the number of patients treated to prevent one TB case was 22 for the ELISA-based QuantiFERON assay, and 40 for the tuberculin skin test at a cutoff induration size of 10 mm (DIEL et al., 2007). These low positive predictive values of both skin test and IFNγ based assays emphasise the need for alternative biomarkers to minimize unneeded treatment and treatment costs. Potential biomarkers would be the

above described T cell cytokine profiles and cell surface markers (HARARI et al., 2011; NEMETH et al., 2012; SCHUETZ et al., 2011; SESTER et al., 2011b; STREITZ et al., 2012; STREITZ et al., 2007) as well as chemokines such as IP-10 or MCP-2 which are produced by cells of the myeloid lineage after *M. tuberculosis* specific stimulation (RUHWALD et al., 2009). Another promising approach is the use of novel *M. tuberculosis* antigens or peptide pools which seem to correlate with different *M. tuberculosis* infection states (DEMISSIE et al., 2006; DOSANJH et al., 2011; VINCENTI et al., 2003). Yet, all these markers still need validation in larger clinical studies and to date there is no biomarker which can predict progression from latent to active *M. tuberculosis* infection with a satisfyingly high positive predictive value.

To further address this issue, we performed IFNγ/IL-2 cytokine profiling in parallel with CTLA-4 and CD27 expression analyses on PPD specific CD4 T cells in a subset of latently infected individuals with known recent exposure to *M. tuberculosis* or clinical risk factors for progression. Our analysis revealed that more than half (52%) of these individuals were below the threshold for active disease for all three markers analysed (Figure 26). Although, we do not have any clinical follow-up data of these individuals, it is tempting to speculate whether preventive chemotherapy might not have been necessary for these individuals, yet immunological and radiological monitoring would be advisable. Because of therapy duration, costs and possible side effects of preventive chemotherapy, biomarkers with higher positive predictive values would be of great benefit to the individual patient as well as the healthcare system in general. If future studies confirmed that immunological markers such as cytokine profiling, CTLA-4 and CD27 indeed were able to more precisely assess the risk for progression to active disease, they would be powerful tools to facilitate clinical decision making on initiation of preventive chemotherapy in the setting of contact tracing.

4.3 Influence of pathogen prevalence on antigen specific immunity in immunocompromised patients

In HIV infected individuals, the ongoing HIV replication and virus induced cell death during acute HIV-infection results in massive depletion of memory CD4 T cells at mucosal sites that can be partially restored, in particular under antiretroviral therapy (ART) (GELDMACHER et al., 2008; GUIHOT et al., 2011). Similarly, an increased turnover in the patients T cell pool occurs in stem cell transplant recipients or in solid organ transplant recipients after anti-

thymocyte-globulin (ATG) induction therapy (CHANG et al., 2014; GUILLAUME et al., 1998; PAGE et al., 2013). We have assessed the frequencies of antigen specific T cells towards CMV and *M. tuberculosis* in HIV infected individuals in tuberculosis high and low endemic countries to elucidate the influence of exogenous and endogenous antigen re-challenge on replenishment of antigen specific immune responses after initial HIV mediated T cell depletion. Our results showed that PPD reactive T cell frequencies were higher in tuberculosis high than low endemic countries (Figure 29). In line with this evidence and with results from immunocompetent individuals (SESTER et al., 2011b), an even stronger mycobacterial challenge during active tuberculosis was associated with a further increase in PPD specific CD4 T cell frequencies. Consequently, immunity towards a persistent pathogen such as CMV that poses a constant antigenic challenge to the immune system should not differ in magnitude between the two groups. Indeed, T cell immunity toward CMV or the bacterial superantigen SEB was detectable in patients and controls from both low and high tuberculosis endemic areas (Figure 29). Together, these data suggest a constant skewing and restriction of specific T cell immunity toward environmental antigens in HIV infected individuals, where either endogenous or exogenous antigen re-challenge is needed to expand T cells with defined specificities from few pre-existing memory T cell clones.

In general, PPD specific immunity wanes in the absence of re-exposure, but this is rather a slow process that occurs over decades (GUIHOT et al., 2011). Hence, the relative maintenance of PPD specific immunity in healthy controls is a common observation even in low endemic countries such as Germany where frequent exposure to *M. tuberculosis* is unlikely. Measurable PPD specific immunity is observed not only in immunocompetent controls but also in patients with other types of immunodeficiency such as chronic renal failure or renal transplant recipients (SESTER et al., 2004; SESTER et al., 2006). Thus, given that HIV infection is usually acquired later in life and that HIV infected individuals were recruited from the same geographic region as controls, one would primarily expect similar levels of PPD specific T cells as in controls or other immunocompromised groups. As there were no differences in ethnicity and countries of origin between controls and HIV infected individuals in Germany, the lower frequency of PPD specific T cells is a particular feature of HIV related immunosuppression in low-prevalence regions and generally indicates a loss of T cell specificities that are not urgently needed to control environmental or persistent pathogens. In line with this evidence, results from HIV infected patients in Tanzania show

that PPD reactive T cells are maintained in areas of higher *M. tuberculosis* endemicity. Our observations are compatible with current views of HIV-pathogenesis and maintenance of antigen specific memory CD4 T cells in long-term HIV infected patients under antiretroviral therapy (GUIHOT et al., 2011). While HIV infection leads to a profound depletion of CD4 T cells already during the primary infection phase (BRENCHLEY et al., 2004; MEHANDRU et al., 2004), subsequent treatment restores antigen specific responses that mediate protection against opportunistic agents (GUIHOT et al., 2011). The trigger that determines the antigen specificity of the recovering immunity seems to be environmental exposure to the antigen itself or other cross-reacting antigens. In support of this, antiretroviral therapy in vaccinia vaccinated patients did not lead to a full recovery of memory T cells specific for smallpox, a now eradicated virus (PUISSANT-LUBRANO et al., 2009), whereas T cell responses against CMV, a persistent viral pathogen, were readily restored (PUISSANT-LUBRANO et al., 2009; SESTER et al., 2007). A similar dependence on antigen has been shown for specific T cell responses recovering after profound lymphopenia in allogeneic stem-cell transplant recipients, where early expansion of donor-derived CMV specific T cell clones in a recipient was observed only when the recipient was CMV seropositive (GANDHI et al., 2003). Thus, exposure to antigen is the key determinant of a measurable T cell response in HIV seropositive individuals.

In conclusion, the antigen specific T cell repertoire in chronically HIV infected individuals seems to be skewed towards environmentally present antigens. Thus, pathogen prevalence may critically influence the specificity of this recovering repertoire in HIV infected individuals. This could have important implications for future vaccination strategies and risk assessment of infectious complications in HIV patients and other immunocompromised patient groups. Whether the observed differences in the levels of PPD specific immunity in HIV infected individuals from low and high endemic regions may translate into differences in the control of *M. tuberculosis* after re-exposure merits further study.

4.4 Concluding remarks on chances and limitations of flow cytometry based phenotypical and functional characterisation of antigen specific immune responses

The exclusive quantitation of antigen specific immunity has proven valuable in assigning the infection status in several chronic infections, yet it is only of limited value to determine disease activity or predict reactivation episodes. As demonstrated in this thesis, phenotypical

and functional characterisation of antigen specific T cells allows assessment of the quality of the patients' pathogen specific immunity. It can be applied not only to determine the patients' infection state but also to assess disease activity and severity. This may in future be exploited to improve diagnosis of infectious diseases, to identify patients with increased risk for complications and to monitor therapy response and success. Our data show that a meaningful combination of phenotypical and functional biomarkers on antigen specific T cells may considerably improve power to diagnose active disease, compared to mere quantitative analysis. Additionally, our 6 hour stimulation-based assay needs less time and blood volume than the commercially available ELISA and ELISPOT assays, which makes it attractive for routine diagnostics. Apart from specific immunity towards CMV and *M. tuberculosis*, the assay principle can also be applied to monitoring immunity towards other pathogens such as HIV, adenovirus, human herpesvirus-8, varicella zoster virus, seasonal and pandemic influenza, and BK polyomavirus (LÖNARD et al., 2007; SCHMIDT et al., 2014; SCHMIDT et al., 2012a; SCHUB et al., 2012; SESTER et al., 2002a; SESTER et al., 2000) or vaccine antigens (SCHMIDT et al., 2012a; SESTER et al., 2013). The phenotypical characterisation of $CD28^{-}CD27^{-}$ CD4 T cells for assessment of CMV associated T cell immunity further reduces assay complexity, costs, sample volumes and logistical limitations, which should facilitate a broader, multicentre application.

One possible limitation of using multiparameter analyses for widespread clinical application is the need for access to a flow cytometer, yet in most university hospitals and diagnostic laboratories flow cytometry is already used since decades for diagnostics of hematologic diseases (ORFAO et al., 1995). Another possible limitation of flow cytometry often discussed is the assumed lack of standardisation between centres and individual devices. However, the development of fluorophore-coated calibration beads, together with standard operating procedures enable standardisation between different sites and virtually all available cytometer platforms (MAECKER et al., 2010; PERFETTO et al., 2012). Furthermore, recently available software tools for automated data analysis may prove helpful to facilitate analyses of multicolour experiments across several centers and avoid operator bias (COSTA et al., 2010; QIU et al., 2011). A combination of the above was used by the "EuroFlow" consortium aimed at standardising flow cytometric diagnostics in the area of hematologic diseases. The consortium achieved standardisation of staining protocols and instrument setups in 18 diagnostic research groups to a level that allowed storage, management and subsequent

analysis of flow cytometry raw data in a shared data base (KALINA et al., 2012; VAN DONGEN et al., 2012a; VAN DONGEN et al., 2012b). In the future the described or similar approaches can be used to synchronize multicentre studies to define universal diagnostic thresholds or detection limits, and to facilitate the exchange of scientific outcome between laboratories.

In conclusion, this thesis highlights the potential of combined functional and phenotypical analysis of pathogen specific T cell immunity via flow cytometry to improve diagnostics of active infections and to facilitate clinical decision making by monitoring treatment response and success. As a future perspective, the diagnostic value of the presented results needs to be validated by multicentre clinical trials where clinical decisions will be based upon the results of the presented assays.

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seasonal antigens and functionally differs from active influenza infection. Eur J Immunol 42:1755-1766
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6 Publications

6.1 First author publications

1. **Dirks, J.**, Tas, H., Schmidt, T., Kirsch, S., Gärtner, B. C., Sester, U., & Sester, M. 2013. PD-1 analysis on CD28(-) CD27(-) CD4 T cells allows stimulation-independent assessment of CMV viremic episodes in transplant recipients.
   Am J Transplant, 13(12): 3132-3141.

2. **Dirks, J.***, Egli, A.*, Sester, U., Sester, M., & Hirsch, H. H. 2013. Blockade of programmed death receptor-1 signaling restores expression of mostly proinflammatory cytokines in anergic cytomegalovirus-specific T cells.
   Transpl Infect Dis, 15(1): 79-89. - *equal contribution

3. Schuetz, A.*, **Dirks, J.***, Sester, U., Haule, A., Elias, N., Geldmacher, C., Sanga, E., Maboko, L., Reither, K., Hoelscher, M., Meyerhans, A., & Sester, M. 2012. Pathogen prevalence may determine maintenance of antigen-specific T cell responses in HIV-infected individuals.
   AIDS, 26(6): 695-700. - *equal contribution

6.2 Co-author publications

1. Schmidt, T., Schub D., Wolf, M., **Dirks, J.**, Ritter, M., Leyking, S., Singh, M., Zawada, A., Blaes-Eise, A.-B., Samuel, U., Sester U. and Sester M. 2014. Comparative analysis of assays for detection of cell-mediated immunity toward cytomegalovirus and M. tuberculosis in samples from deceased organ donors.
   Am J Transplant, in press.

2. Schmidt, T., Adam, C., Hirsch, H. H., Janssen, M. W., Wolf, M., **Dirks, J.**, Kardas, P., Ahlenstiel-Grunow, T., Pape, L., Rohrer, T., Fliser, D., Sester, M., & Sester, U. 2014. BK polyomavirus-specific cellular immune responses are age-dependent and strongly correlate with phases of virus replication.
   Am J Transplant, 14(6): 1334-1345.

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Traffic, 14(7): 798-809.

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Eur J Immunol, 43(4): 1099-1108.

5. Schmidt, T., **Dirks, J.**, Enders, M., Gärtner, B. C., Uhlmann-Schiffler, H., Sester, U., & Sester, M. 2012. CD4 T cell immunity after pandemic influenza vaccination cross-reacts with seasonal antigens and functionally differs from active influenza infection.
Eur J Immunol, 42(7): 1755-1766.

6. Schmidt, T., Ritter, M., **Dirks, J.**, Gärtner, B. C., Sester, U., & Sester, M. 2012. Cytomegalovirus-specific T cell immunity to assign the infection status in individuals with passive immunity: a proof of principle.
J Clin Virol, 54(3): 272-275.

7. Sester, U., Fousse, M., **Dirks, J.**, Mack, U., Prasse, A., Singh, M., Lalvani, A., & Sester, M. 2011. Whole-blood flow cytometric analysis of antigen-specific CD4 T cell cytokine profiles distinguishes active tuberculosis from non-active states.
PLoS One, 6(3): e17813.

Publication from diploma thesis

Sester, U., Presser, D., **Dirks, J.**, Gärtner, B. C., Köhler, H., & Sester, M. 2008. PD-1 expression and IL-2 loss of cytomegalovirus-specific T cells correlates with viremia and reversible functional anergy.
Am J Transplant, 8: 1486-1497.

Manuscript in preparation

Dirks, J., Schmidt, T., Leyking, S., Gärtner, B.C., Sester, U. & Sester, M. Combined analysis of multiple inhibitory T cell receptors on cytomegalovirus specific CD4 T cells allows identification of patients at risk for viral reactivation after transplantation, in preparation.

6.3 Presentations on scientific meetings (selection)

1. **Dirks, J.**, Sester, U., Mack, U., Fousse, M., Sybrecht, G., Lalvani, A., Sester, M.
   Flow cytometric analysis of antigen-specific CD4 T cell cytokine profiles distinguishes active tuberculosis from successfully treated disease
   Poster presentation at the Annual meeting of the Society of Internal Medicine in Saarland and Rhineland-Palatinate in Neustadt, Germany, 03.2009
2. **Dirks, J.**, Sester, U., Gärtner, B., Sester, M.
   Novel phenotypical and functional markers on CMV specific T cells as predictors of CMV viremia after renal transplantation
   Poster presentation at the American Transplant Congress in San Diego, USA, 05.2010
3. **Dirks, J.**, Schuetz, A., Sester, A., Haule, A., Geldmacher, C., Elias, N., Schindler, K., Sanga, E., Maboko, L., Minja, F., Samky, E., Reither, K., Hoelscher, M., Meyerhans, A., Sester, M.
   Pathogen prevalence may determine maintenance of antigen specific T cell responses in HIV infected individuals
   Oral presentation at the annual meeting of the European Respiratory Society in Barcelona, Spain, 09. 2010
4. **Dirks, J**., Tas, H., Schmidt, T., Kirsch, S., Gärtner, B,. Sester, U., Sester, M.
   Combined analysis of multiple inhibitory T cell receptors on cytomegalovirus specific CD4 T cells allows identification of patients at risk for viral reactivation after transplantation
   Oral presentation at the 24th International Congress of the Transplant Society in Berlin, Germany, 07.2012
5. **Dirks, J.,** Tas, H., Schmidt, T., Kirsch, S., Gärtner, B., Sester, U., Sester, M.

   PD-1 analysis on $CD28^{-}CD27^{-}$ CD4 T cells allows rapid stimulation-independent assessment of CMV-viremic episodes in transplant recipients

   Plenary session talk at the American Transplant Congress in Seattle, USA, 05.2013

Upcoming presentation

6. **Dirks, J**., Fousse, M., Sester, U., Sester, M.

   Combined phenotypical and functional analysis of *M. tuberculosis* specific immunity for diagnosing active infection and risk assessment in latently infected individuals

   Oral presentation at the Annual Meeting of the European Respiratory Society in Munich, Germany, 09.2014

6.4 Scientific awards

03.2009: **Poster award**

Flow cytometric analysis of antigen-specific CD4 T cell cytokine profiles distinguishes active tuberculosis from successfully treated disease

Annual meeting of the society of internal medicine in Saarland and Rhineland-Palatinate

09.2010: **Young investigator award**

Pathogen prevalence may determine maintenance of antigen-specific T cell responses in HIV-infected individuals

Annual meeting of the European Respiratory Society (ERS) in Barcelona, Spain

06.2011: Invited as a young researcher to the **Lindau Nobel Laureate Meeting** dedicated to Physiology or Medicine, Lindau, Germany

05.2013: **Young investigator award** and oral presentation in plenary session

PD-1 analysis on $CD28^{-}CD27^{-}$ CD4 T cells allows rapid stimulation-independent assessment of CMV-viremic episodes in transplant recipients

Annual meeting of the American Transplantation Society (ATC) in Seattle, USA

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8 Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form in einem anderen Verfahren zur Erlangung des Doktorgrades einer anderen Prüfungsbehörde vorgelegt.

Homburg, den 1.08.2014

Jan Dirks